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BAS 750 F

Document M-CA, Section 1

IDENTITY OF THE ACTIVE SUBSTANCE

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[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 1 IDENTITY OF THE ACTIVE SUBSTANCE

CA 1.1 Applicant

BASF Agro B.V. Arnhem (NL)
Zürich Branch
Im Tiergarten 7
8055 Zürich
Switzerland

Contact person:

[redacted]
Telephone
Telefax
e-mail [redacted]

Alternative contact:

[redacted]
Telephone:
Telefax:
E-mail: [redacted]

CA 1.2 Producer

Producer of BAS 750 F (legal entity)

BASF Agro B.V. Arnhem (NL)
Zürich Branch
Im Tiergarten 7
8055 Zürich
Switzerland

Contact person: Please refer to CA 1.1 Applicant.

Location of manufacturing site for BAS 750 F

CONFIDENTIAL information - data provided separately (Document J)

CA 1.3 Common Name Proposed or ISO-accepted and synonyms

ISO common name: Mefentrifluconazole (ISO proposed)

CA 1.4 Chemical Name (IUPAC and CA nomenclature)

IUPAC name: (2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol

CA nomenclature: alpha-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-alpha-methyl-1H-1,2,4-triazole-1-ethanol

CA 1.5 Producer's Development Code Numbers

BASF Number: BAS 750 F
BASF Registry Number: Reg.No. 5834378

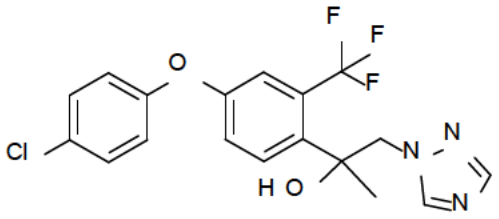
CA 1.6 CAS, EC and CIPAC Numbers

CAS No.: 1417782-03-6
CIPAC No.: not assigned
EC No.: not assigned

CA 1.7 Molecular and Structural Formula, Molar Mass

Molecular formula: $C_{18}H_{15}ClF_3N_3O_2$
Molar mass: 397.8 g/mol

Structural formula:



CA 1.8 Method of Manufacture (synthesis pathway) of the active substance

CONFIDENTIAL information - data provided separately (Document J)

CA 1.9 Specification of Purity of the Active Substance in g/kg

Minimum purity: 965 g/kg

CA 1.10 Identity and Content of Additives (such as Stabilisers) and impurities

CA 1.10.1 Additives

CONFIDENTIAL information - data provided separately (Document J)

CA 1.10.2 Significant impurities

CONFIDENTIAL information - data provided separately (Document J)

CA 1.10.3 Relevant impurities

CONFIDENTIAL information - data provided separately (Document J)

CA 1.11 Analytical Profile of Batches

CONFIDENTIAL information - data provided separately (Document J)

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BAS 750 F

Document M-CA, Section 2

PHYSICAL AND CHEMICAL PROPERTIES OF THE ACTIVE SUBSTANCE

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E-mail:

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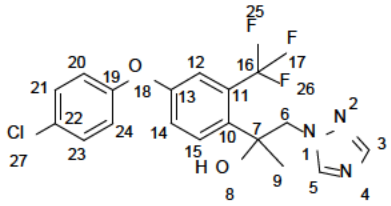
CA 2 PHYSICAL AND CHEMICAL PROPERTIES OF THE ACTIVE SUBSTANCE

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.1 Melting point and boiling point	OPPTS 830.7200 DSC/TG method (equivalent to OECD 102)	L84-238: 99.7 %	Melting point (onset): 126 °C Decomposition temp. (onset): approx. 300 °C (onset of exothermic peak)	Y	[see 2014/1117052 Kroehl T. 2014 a]
	OPPTS 830.7200 DSC/TG method (equivalent to OECD 102)	COD-001740: 98.8 %	Melting point (onset): 125 °C Decomposition temp. (onset): approx. 300 °C (onset of exothermic peak)	Y	[see 2014/1117053 Kroehl T. 2014 b]
CA 2.2 Vapour pressure, volatility	EEC A.4 OECD 104	L84-238: 99.7 %	Vapour pressure: $p = 3.2 \cdot 10^{-6}$ Pa (20 °C) $p = 6.5 \cdot 10^{-6}$ Pa (25 °C)	Y	[see 2014/1117052 Kroehl T. 2014 a]
	calculation	not relevant	Henry Constant: $H = 1.6 \cdot 10^{-3}$ Pa · m^3 · mol^{-1}	N	[see 2014/1173597 Kroehl T. 2014 c]

<table>
<tr><th>Test or Study & Data Point</th><th>Guideline and method</th><th>Test material purity and specification</th><th>Findings</th><th>GLP Y/N</th><th>Reference</th></tr>
<tr><td rowspan="2">CA 2.3 Appearance (Physical state, colour)</td><td rowspan="2">OPPTS 830.6302 to 830.6304 (visual and olfactory examina-tion)</td><td>L84-238: 99.7 %</td><td>white, solid and odorless crystalline powder</td><td>Y</td><td>[see 2014/1117052 Kroehl T. 2014 a]</td></tr>
<tr><td>COD-001740: 98.8 %</td><td>fine powdered, off-white solid of moderate thiolic odour</td><td>Y</td><td>[see 2014/1117053 Kroehl T. 2014 b]</td></tr>
<tr><td rowspan="2">CA 2.4 Spectra (UV/VIS, IR, NMR, MS), molar extinction at relevant wavelengths, optical purity</td><td>OECD 101, UV-VIS</td><td rowspan="2">L84-238: 99.7 %</td><td>molar extinction coefficients ε in L · mol^{-1} · cm^{-1} (at relevant wavelengths)
<table>
<tr><th>Methanol solution

(pH 6.1)</th><th>Methanol/Water solution

10:90
(pH 6.4)</th><th>Methanol/HCl (1M)/Water solution
10:5:85
(pH 1.4)</th><th>Methanol/NaOH (1M)/Water solution
10:5:85
(pH 12.2)</th></tr>
<tr><td>37761 (202 nm)
17322 (232 nm)
2774 (272 nm)
1727 (290 nm)
439 (295 nm)</td><td>54636 (194 nm)
16618 (231 nm)
2780 (275 nm)
1504 (290 nm)
420 (295 nm)</td><td>43245 (199 nm)
16553 (231 nm)
2759 (272 nm)
1438 (290 nm)
402 (295 nm)</td><td>
16668 (231 nm)
2784 (277 nm)
1653 (290 nm)
615 (295 nm)</td></tr>
</table></td><td rowspan="2">Y</td><td rowspan="2">[see 2014/1173598 Kroehl T., Behnken H. 2014 a]
[see 2015/1183750 Kroehl T. 2015 a]</td></tr>
<tr><td>IR</td><td>wave numbers ῡ in cm^{-1}
3116 C-H stretching vibration in aromatic methine groups
2977 C-H stretching vibration in alkane groups
1484 C-C ring stretching in C-6 aromatic groups
1251 C-O stretching vibration in diaryl ethers
1158 C-O-C stretching vibration in ethers</td></tr>
</table>

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
			1127 C-H rocking vibration in methyl groups 1087 C-O stretching vibration in alcohols 831 C-H stretching vibration in aromatic methine groups The spectrum is in accordance with the proposed structure.		
	NMR		 Samples are dissolved in deuterated dimethyl sulfoxide (DMSO-d_6), shifts are given in ppm (assignments in brackets). **^{1}H**: 7.9 (**3**), 8.3 (**5**), 4.5 (**6**), 5.7 (**8**), 1.5 (**9**), 7.4 (**12**), 7.2 (**14**), 7.6 (**15**), 7.1 (**20**, **24**), 7.5 (**21**, **23**) **^{13}C**: 150.5 (**3**), 145.0 (**5**), 59.0 (**6**), 73.3 (7), 27.7 (**9**), 139.8 (**10**), 128.4[1] (**11**), 117.5[3] (**12**), 154.6 (**13**), 121.0 (**14**), 131.2 (**15**), 123.9[2] (**16**), 155.3 (**19**), 120.8 (**20**, **24**), 130.1 (**21**, **23**), 128.1 (**22**) [1] Quartet at 128.7, 128.5, 128.3 and 128.1 ppm due to $^{2}J_{C,F}$ coupling [2] Quartet at 126.7, 124.8, 123.0 and 121.2 ppm due to $^{1}J_{C,F}$ coupling [3] Quartet at 117.6, 117.5, 117.5 and 117.4 ppm due to $^{3}J_{C,F}$ coupling **^{19}F**: -53.1 (**17**, **25**, **26**)		

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
	MS-ESI GC/MS-EI GC/MS-CI		Electrospray ionization (ESI): *m/z* = 397.7 $[M+H]^+$ (protonated ion of BAS 750 F) characteristic fragments of BAS 750 F: *m/z* = 69.8, 182.0, 241.1, 269.0, 289.1, 309.1 Electron ionization (EI): *m/z* = 379 $[M-H_2O]^+$ characteristic fragments of BAS 750 F: *m/z* = 83, 111, 185, 235, 295, 340 Chemical ionization (CI): *m/z* = 398 $[M]^+$ characteristic fragments of BAS 750 F *m/z* = 340, 380		
CA 2.5 Solubility in water	EEC A.6 OECD 105	L84-283: 99.7 %	Results were determined applying the column elution method. water: 0.81 mg/L (pure water, resulting pH value: 6.8) pH 4: 0.66 mg/L (acetate buffer) pH 7: 0.71 mg/L (phosphate buffer)	Y	[see 2013/1397136 Wilbrand S. 2013 a]

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.6 Solubility in organic solvents	CIPAC MT 181	COD-001740: 98.8 %	Results (in g/L) were obtained at 20 °C (± 0.5 °C) applying the flask method. Acetone 93.2 (± 1.6) Ethyl acetate 116.2 (± 1.8) Methanol 73.2 (± 3.2) 1,2-Dichloroethane 55.3 (± 0.4) Acetonitrile 49.4 (± 0.7) Xylene 8.5 (± 0.1) n-Heptane $9.46 \cdot 10^{-2}$ ($\pm 0.9 \cdot 10^{-3}$)	Y	[see 2013/1391669 Wilbrand S. 2013 b]
CA 2.7 Partition co-efficient n-octanol/water	EEC A.8 OECD 117	L84-238: 99.7 %	Results determined at 20 °C applying the HPLC method. pH 4*: log P_{OW} = 3.4 pH 7: log P_{OW} = 3.4 pH 7*: log P_{OW} = 3.3 pH 9*: log P_{OW} = 3.4 * buffered	Y	[see 2013/1382370 Wilbrand S. 2013 c]
CA 2.8 Dissociation in water - dissociation constant(s) (pKa values) - identity of dissociated species - dissociation constant(s) (pKa values) of the active principle	OECD 112	L84-238: 99.7 %	Due to the low solubility of the test item in water, the titration method is not suitable for the determination of the dissociation constant. Instead of the titration method the spectroscopic method was used in this study. pK_a at 20 °C: 2.7 ± 0.5 pK_a at 30 °C: 2.5 (± 0.5) pK_a (calculated; ACD Lab 12.01): 3.0 Due to the high variation of the test results within each measurement as well as to the high relative standard deviations, the pKa value calculated is reported as the final result of the dissociation constant of Reg. No. 5834378.	Y	[see 2013/1397719 Wilbrand S. 2013 d]

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.9 Flammability and self-heating	EEC A.10 EEC A.16	COD-001740: 98.8 %	Flammability: No ignition of the test item by flame in the preliminary test (the test item melted). Thus, the main test was omitted. Relative self-ignition of solids: Test not performed (melting point < 160 °C).	Y	[see 2014/1109962 Moeller M. 2014 a]
CA 2.10 Flash point		COD-001740: 98.8 %	Not applicable (melting point > 40 °C).	Y	[see 2014/1109962 Moeller M. 2014 a]
CA 2.11 Explosive properties	OECD 113 EEC A.14	COD-001740: 98.8 %	Thermal Stability (DSC method): 1st reaction: onset 110 °C, energy intake 110 J/g (endothermic) 2nd reaction: onset 340°C, energy release 580 J/g (exothermic) Mechanical sensitivity, friction: No reaction observed in six tests using BAM friction apparatus with a force of 360 N. Mechanical sensitivity, impact. No reaction observed in six tests using BAM drop hammer (mass 10 kg, drop height 40 cm). Thermal sensitivity, Koenen-test:	Y	[see 2014/1109962 Moeller M. 2014 a]

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
			The results of the test series with a 2.0 mm diameter orifice plate showed no explosion. Therefore, further tests were not required. Final conclusion: not explosive		
CA 2.12 Surface Tension	OECD 115		Not applicable, substances with a water solubility < 1 mg/L need not be tested.		
CA 2.13 Oxidizing properties	EEC A.17	COD-001740 : 98.8 %	The highest burning rate of a test mixture of test item with cellulose (1.07 mm/s) is lower than the highest burning rate of a mixture of barium nitrate with cellulose (1.27 mm/s). Conclusion: Not oxidizing.	Y	[see 2014/1109962 Moeller M. 2014 a]

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.14 Other studies:					
Vapour pressure of the metabolites M750F003 (Reg.No. 5924326) M750F005 (Reg.No. 6003433) M750F006 (Reg.No. 5863469) M750F007 (Reg.No. 6003432) M750F008 (Reg.No. 6010286)	EEC A.4 OECD 104	Reg.No. 5924326, L84-250: 99.6 % Reg.No. 6003433, L87-34: 99.4 % Reg.No. 5863469, L87-30: 98.9 % Reg.No. 6003432, L87-32-1: 97.0 % Reg.No. 6010286, L85-94: 96.5 %	<u>M750F003 (Reg.No. 5924326):</u> $p = 3.4 \cdot 10^{-06}$ Pa (20 °C) $p = 7.1 \cdot 10^{-06}$ Pa (25 °C) <u>M750F005 (Reg.No. 6003433):</u> $p = 2.3 \cdot 10^{-09}$ Pa (20 °C) $p = 6.1 \cdot 10^{-09}$ Pa (25 °C) <u>M750F006 (Reg.No. 5863469):</u> $p = 4.5 \cdot 10^{-08}$ Pa (20 °C) $p = 1.0 \cdot 10^{-07}$ Pa (25 °C) <u>M750F007 (Reg.No. 6003432):</u> $p = 3.7 \cdot 10^{-11}$ Pa (20 °C) $p = 1.0 \cdot 10^{-10}$ Pa (25 °C) <u>M750F008 (Reg.No. 6010286):</u> $p = 2.7 \cdot 10^{-13}$ Pa (20 °C) $p = 9.1 \cdot 10^{-13}$ Pa (25 °C)		[see 2015/1205970 Daum A. 2015 a] [see 2015/1205971 Daum A. 2015 b] [see 2015/1205972 Daum A. 2015 c] [see 2015/1205973 Daum A. 2015 d] [see 2015/1205976 Daum A. 2015 e]

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
Water solubility of the metabolites M750F003 (Reg.No. 5924326) M750F005 (Reg.No. 6003433) M750F006 (Reg.No. 5863469) M750F007 (Reg.No. 6003432) M750F008 (Reg.No. 6010286)	EEC A.6 OECD 105		<u>M750F003 (Reg.No. 5924326): Flask method</u> Double Distilled Water 2.46 (± 0.04) g/L, pH 6.5 Acetate Buffer 2.42 (± 0.11) g/L, pH 4.4 Borate Buffer 2.48 (± 0.03) g/L, pH 8.3* *the pH of the buffer solution without test item was 8.92 <u>M750F005 (Reg.No. 6003433): Column elution method</u> Double Distilled Water 11.3 (± 0.6) mg/L, pH 6.8 Acetate Buffer 9.9 (± 1.5) mg/L, pH 4.3 Borate Buffer 13.8 (± 1.3) mg/L, pH 9.0 <u>M750F006 (Reg.No. 5863469): Column elution method</u> Double Distilled Water 11.2 (± 1.3) mg/L, pH 7.1* Acetate Buffer 18.9 (± 2.4) mg/L, pH 4.2* Borate Buffer 13.9 (± 2.3) mg/L, pH 9.0 *pH-value of the buffer solution, used for the elution of the test item <u>M750F007 (Reg.No. 6003432): Flask method</u> Double Distilled Water 72.7 (± 11.4) mg/L, pH 6.4 Acetate Buffer 73.4 (± 1.2) mg/L, pH 4.3 Borate Buffer 71.8 (± 2.5) mg/L, pH 8.9 <u>M750F008 (Reg.No. 6010286): Column elution method</u> Double Distilled Water 1.96 (± 0.10) mg/L, pH 6.4 Acetate Buffer 2.43 (± 0.21) mg/L, pH 4.4 Borate Buffer 3.67 (± 0.34) mg/L, pH 9.1	Y	[see 2015/1139989 Wilbrand S. 2015 a] [see 2015/1252305 Wilbrand S. 2016 a] see 2016/1030230 Wilbrand S. 2016 b] [see 2015/1139993 Wilbrand S. 2015 b] [see 2015/1139994 Wilbrand S. 2015 c] [see 2015/1139997 Wilbrand S. 2015 d] [see 2015/1139998 Wilbrand S. 2015

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
			Note: unless otherwise flagged, pH values reflect average values of the saturated solutions. Determinations of water solubility were performed at 20 (±0.5) °C.		e]
log Pow of the metabolites M750F003 (Reg.No. 5924326) M750F005 (Reg.No. 6003433) M750F006 (Reg.No. 5863469) M750F007 (Reg.No. 6003432) M750F008 (Reg.No. 6010286)	EEC A.8 (HPLC method) OECD 117		<u>M750F003 (Reg.No. 5924326):</u> Water P_{OW} 2.59, log P_{OW} 0.41, pH 6.8 Acetate Buffer P_{OW} 2.35, log P_{OW} 0.37, pH 4.4 Borate Buffer P_{OW} < 1, log P_{OW} < 0, pH 9.0 <u>M750F005 (Reg.No. 6003433):</u> Water P_{OW} 48.98, log P_{OW} 1.69, pH 6.8 Acetate Buffer P_{OW} 25.29, log P_{OW} 1.41, pH 4.4 Borate Buffer P_{OW} 9.22, log P_{OW} 0.96, pH 9.0 <u>M750F006 (Reg.No. 5863469):</u> Water P_{OW} 538.1, log P_{OW} 2.73, pH 6.8 Acetate Buffer P_{OW} 305.9, log P_{OW} 2.49, pH 4.3 Borate Buffer P_{OW} 352.9, log P_{OW} 2.55, pH 9.0 <u>M750F007 (Reg.No. 6003432):</u> Water P_{OW} 7.91, log P_{OW} 0.90, pH 6.8 Acetate Buffer P_{OW} 7.82, log P_{OW} 0.89, pH 4.4 Borate Buffer P_{OW} < 1, log P_{OW} < 0, pH 9.0 <u>M750F008 (Reg.No. 6010286):</u> Water P_{OW} 57.36, log P_{OW} 1.76, pH 6.8 Acetate Buffer P_{OW} 31.35, log P_{OW} 1.49, pH 4.4	Y	

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
			Borate Buffer P_{OW} < 1, log P_{OW} < 0, pH 9.0 Note: pH values reflect values of Millipore water resp. the buffer solution, used for the preparation of the HPLC Eluent. Determinations of log P_{OW} values were performed at 25 (±1) °C.		
Dissociation constant of the metabolites M750F003 (Reg.No. 5924326) M750F005 (Reg.No. 6003433) M750F006 (Reg.No. 5863469) M750F007 (Reg.No. 6003432) M750F008 (Reg.No. 6010286)	OECD 112		<u>M750F003 (Reg.No. 5924326):</u> pKa = 8.7 (± 0.1) No temperature dependence. <u>M750F005 (Reg.No. 6003433):</u> pKa = 9.84 (± 0.05) No temperature dependence. <u>M750F006 (Reg.No. 5863469):</u> No dissociation constant found in aqueous solution for 2 < pH < 12. Reg.No. 5863469 contains a lactone structure which might be hydrolyzed under basic conditions by ring-opening. The different UV/Vis spectra in the range above pH 10 are caused by this hydrolysis product. <u>M750F007 (Reg.No. 6003432):</u> pKa = 9.5 (± 0.1) No temperature dependence. <u>M750F008 (Reg.No. 6010286):</u>	Y	

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
			pKa = 9.09 (± 0.15) No temperature dependence. Note: The dissociation constants were determined with the spectroscopic method (OECD 112) at 20°C (±1°C) and, to evaluate the temperature-dependence of this value at 30°C (±1°C).		

Summary:

BAS 750 F (PAI) is a white, solid, odourless, crystalline powder. BAS 750 F Technical is an off-white powder with moderate thiolic odour. The active substance is neither flammable, nor explosive, nor oxidising. The vapour pressure amounts to $3.2 \cdot 10^{-6}$ Pa at 20°C. BAS 750 F has a solubility in pure water of 0.81 g/L at 20 °C. Its n octanol/water partition coefficient (log P_{OW}) is 3.4. The dissociation constant (pKa) is reported to be 3.0. The active substance absorbs light in the UV area (absorption bands around 202, 232, 272, 290 and 295 nm). IR, NMR and MS spectra are in line with the molecular structure.

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BAS 750 F

Document M-CA, Section 3

FURTHER INFORMATION ON THE ACTIVE SUBSTANCE

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Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

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CA 3 FURTHER INFORMATION ON THE ACTIVE SUBSTANCE

CA 3.1 Use of the Active Substance

BAS 750 F (Mefentrifluconazole, proposed) is a triazole fungicide under development for worldwide use in several crops for the control of a broad range of important pathogens. BAS 750 F is active against different fungal stages both on the plant surface and in the plant tissue. After application to the plant, the active ingredient is taken up via the leaf and then translocated apically via the transpiration flow. Due its mobility, it shows systemic and translaminar activity. As a result, it can control fungal stages which have already become established in deeper tissue layers. BAS 750 F is thus suitable for preventative and curative treatments.
Since the vapour pressure of BAS 750 F is very low, a gas phase activity was not observed.

CA 3.2 Function

BAS 750 F is a fungicide to control harmful diseases in a broad range of crops.

BAS 750 F (LS 5834378) is a racemic mixture of a (R)-enantiomer (LS 5934591) and a (S)-enantiomer (LS 5934588) at a 1 : 1 ratio. The biological efficacy of the two enantiomers and the BAS 750 F have been compared under greenhouse conditions against the cereal diseases *Puccinia triticina*, *Blumeria graminis*, *Zymoseptoria tritici* (sensitive and azole shifted) and *Rhynchosporium secalis* (Table 3.2-1). The compounds were dissolved in an aceton/wettol water solution (5% aceton) and applied at a water volume of 200 L/ha and a constant adjuvant rate of 200 g/ha.

Both enantiomers as the racemate showed biological activity on all tested pathogens.
Depending on the disease the (R)-enantiomer (*P. triticina*, *B. graminis*, *Z. tritici*) or the (S)-enantiomer (*R. secalis*) was the more active compound, but in all cases the racemate showed high level of activity.

Table 3.2-1: Efficacy of BAS 750 F (LS 5834378) and the enantiomers LS 5934591 and LS 5934588 against various cereals diseases

Disease	Untreated control [% infection]	application rate BAS 750 F (racemate) [g a.i./ha]	% efficacy		
				application rate of enantiomers: 50% of racemate	
			BAS 750 F (racemate)	LS 5934591 (R)-enantiomer	LS 5934588 (S)-enantiomer
Puccinia triticina	81	150	100	100	82
		37.5	100	100	11
		9	98	98	0
Blumeria graminis	75	150	99	100	67
		37.5	97	99	17
		9	91	82	0
Zymoseptoria tritici (azole shifted)	39	300	92	93	39
		75	75	61	6
		18.8	0	10	0
Zymoseptoria tritici (azole sensitive)	55	300	100	100	72
		75	97	100	25
		18.8	82	81	16
Rhynchosporium secalis	50	150	94	74	100
		37.5	81	50	87

CA 3.3 Effects on Harmful Organisms

BAS 750 F is active against different fungal stages on and in the plant. When applied protectively, BAS 750 F inhibits further pathogen development after germination of fungal spores. Due to its ability to enter into the leaf, its further translocation as well as its high intrinsic activity, it can also control fungal stages that have already become established in deeper tissue layers. BAS 750 F is thus suitable for preventative and curative treatments.

CA 3.4 Field of Use Envisaged

Agriculture

CA 3.5 Harmful Organisms Controlled and Crops or Products Protected or Treated

BAS 750 F containing products are under development to control a broad range of important fungal diseases in several crops worldwide. As a most representative major crop / pest combination for Europe, the control of *Septoria tritici* (*Zymoseptoria tritici, Mycosphaerella graminis*) in wheat is described. Efficacy evaluation for further uses in cereals and other crops are currently under development or evaluation and will be addressed with the individual biological assessment dossiers for the corresponding product evaluations.

CA 3.6 Mode of Action

According to the fungicide mode of action classification of the Fungicide Resistance Action Committee (FRAC), BAS 750 F is a fungicide belonging to the group of the sterol biosynthesis inhibitors (SBI, mode of action class G). Within the SBIs, it belongs to the sub group of demethylation inhibitor (DMI, G1) and the chemical group of triazoles. Due to its unique isopropanol moiety, it will be proposed to belong to a new sub-group of triazole fungicides, the isopropanol azoles.

The primary mode of action of DMIs is the blocking of ergosterol biosynthesis through inhibition of cytochrome P450 sterol 14α-demethylase (CYP51). The depletion of ergosterol and accumulation of non-functional 14α-methyl sterols results in inhibition of growth and cell membrane disruption.

CA 3.7 Information on Occurrence or Possible Occurrence of the Development of Resistance and Appropriate Management Strategies

Mechanism of Resistance

Three major resistance mechanisms are associated with changes in DMI-sensitivity:

- Mutations in the target gene (cyp51), as described e.g. for *Zymoseptoria tritici* (Leroux *et al.* 2006, Stammler *et al.* 2008), *Puccinia triticina* (Stammler *et al.* 2009) and *Phakopsora pachyrhizi* (Schmitz *et al.* 2013).
- Overexpression of the target protein, as described e.g. for *Z. tritici* (Cools *et al.* 2012), *P. pachyrhizi* (Schmitz *et al.*) and *Blumeriella jaapii* (Ma *et al.* 2006), *P. triticina* (Stammler *et al.* 2009).
- Reduced intracellular accumulation of DMIs by overexpression of efflux-pumps, as described e.g. for *Z. tritici* (Leroux and Walker 2011) and *Botrytis cinerea* (Kretschmer *et al.* 2009, Grabke and Stammler 2014).

Various mutations in the target gene have been shown to have different effects on different DMIs (Fraaije *et al.* 2007, Stammler *et al.* 2008). Target gene mutations can be combined and accumulated resulting in higher levels of resistance (Cools and Fraaije 2013). In addition, target site overexpression and/or enhanced efflux, although rare in the field, can also be found simultaneously in a small number of isolates (Stammler and Semar 2011, Cools and Fraaije 2013, Strobel *et al.* 2014).

The accumulation of different resistance mechanisms results in a quantitative (directional) type of resistance and thus changes in the sensitivity of a population are gradual over time.

Evidence of Resistance

Some pathogens have shown a shift towards lower sensitivity in the period since DMI introduction. For several plant pathogenic fungi, the situation has stabilized after a period of adaptation (FRAC 2015).

European DMI sensitivity monitoring has been intensified for *Z. tritici* since 2003 following the occurrence and identification of QoI resistance in this pathogen in Western Europe. A shift to a reduced sensitivity towards different DMIs has been shown with isolates taken from the most important cereal-growing regions in Western Europe (FRAC 2015, Strobel *et al*. 2014).

Mutations and combinations of mutations in the target gene, and to a much lesser extent also enhanced efflux and target protein overexpression, can be linked to the sensitivity changes observed (Cools and Fraaije 2013).

Isolates belonging to different cyp51-haplotypes showed variation in their sensitivity response to different DMIs, such that the correlation of sensitivity between various DMIs can be low or even in some cases negative (Stammler and Semar 2011). This was confirmed from frequency analyses of cyp51-haplotypes in the field after various DMI applications, which showed that DMIs select cyp51-haplotypes differently (Fraaije *et al.* 2007, Stammler *et al.* 2008).

Sensitivity changes observed in the laboratory do not always correlate with DMI efficacy observed in the field, this is due to other factors such as application timing, weather conditions and disease pressure (Mehl *et al.* 2010, Strobel *et al.* 2014).

Despite sensitivity changes measured in microtiter plates, some DMIs at registered dose rates have shown reliable field performance against *Z. tritici* throughout the past decade, whereas the efficacy of some other DMIs has significantly decreased (Defra 2007, Strobel *et al.* 2014).

There are several examples in various crops (*e.g.* apples, cereals) and pathogenic fungi (*e.g. Venturia inaequalis, Blumeria graminis, Z. tritici*) where newly introduced DMIs gave improved performance against various already "shifted" pathogens and where the new DMIs were less affected by resistance mechanisms prevalent at the time of market launch.

Sensitivity data

It is now more than 40 years since the first DMI fungicides were introduced into the European market for the control of various pathogens in a large number of crops. As many field populations of plant pathogens have adapted to DMIs over time they no longer can be considered to reflect the "wild type" or "baseline" sensitivity of the population before the introduction of DMIs.

As a result, sensitivity studies nowadays cannot be seen to reflect the baseline of a "wildtype" population but show the current sensitivity situation against an adapted population from the field. Therefore, the sensitivity of old wild type isolates obtained from internal or external fungal culture collections which are maintained in the lab cannot be used for a realistic analysis of the present situation.

What is important is to establish whether the current field population of *Z. tritici* is sufficiently controlled with registered field rates of the applied product. For BAS 750 F this has been clearly documented (see BAS 750 01 F Document MCP Section 3; BASF DocID 2016/1000846). Moreover, efficacy studies with a range of highly adapted strains of *Z. tritici* were done in the glasshouse confirming the high level of efficacy of BAS 750 F against these isolates (Figure 3.7-1).

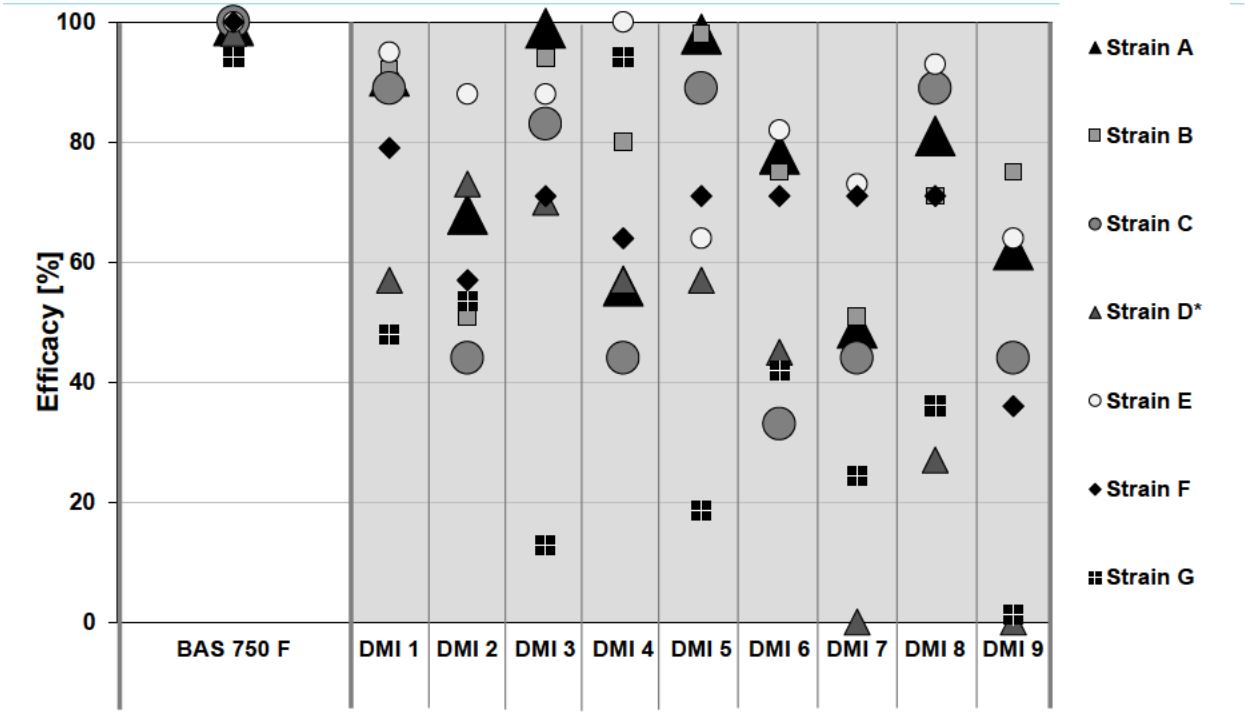


Figure 3.7-1: **Efficacy of BAS 750 F versus commercially used DMI compounds against highly adapted strains of *Zymoseptoria tritici* in the glasshouse (inoculation one day after application; 33% of the registered dose rate). * Strain D = MDR-phenotype.**

Broad European field monitoring for BAS 750 F was started in 2014. The frequency distribution of isolates with different sensitivities to BAS 750 F, metconazole and epoxiconazole are shown in Figure 3.7-2. The test method was a microtiter assay as previously published (Stammler *et al.* 2008) with tests carried out by the external company Epilogic (Freising, Germany). Isolates of *Z. tritici* were randomly sampled over several European countries, mainly from intensive wheat growing areas and from commercial sites. Data show that sensitivity of the European population in 2014 towards BAS 750 F is much higher than towards epoxiconazole and metconazole. Analyses for 2015 populations are ongoing.

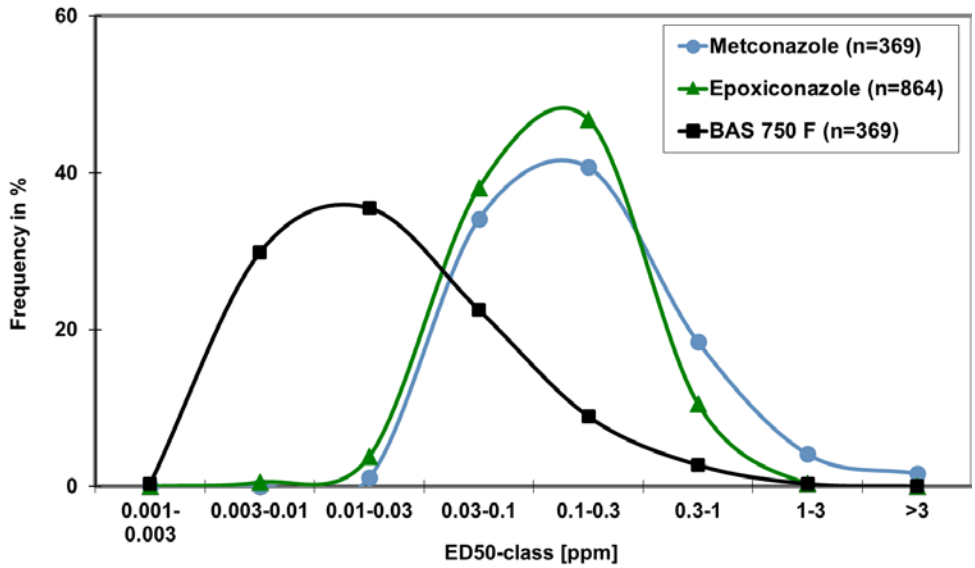


Figure 3.7-2: **Sensitivity of European populations of *Zymoseptoria tritici* from 2014 towards BAS 750 F (square), epoxiconazole (triangle) and metconazole (dot). Method was a microtiter test, ED_{50} was determined by Probit analysis and classification was done according to the concentrations used in the test.**

Cross-resistance

Within the SBI group, there is no cross-resistance between DMIs (G1), amines/morpholines (G2), keto reductase inhibitors (G3) and squalene reductase inhibitors (G4) (FRAC 2015).

A sensitivity analysis (microtiter plate assays) with a high number of European isolates from 2014 showed that the sensitivity towards BAS 750 F does not correlate with the sensitivity to SDHIs. (Figure 3.7-3)

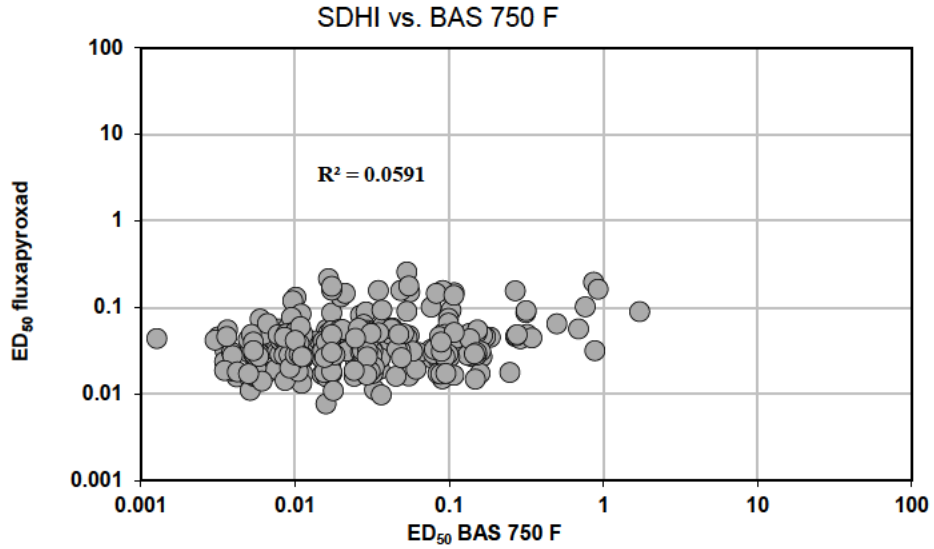
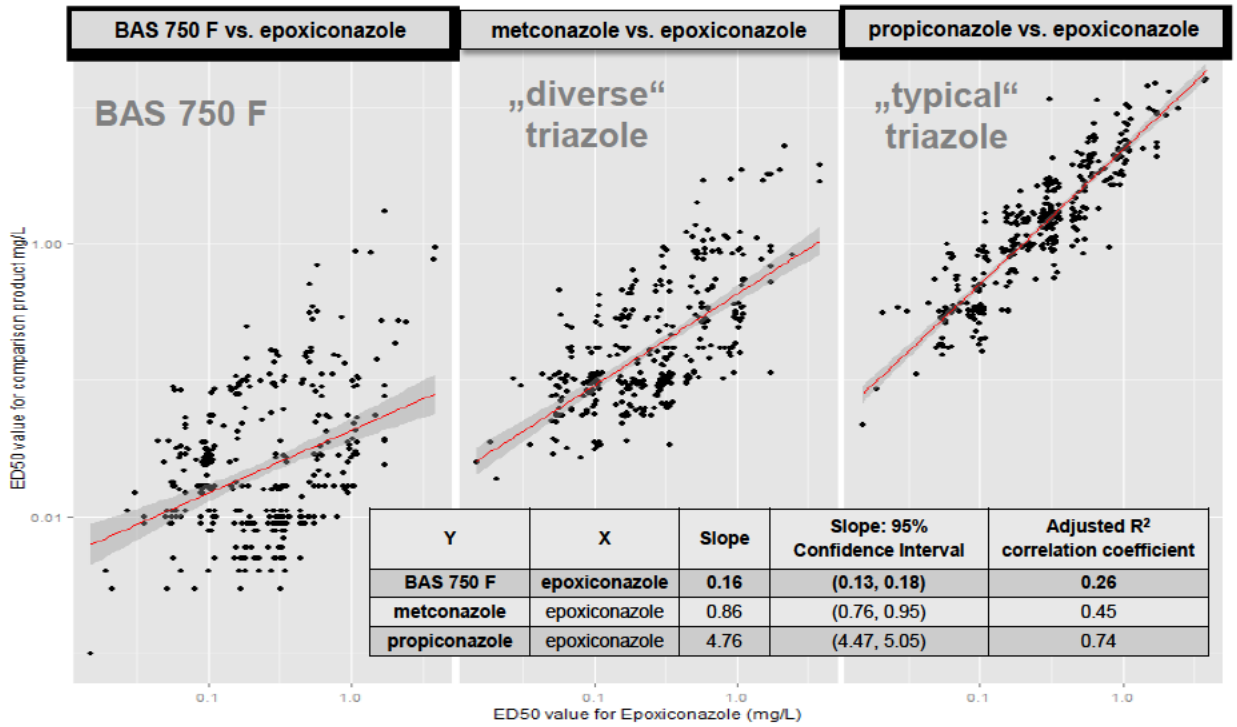


Figure 3.7-3: Correlation of the sensitivity of a representative isolate European collection from 2014 (n=359) of *Zymoseptoria tritici* to BAS 750 F and the SDHI fluxapyroxad. Data show that sensitivities do not correlate.

Studies on cross-resistance between different DMIs indicated that a clear statement was not possible. Such studies were carried out with different pathogens and different DMIs. There are DMIs where in cross-resistance studies there were good correlations of the sensitivities to *Z. tritici*, but in other cases correlation between different DMIs showed a low to moderate relationship, especially when the sensitivities of imidazoles and triazoles were correlated (Stammler and Semar 2011). These observations confirm older reports on other fungal pathogens that complete cross-resistance within DMI fungicides is not always observed (Leroux *et al.* 2000, 2006, Kendall *et al.* 1986, Steva *et al*. 1990).

Although BAS 750 F is a triazole, and in general cross-resistance to other triazole compounds exists, the correlation of sensitivities of *Z. tritici* compared with other DMIs was found to be much lower than with other comparisons (Figure 3.7-4). Whether this was related to the unique isopropanol group of BAS 750 F is currently under further investigation.



Y	X	Slope	Slope: 95% Confidence Interval	Adjusted R^2 correlation coefficient
BAS 750 F	**epoxiconazole**	**0.16**	**(0.13, 0.18)**	**0.26**
metconazole	epoxiconazole	0.86	(0.76, 0.95)	0.45
propiconazole	epoxiconazole	4.76	(4.47, 5.05)	0.74

Figure 3.7-4: Correlations of ED_{50} values for epoxiconazole vs. BAS 750 F (left), metconazole (middle) or propiconazole (right) in a representative European isolate collection of *Zymoseptoria tritici* (n=359).

Each data point depicts the calculated ED_{50} value for a specific isolate, when tested with the respective substances. For each graph a linear regression has been fitted; the results of this are summarized in the table. BAS 750 F has the shallowest estimated slope of the three linear regressions fitted. This indicates that for each milligram extra of epoxiconazole per liter required to achieve the ED_{50} level for a given isolate, only an estimated 0.16 milligrams per liter of BAS 750 F would be required to achieve the same level. For metconazole and propiconazole, the estimated increases are 0.86 and 4.76 milligrams per liter respectively. Substances, which have a higher resistance are required to be applied in larger doses, in order to achieve the same level of inhibition. From this, it can be inferred that there is less resistance to BAS 750 F, compared to epoxiconazole. Metconazole has a similar resistance to epoxiconazole and propiconazole has shown a higher relative resistance. The correlation coefficient between BAS 750 F and epoxiconazole is only 0.26, compared with 0.45 for metconazole and 0.74 for propiconazole. This shows a weaker relationship between the two substances than for the other two pairs analyzed, which are more similar to epoxiconazole in their effect on the sample of isolates.

Resistance risk associated with unrestricted use pattern

Fungicide resistance risk: FRAC describes the DMI fungicides in general as medium-risk compounds, according to the principles described in FRAC Monographs 1 and 2 (Brent 1995, Brent and Hollomon 1998).

Pathogen resistance risk: FRAC classified a number of plant pathogenic fungi in classes with a low, medium and high resistance risk. This list is the result of an evaluation of the FRAC member companies. *Z. tritici* is seen as a medium risk pathogen (Table 3.7-1).

Table 3.7-1: Plant pathogens accepted as showing a medium risk of development of resistance to fungicides (after FRAC, FRAC 2015). *Z. tritici* is marked in bold.

Pathogen	Crop	Disease
Alternaria solani	potato, tomato	early blight
Ascochyta pisi	peas	Ascochyta blight
Bipolaris maydis	maize	leaf blight
Bremia lactucae	lettuce	downy mildew
Cercospora beticola	sugar beet	leaf spots
Cercospora kikuchii	peanuts, beans, various	leaf blight
Cercospora sojina	soybean	frogeye leaf spot
Colletotrichum gloeosporoides	various	anthracnose
*Erysiphe necator**	grapevine	powdery mildew
*Gibberella fujikuori**	rice	bakanae
Leveillula taurica	pepper	powdery mildew
Microdochium nivale	cereals, turf	snow mold
Monilinia spp.	various	blossom and fruit rot
Mycosphaerella graminicola (Zymoseptoria tritici)	**wheat**	**leaf spot**
Mycosphaerella musicola	banana	yellow sigatoka
Mycosphaerella pinodes	pea	blight, purple spot
Mycovellosiella nattrassii	eggplant	leaf mold
Oculimacula spp.	wheat/barley	eyespot
Penicillium digitatum	various	green mold
Penicillium expansum	various	blue mold
Peronospora spp.	various	downy mildews
Pestalotiopsis longiseta	tea, various	grey blight
Phyllosticta citricarpa	citrus	black spot
Phytophthora infestans	potato/tomato	late blight
Pseudoperonospora humuli	hops	downy mildew
Pyrenophora teres	barley	net blotch
Pyrenophora tritici-repentis	wheat	tan spot
Sclerotinia homoeocarpa	turf, various	dollar spot
Setosphaeria turcica	maize	Northern leaf blight
Sphaerotheca macularis	strawberry, various	powdery mildew
Stemphylium vesicarium	asparagus	purple spot
Venturia carpophila	stone fruits, almonds	scab
Venturia cerasi	cherry	scab
Venturia nashicola	Chinese pear	scab
Venturia pirina	pear	scab

** The EPPO Guideline lists these pathogens as high risk pathogens of which baseline sensitivity is normally requested*

Combined resistance risk: The combined risk of the BAS 750 F and *Z. tritici* is visualized in Figure 3.7-5. This model is proposed by EPPO in a new and updated version (revision 2012-09, EPPO 2012) of the original paper (EPPO 2003). It shows the resistance risk of a fungicide and a pathogen under unrestricted use of the fungicide for pathogen control.

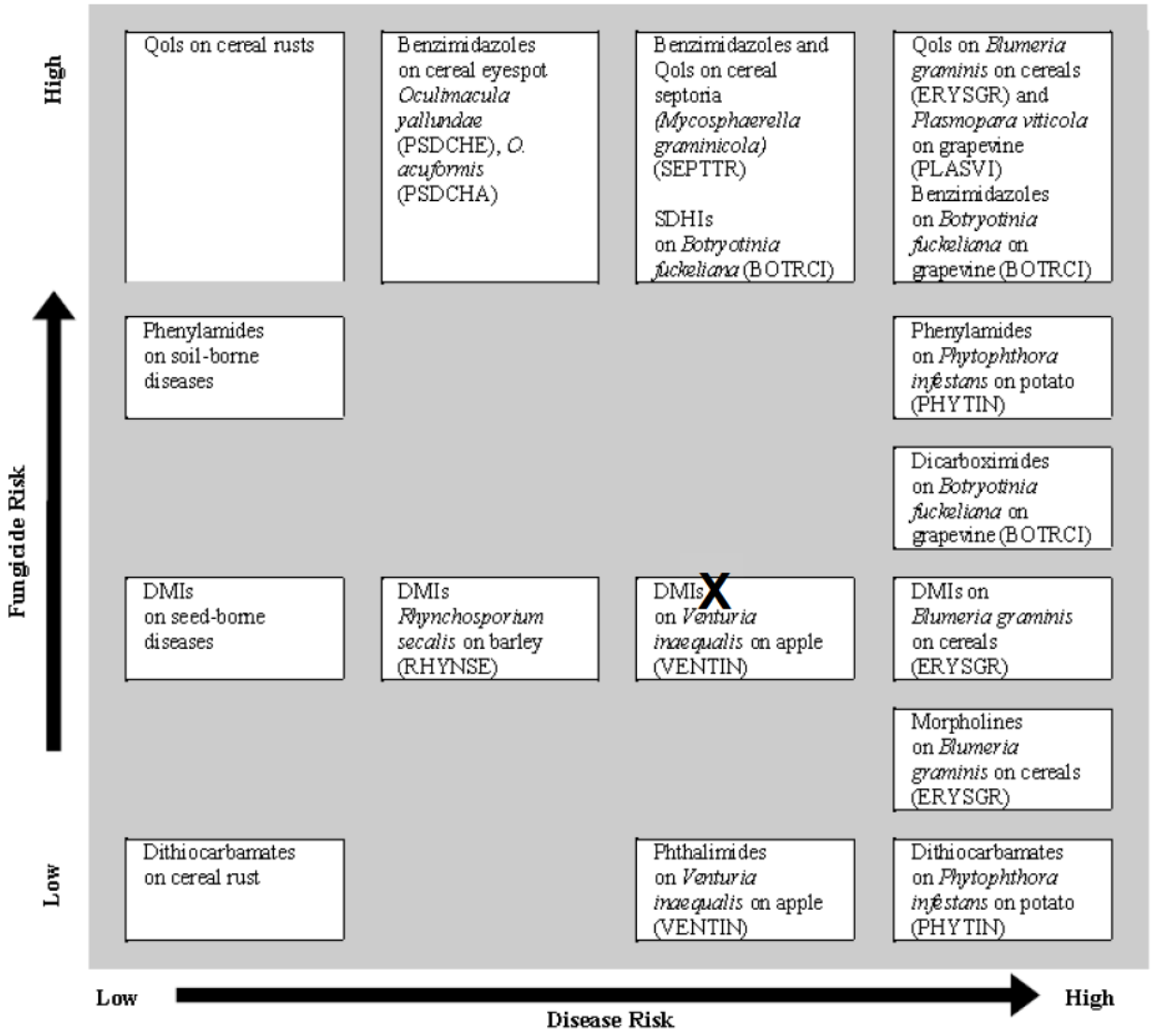


Figure 3.7-5: Combined resistance risk analysis according to EPPO. The "X" indicates the resistance risk of BAS 750 F and *Z. tritici* under unrestricted use

The resistance risk analysis indicates combined resistance risk for BAS 750 F and *Z. tritici* under unrestricted use is medium. Modifiers for reduction of the resistance risk are described.

Appropriate Management Strategies

- Resistance Management shall focus on reduction of selection pressure.
- BASF will conduct a broad sensitivity monitoring of *Z. tritici* in all EPPO climatic zones with focus on “Maritime” and “North East”
- Good agricultural practice leads to less infection pressure (e.g. phytosanitary measurements, cultivation of less susceptible varieties, appropriate crop cultivation unfavourable for the target pathogens).
- Limiting the number of sprays is also an important factor in delaying the build-up of resistant pathogen populations.
- A further tool is the use of fungicide mixtures. Recent studies showed that especially mixtures help in delaying the selection of resistance (Hobbelen *et al.* 2013, 2014, van den Bosch *et al.* 2014). A sound resistance management approach is provided by the combination of mixing partners with a good *Z. tritici* activity.
- Since population size of pathogens is lower at disease onset than when already established in the field, selection pressure is less when using preventive applications rather than curative or eradicative spray schemes. An optimal timing is also an effective resistance management (van den Berg *et al.* 2013).
- Follow FRAC guidelines of the SBI-Working Group

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CA 3.8 Methods and Precautions Concerning Handling, Storage, Transport or Fire

Report: CA 3.8/1
Anonymous, 2016 b
Safety data sheet - BAS 750 F
2016/1052696
Guidelines: EEC 1907/2006
GLP: no

Handling and Storage

Precautions for safe handling
No special measures necessary if stored and handled correctly. Ensure thorough ventilation of stores and work areas. When using do not eat, drink or smoke. Hands and/or face should be washed before breaks and at the end of the shift.
Protection against fire and explosion:
Avoid dust formation. Dust can form an explosive mixture with air. Prevent electrostatic charge - sources of ignition should be kept well clear - fire extinguishers should be kept handy.

Conditions for safe storage, including any incompatibilities
Segregate from foods and animal feeds.
Further information on storage conditions: Keep away from heat. Protect against moisture.
Protect from direct sunlight.
Protect from temperatures below: 4 °C
Changes in the properties of the product may occur if substance/product is stored below indicated temperature for extended periods of time.
Protect from temperatures above: 25 °C
Changes in the properties of the product may occur if substance/product is stored above indicated temperature for extended periods of time.

Specific end use(s)
For the relevant identified use(s) listed in Section 1 the advice mentioned in this section 7 is to be observed.

Transport

Land transport

ADR

UN number	UN3077
UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains TRIAZOLE DERIVATIVE)
Transport hazard class(es):	9, EHSM
Packing group:	III
Environmental hazards:	yes
Special precautions for user:	Tunnel code: E

RID

UN number	UN3077
UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains TRIAZOLE DERIVATIVE)
Transport hazard class(es):	9, EHSM
Packing group:	III
Environmental hazards:	yes
Special precautions for user:	None known

Inland waterway transport

ADN

UN number	UN3077
UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains TRIAZOLE DERIVATIVE)
Transport hazard class(es):	9, EHSM
Packing group:	III
Environmental hazards:	yes
Special precautions for user:	None known

Transport in inland waterway vessel
Not evaluated

Sea transport

IMDG

UN number:	UN 3077
UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains TRIAZOLE DERIVATIVE)
Transport hazard class(es):	9, EHSM
Packing group:	III
Environmental hazards:	yes
Marine pollutant:	YES
Special precautions for user:	None known

Air transport
IATA/ICAO

UN number:	UN 3077
UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains TRIAZOLE DERIVATIVE)
Transport hazard class(es):	9, EHSM
Packing group:	III
Environmental hazards:	yes
Special precautions for user:	None known

Transport in bulk according to Annex II of MARPOL and the IBC Code

Regulation:	Not evaluated
Shipment approved:	Not evaluated
Pollution name:	Not evaluated
Pollution category:	Not evaluated
Ship Type:	Not evaluated

Further information
Caution - substance not yet fully tested.

Fire

Extinguishing media
Suitable extinguishing media: water spray, dry powder, foam
Unsuitable extinguishing media for safety reasons: carbon dioxide

Special hazards arising from the substance or mixture:
carbon monoxide, carbon dioxide, nitrogen oxides
The substances/groups of substances mentioned can be released in case of fire.

Advice for fire-fighters
Special protective equipment:
Wear self-contained breathing apparatus and chemical-protective clothing.

Further information:
Keep containers cool by spraying with water if exposed to fire. In case of fire and/or explosion do not breathe fumes. Collect contaminated extinguishing water separately, do not allow to reach sewage or effluent systems. Dispose of fire debris and contaminated extinguishing water in accordance with official regulations.

CA 3.9 Procedures for Destruction or Decontamination

Waste treatment methods

For purposes of disposal, combustion of BAS 750 F or its pesticide products in a licensed incinerator is recommended. This method of disposal applies also to contaminated packages, which cannot be cleaned or reused.

Although it is possible to incinerate the product at lower temperatures, combustion at approximately 1100°C with a residence time of about 2 seconds is advised.
By doing so, i.e., operating the incinerator according to the conditions laid down in council directive 94/67/EEC resp. directive 2000/76/EC of the European Parliament, one will achieve complete combustion and minimize the formation of undesired by-products in the off-gases.

Contaminated packaging:
Contaminated packaging should be emptied as far as possible and disposed of in the same manner as the substance/product.

Decontamination methods

Report:	CA 3.9/1 Collet K., 2014 a Adsorption of BAS 750 F onto activated charcoal 2015/1001401
Guidelines:	<none>
GLP:	no

Principle of method

Organic substances can be adsorbed onto the surface of activated charcoal. The efficiency of adsorbance can be evaluated by means of the adsorption isotherm according to FREUNDLICH. Experiments have been conducted to demonstrate the feasibility of water decontamination using activated charcoal.

Experimental conditions

- Initial conditions: 20-50 mg/L Dissolved Organic Carbon (DOC; alternatively a suitable peak of the UV-spectrum of the organic compound can be used for the determination of concentration, e.g. at DOC << 20 mg/l). DOC concentrations < 5 mg/l require single substance analysis by means of GC and HPLC or by means of more sensitive summary parameters like SAC (Spectral Adsorption Coefficient) or AOX (Absorbable Organic Halides (X)).
- Filter for DOC evaluation and for the separation of the loaded activated carbon: membrane filter of polycarbonate type with a pore width of 0.45 µm.

- Weighed amounts of activated carbon (Chemviron F300, 40 – 100 µm): 2.5, 5, 12.5, 25, 50, 100, 150, 250, 500, 1000, 2000, 4000, 8000 and 16000 mg/l. According to the initial concentration resp. water solubility of the organic species 10 different amounts of the activated carbon in the lower, middle or upper range should be chosen. Alternations of these amounts are possible.
- Highly purified water for HPLC etc.: fully deionized and final passage through activated carbon column.
- Double measurement of 100 ml solution volume each.
- Shaking flask: 100 ml shaking glass bottles.
- Shaking device: IKA Labortechnik Type KS 250 basic (180 min-1 frequency)
- Perkin Elmer 550 UV/VIS Spectrophotometer
- Standard adsorption time: ≥ 24 hrs

Evaluation of the adsorption experiment

Because the solubility of BAS 750 F proved to be very poor (3.7 mg/l, pH = 7) after 14 days of stirring in high purified water, evaluation of the adsorption isotherm by means of the DOC or single substance determination by HPLC was not feasible. Analysis of these parameters is not possible regarding the detection limit of 0.1 mg/l DOC and the uncertainty of the HPLC-Determination at the given concentration range.
However looking at the low solubility of BAS 750 F and the hydrophobic structure of the molecule, a good absorbability of the soluble part of the compound onto activated carbon can be foreseen based on experiences with other molecules of comparable polarity.

Conclusion

BAS 750 F, dissolved in neutral water, is to be classified as efficiently absorbable onto activated charcoal.

CA 3.10 Emergency Measures in Case of an Accident

Report: CA 3.10/1
Anonymous, 2016 b
Safety data sheet - BAS 750 F
2016/1052696
Guidelines: EEC 1907/2006
GLP: no

Personal precautions, protective equipment and emergency procedures
Avoid dust formation. Use personal protective clothing. Avoid contact with the skin, eyes and clothing.

Environmental precautions
Do not discharge into the subsoil/soil. Do not discharge into drains/surface waters/groundwater.

Methods and material for containment and cleaning up
For small amounts: Contain with dust binding material and dispose of.
For large amounts: Sweep/shovel up.
Avoid raising dust. Dispose of absorbed material in accordance with regulations. Collect waste in suitable containers, which can be labeled and sealed. Clean contaminated floors and objects thoroughly with water and detergents, observing environmental regulations.

Description of first aid measures
Remove contaminated clothing.
If inhaled: Keep patient calm, remove to fresh air, and seek medical attention.
On skin contact: Wash thoroughly with soap and water.
On contact with eyes: Wash affected eyes for at least 15 minutes under running water with eyelids held open.
On ingestion: Immediately rinse mouth and then drink 200-300 ml of water, seek medical attention.

Most important symptoms and effects, both acute and delayed
Symptoms: The most important known symptoms and effects are described in the labelling section
Further important symptoms and effects are so far not known.

Indication of any immediate medical attention and special treatment needed
Treatment: Treat according to symptoms (decontamination, vital functions), no known specific antidote.

We create chemistry

BAS 750 F

Document M-CA, Section 4

ANALYTICAL METHODS

Compiled by:

[redacted]

Telephone: [redacted]
E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 4 ANALYTICAL METHODS

CA 4.1 Methods used for the generation of pre-approval data

CA 4.1.1 Methods for the analysis of the active substance as manufactured

(a) Determination of the pure active substance in the active substance as manufactured and specified in the dossier submitted in support of approval under Regulation (EC) No 1107/2009

Report:	CA 4.1.1/1 Bentz A., 2013 a Analytical method APL0669/01 - Determination of the active ingredient Reg.No.5834378 in Reg.No. 5834378 TGAI 2013/1140545
Guidelines:	none
GLP:	no

Report:	CA 4.1.1/2 Bentz A.,Harsch M., 2013 a Validation of the analytical method APL0669/01: Determination of the active ingredient Reg.No. 5834378 in Reg.No. 5834378 TGAI 2013/1140546
Guidelines:	OECD Principles of Good Laboratory Practice, GLP Principles of the German Chemikaliengesetz (Chemicals Act), 2004/10/EC, EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, CIPAC Guidelines on method validation, SANCO/3030/99 rev. 4 (11 July 2000), US EPA OPPTS Harmonized Test Guideline 830.1000, US EPA OPPTS Harmonized Test Guideline 830.1800
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the methods

The active ingredient content in BAS 750 F TGAI (technical grade active ingredient) is quantified by using method APL0669/01. The samples are analyzed using a high pressure liquid chromatographic procedure that employs UV detection and external standardization.

Method parameters (according to DocID 2013/1140545)

<table>
<tr><th colspan="2">HPLC parameters</th></tr>
<tr><td>Column</td><td>Aquity BEH C18, 50 mm x 2.1 mm, 1.7 µm (or equivalent type)</td></tr>
<tr><td>Mobile phase</td><td>Solvent A: 1000 mL water + 0.5 mL formic acid
Solvent B: 1000 mL acetonitrile + 0.5 mL formic acid</td></tr>
<tr><td>Column temperature</td><td>40 °C</td></tr>
<tr><td>Injection volume</td><td>1 µL</td></tr>
<tr><td>Detection</td><td>230 nm</td></tr>
<tr><td>Gradient</td><td>
<table>
<tr><th>Time [min]</th><th>Solvent A [%]</th><th>Solvent B [%]</th><th>Flow [mL/min]</th></tr>
<tr><td>0.00</td><td>53</td><td>47</td><td>0.7</td></tr>
<tr><td>2.00</td><td>53</td><td>47</td><td>0.7</td></tr>
<tr><td>2.01</td><td>0</td><td>100</td><td>0.7</td></tr>
<tr><td>3.00</td><td>0</td><td>100</td><td>0.7</td></tr>
<tr><td>3.01</td><td>53</td><td>47</td><td>0.7</td></tr>
<tr><td>4.00</td><td>53</td><td>47</td><td>0.7</td></tr>
</table>
</td></tr>
<tr><td>Retention time</td><td>Reg.No. 5834378: approx. 1.3 min</td></tr>
<tr><td>Total running time</td><td>4 min</td></tr>
</table>

Identity
The identity was confirmed by comparison of the retention time, the UV-spectra, IR-spectra and MS-spectra of the analyte (Reg.No. 5834378) in the reference item and the analyte (Reg.No. 5834378) in the test item. The retention time as well as the spectra were found to be identical.

Specificity
The specificity of the method was demonstrated by HPLC/UV, HPLC/MS and IR-spectroscopy. It was verified by comparing the retention time, UV-spectra, IR-spectra and the corresponding MS- fragmentation of the given reference item with that of the test item. There were no indications of interferences due to other components.

Linearity
Linearity was measured using the reference item Reg.No. 5834378 in a series of five concentrations.

range:	86 – 312 mg/L (ca. 50 - 150 % of the nominal concentration)
slope (m):	8855533
y-axis intercept (b):	397542
correlation factor:	1.0000

Accuracy
Not required for the active substance.

Precision (Repeatability)
Precision is calculated as the relative standard deviation of five individual sample weights of the test item. Each sample solution was injected twice. The acceptability of the % RSD values (relative standard deviation) for precision was proved by the Horwitz equation, an exponential relationship between the inter laboratory relative standard deviation (RSDR) and concentration C (expressed as decimal fraction):

$$\%RSDR = 2^{(1-0.5\log C)}$$

which is modified for the estimation of repeatabilities (RSDr internal laboratory) to:

$$\%RSDr = \%\ RSDR \times 0.67$$

Horwitz results for the repeatability test with BAS 750 F TGAI:

Item	**nominal conc. [%]**	**corresp. conc. `C`**	**%RSDR Horwitz (Inter Lab. RSD)**	**%RSDr Horwitz (Intra Lab. RSD)**	**%RSD analyzed**	**%RSD accepted**
BAS 750 F	99.387	0.99387	2.002	1.341	0.364	yes

No outliers or stragglers were determined.

CONCLUSION
The present study has shown that the conditions employed in the analytical HPLC method APL0669/01 are suitable for the quantification of Reg.No. 5834378 in technical grade active ingredient BAS 750 F (TGAI).

(b) Determination of significant and relevant impurities and additives (such as stabilisers) in the active substance as manufactured

CONFIDENTIAL information - data provided separately (Document J)

CA 4.1.2 Methods for risk assessment

(a) Methods in soil, water, sediment, air and any additional matrices used in support of environmental fate studies

Soil

Report:	CA 4.1.2/1 Studenroth S.,Lueer D., 2015 a Validation of analytical method L0214/01 for the determination of BAS No. 750 F (Reg.No. 5834378) and metabolites of Reg.No. 5924326 and 1,2,4-Triazole (Reg.No. 87084) in soil by LC-MS/MS 2015/1039006
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 850.7100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: A 5 g soil sample is extracted with a mixture of acetonitrile/water (70/30, v/v) for 30 min on a mechanical shaker at 225 rpm. After centrifugation (10 min at 4000 rpm) an aliquot of 10 mL is taken (extract 1) and the remaining supernatant is decanted and discarded. The same extraction procedure is repeated once and after centrifugation a second aliquot of 10 mL (extract 2) is combined with extract 1 and thoroughly mixed. Residues of Reg.No. 5834378 (BAS 750 F) and metabolite Reg.No. 5924326 are directly analysed by LC-MS/MS. For analysis of Reg.No. 87084 (1,2,4-Triazole), 5 mL of the combined extracts 1 and 2 are transferred into a tared glass tube and the volume is reduced in a nitrogen evaporator to a volume less than 1 mL (confirmation by weighing, assuming a density of 1 g/cm3). The concentrated extract is filled up to a volume of 1 mL with ultra-pure water and analysed by LC-MS/MS. For the investigation of Reg.No. 5834378 (BAS 750 F) and metabolite Reg.No. 5924326 an Aquasil C-18 column is used. Reg. No. 87084 is investigated on two different columns, a Hypercarb and a Synergy Hydro-RP column. For all analyses a water/acetonitrile gradient is used with 0.1 % formic acid as modifier.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the table below.

Table 4.1.2-1: Validation results of method L0214/01: Determination of Reg. No. 5834378 (BAS 750 F), Reg. No. 5924326 and Reg. No. 87084 (1,2,4-Triazole) in soil

Test substance	Soil type	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				**398 → 182**	**398 → 133**	**398 → 182**	**398 → 133**
Reg. No. 5834378 (BAS 750 F)	Lufa 2.2	0.002	5	102	98	1.6	2.1
		0.02	5	102	102	1.4	1.0
	Lufa 2.3	0.002	5	109	107	2.4	3.2
		0.02	5	96	96	12.1	15.0
Transition				**288 → 159**	**288 → 103**	**288 → 159**	**288 → 103**
Reg. No. 5924326	Lufa 2.2	0.002	5	101	98	2.3	3.7
		0.02	5	102	103	1.6	2.7
	Lufa 2.3	0.002	5	99	99	11.2	7.3
		0.02	5	82	82	2.7	4.1
Transition				**70 → 43 (Hypercarb)**	**70 → 43 (Synergy)**	**70 → 43 (Hypercarb)**	**70 → 43 (Synergy)**
Reg. No. 87084 (1,2,4-triazole)	Lufa 2.2	0.002	5	102	93	3.1	0.5
		0.02	5	88	97	7.5	2.5
	Lufa 2.3	0.002	5	88	95	8.4	5.6
		0.02	5	85	96	3.7	5.6

Linearity: Good linearity (r > 0.995) was observed in the range of 0.025 ng/mL to 3.0 ng/mL for the two mass transitions of Reg.No. 5834378 and metabolite Reg.No. 5924326 and in the range of 0.125 ng/mL to 15 ng/mL for the one mass transition of metabolite Reg. No. 87084. At least seven calibration points were determined.

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required for BAS 750 F and Reg. No. 5924326, because the analysis is possible at two different mass transitions. For Reg. No. 87084 a second LC column with different chemistry was used as confirmatory technique.

Matrix effects: It was demonstrated that the matrix-load in the tested matrix-matched standards of Reg.No. 5834378 had an influence on the detection of Reg. No. 5834378 (BAS 750 F) which could not be neglected (up to 21 %). No matrix effect was observed for both metabolites Reg. No. 5924326 and Reg. No. 87084.

Interference: The method determined residues of Reg.No. 5834378 and its metabolites Reg.No. 5924326 and Reg.No. 87084 in soil. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions considered.

Limit of quantitation: The limit of quantitation is defined by the lowest fortification level successfully tested, hence 0.002 mg/kg, corresponding to a concentration of 0.125 ng/mL for Reg. No. 5834378 and Reg. No. 5924326 and 0.625 ng/mL for Reg. No. 87084 in the soil extract.

Limit of detection: The limit of detection is estimated as 20% of the limit of quantitation, equivalent to 0.0004 mg/kg, corresponding to a concentration of 0.025 ng/mL Reg. No. 5834378 and Reg. No. 5924326 and 0.125 ng/mL Reg. No. 87084 in the soil extract.

Repeatability: The relative standard deviations (RSD, %) for all fortification levels were below 20%.The detailed values are shown in Table 4.1.2-1.

Reproducibility: Reproducibility of the method was not determined within this validation study.

Standard stability: Standard solutions of Reg.No. 5834378 and Reg.No. 5924326 in acetonitrile were stable (≤ 10% decline) for 43 days when stored at 4 ± 2°C. Standard solutions of Reg.No. 87084 in ultra-pure water were stable (≤ 10% decline) for 31 days when stored at 4 ± 2°C.

Extract stability: The mean recovery values of Reg.No. 5834378 found during experiments ranged between 90% and 107%. The corrected mean recovery values (time zero recovery values were set as 100% reference value) of the metabolites Reg.No. 5924326 and Reg.No. 87084 found during the experiments ranged between 91% to 111% (uncorrected mean values ranged between 89% and 106%). This demonstrates that Reg.No. 5834378 and its metabolites Reg.No. 5924326 and Reg. No. 87084 were stable in the soil extracts over the tested time period of at least 7 days.

Extractability The extractability of BAS 750 F residues from selected soil samples from metabolism studies was addressed in the BASF Study [see KCA 7.1.2.2.1/5 2015/1182724]. The extraction procedures of the soil/metabolism study and of the residue analytical method were compared. Results showed that the extractability was similar for both extraction schemes ranging between 91.4% and 108%.

Conclusion: **It could be demonstrated that method L0214/01 fulfills the requirements with regard to specificity, linearity, repeatability, limit of quantitation and recoveries and is therefore applicable to correctly determine residues of Reg.No. 5834378 (BAS 750 F) and its metabolites Reg.No. 5924326 and Reg.No. 87084 (1,2,4-Triazole) in both soil types.**

The following method was submitted with the re-registration dossier of BAS 555 F (Metconazole). This is included in the present chapter as supplementary information for supporting the storage stability study of 1,2,4-Triazole in section KCA 7.1 (DocID 2015/1204922). In this study, samples were analyzed using BASF method L0203/01. Therefore, just the 1,2,4-Triazole data is relevant for BAS 750 F and the executive summary of the study is confined only to its results.

Report: CA 4.1.2/2
Geschke S., 2014 a
Validation of an analytical method for determination of BAS 555 F (Metconazole) and its metabolite 1,2,4-(1H)-Triazole in soil
2013/1377001
Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010)
GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method

The analytical method L0203/01 for the determination of 1,2,4-(1H)-triazole (Reg. No. 87084) in soil, was validated at Eurofins Agroscience Services EcoChem GmbH, Niefern-Öschelbronn, Germany.
A 5 g soil sample is extracted twice with a mixture of acetonitrile/water (70/30, v/v). After centrifugation, the extracts are combined and the residues of 1,2,4-(1H) triazole are analyzed by LC-MS/MS monitoring three mass transitions of 1,2,4-(1H)-triazole.

Recovery findings

The method proved to be suitable to determine 1,2,4-(1H)-triazole in soil. Samples were fortified with the analytes at the limit of quantification of 0.002 mg kg^{-1} and 10 times higher (0.02 mg kg^{-1}). For 1,2,4-(1H)-triazole residues were detectable in blank specimens, so blank correction in the recovery data, for one of three mass transitions, was needed.
Mean recovery values (mean of five replicates per fortification level and analyte) were between 79% and 105% (see table below), which fulfils the legal requirements.

Table 4.1.2-2: Summary of the recovery data in soil matrices

Soil	Analyte	m/z	Forti-fication Level [mg kg^{-1}]	Number of replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Soil (L120313)*	1,2,4-(1H)-triazole Reg.No. 87084	70 → 28	0.002	5	79	18	88	16
			0.02	5	97	6		
		70 → 43	0.002	5	87	19	89	14
			0.02	5	92	6		
		70 → 70	0.002	5	92	15	95	12
			0.02	5	98	7		
Soil (L120317)*		70 → 28	0.002	5	95	8	91	9
			0.02	5	87	8		
		70 → 43	0.002	5	105	16	100	13
			0.02	5	94	3		
		70 → 70	0.002	5	84	19	90	14
			0.02	5	95	5		

RSD = Relative standard deviations
* soil originate from a field dissipation study (DocID 2015/1000221)

Linearity

Good linearity ($r \geq 0.999$) was observed in the range of 0.03 ng mL^{-1} to 10 ng mL^{-1} for the quantifier and qualifier mass transitions of 1,2,4-(1H)-triazole ($r \geq 0.997$).
Standards used for calibration curves were prepared in acetonitrile/water (70/30, v/v).

Specificity

Significant interferences (> 30% of LOQ) were observed at the retention time and for one mass transition considered for 1,2,4-(1H)-triazole. Therefore, interferences in the control samples were determined and blank correction in the recovery data was needed for this mass transition.
Due to the high selectivity and specificity of LC-MS/MS, monitoring at least two mass transitions, an additional confirmatory technique was not necessary.

Matrix Effects

To check possible ion enhancement or suppression effects in HPLC/MS-MS analysis, final extracts from soil blank samples were spiked with defined concentrations of 1,2,4-(1H)-triazole. Results demonstrated that the matrix load in the tested soil samples had no significant influence on the analyte analysis. Therefore, calibration was performed with standards in acetonitrile/water (70:30, v/v).

Limit of Quantification	The limit of quantification (LOQ) is defined by the lowest fortification level successfully tested. It is 0.002 mg kg^{-1} for 1,2,4-(1H)-triazole.
Limit of Detection	The limit of detection (LOD) was estimated at 30% of the LOQ; equivalent to 0.0006 mg kg^{-1} 1,2,4-(1H)-triazole, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 20%.
Stability of Working Solutions	Stock solutions of 1,2,4-(1H)-triazole were stable (less than 10% decline) for at least 4.5 months refrigerated. The stock solutions were prepared within an ongoing parallel field study.
Extract Stability	Mean recovery values of the soil extracts after a storage period of 7 days at 1-10°C (nominally) in the dark are within a range of 70-120% recovery, hence soil extracts were considered stable for this period of time.
Reproducibility	Reproducibility of the method was not determined within this validation study.
Conclusion	**The analytical method L0203/01 for analysis of 1,2,4-(1H)-triazole in soil uses LC-MS/MS for final determination, with a limit of quantification of 0.002 mg kg^{-1}.** **It could be demonstrated that the method fulfils the requirements with regard to specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine 1,2,4-(1H)-triazole in soil.**

Water

Report: CA 4.1.2/3
Malinsky D.S., 2016 a
Validation of analytical method D1506/01: Method for the determination of Mefentrifluconazole (BAS 750 F, Reg. No.5834378) and its metabolites M750F003 (Reg. No.5924326), M750F005 (Reg. No.6003433), M750006 (Reg. No.5863469), M750F007 (Reg. No.6003432) and M750F008 (Reg. No.6010286) in surface and drinking water by LC-MS/MS
2015/7001125

Guidelines: EPA 850.6100, SANCO/3029/99 rev. 4 (11 July 2000), SANCO/825/00 rev. 8.1 (16 November 2010)

GLP: yes
(certified by United States Environmental Protection Agency)

Principle of the method

The analytical method D1506/01 is developed for the determination of BAS 750 F and its metabolites M750F003 (Reg. No. 5924326), M750F005 (Reg. No. 6003433), M750F006 (Reg. No. 5863469), M750F007 (Reg. No. 6003432) and M750F008 (Reg. No. 6010286) in surface and ground water by LC-MS/MS.
The water samples are diluted with acetonitrile, mixed, filtered and then analyzed by direct injection onto a high performance liquid chromatography (HPLC) column with detection by positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring the following ion transitions: m/z 398→70 and 400→70 for the parent compound BAS 750 F; m/z 288→70 for M750F003; m/z 380→70 for M750F005; m/z 356→259 and 356→217 for M750F006; m/z 338→241 for M750F007 and m/z 356→259 and 356→241 for M750F008. In lieu of secondary (alternate) ion transitions for M750F003, M750F005 and M750F007, confirmatory analysis is performed using a different LC-MS/MS column (C18 and phenyl column options are available). The results are calculated by direct comparison of the sample peak responses to those of external standards.

Recovery findings

The results show that the method is suitable to determine residues of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in water. Samples spiked with the analytes at the limit of quantification of 30 ng/L and ten times higher (300 ng/L) have overall recovery values (mean of five replicates per fortification level, matrix and analyte) between 83% and 121%. The detailed results are given in the table below (Table 4.1.2-3).

Table 4.1.2-3: Results of the method validation for the determination of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in surface and ground water

Matrix	Analyte	m/z	Fortification level [ng/L]	Number of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	Overall RSD [%]
Surface water	BAS 750 F	398→70	30	10	93	18	94	15
			300	10	95	12		
		400→70	30	10	77	13	83	14
			300	10	89	11		
	M750F003	288→70	30	10	93	7	99	15
			300	10	105	18		
		288→70	30	10	98	7	99	7
			300	10	101	6		
	M750F005	380→70	30	10	104	15	108	15
			300	10	112	15		
		380→70	30	10	100	6	104	9
			300	10	108	11		
	M750F006	356→259	30	10	98	9	102	8
			300	10	105	7		
		356→217	30	10	90	15	94	14
			300	10	98	12		
	M750F007	338→241	30	10	112	10	115	13
			300	10	117	16		
		338→241	30	10	102	7	103	7
			300	10	105	7		
	M750F008	356→259	30	15	92	13	97	18
			300	15	103	19		
		356→241	30	15	92	19	94	17
			300	15	95	15		
Drinking water	BAS 750 F	398→70	30	10	91	9	93	13
			300	10	96	15		
		400→70	30	10	93	18	95	15
			300	10	98	12		
	M750F003	288→70	30	9	107	16	102	17
			300	10	99	19		
		288→70	30	10	97	7	96	9
			300	10	95	10		
	M750F005	380→70	30	5	76	21	83	18
			300	5	89	15		
		380→70	30	15	98	18	108*	18*
			300	10	123	7		
	M750F006	356→259	30	10	102	8	103	9
			300	10	105	10		
		356→217	30	10	91	15	95	14
			300	10	99	13		
	M750F007	338→241	30	10	75	14	80	19
			300	10	86	20		
		338→241	30	15	99	13	106*	13*
			300	10	116	5		
	M750F008	356→259	30	10	97	16	95	16
			300	10	94	16		
		356→241	30	10	90	15	98	17
			300	10	105	15		

RSD = Relative standard deviation
*Matrix-Matched Standards were used.

Linearity	Acceptable linearity of $r \geq 0.9876$ was observed in the range of 0.006 ng/mL to 0.3 ng/mL.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the analytes in water. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions of the analytes. According to the method, at least one mass transition was used and confirmatory analysis was performed using a different LC-MS/MS column (C18 and phenyl column options are available).
Matrix effects	Matrix effects were tested preparing matrix-matched standards for each matrix and analyte. It was shown that the matrix-load in the tested matrix-matched standards of M750F005 and M750F007 in the matrix ground water had an influence on the detection. Therefore, matrix-matched standards are needed for further experiments.
Limit of quantification	The method has a limit of quantification (LOQ) of 30 ng/L per analyte, corresponding to the lowest fortification level.
Limit of detection	The limit of detection (LOD) is 6 ng/L for each analyte, corresponding to 20% of the LOQ.
Repeatability	The overall relative standard deviations (RSD) for all matrices, analytes and fortification levels were ≤ 24%.
Standard stability	The stability of the analytes in standard solutions has been determined. The storage stability data indicate that stock and fortification solutions of each analyte prepared in acetonitrile are stable for at least 3 months when held under refrigeration and in the dark, and that calibration standards prepared in acetonitrile / water (20/80, v/v) are stable for at least 1 month when held under refrigeration and in the dark.
Final volume stability	All analytes were considered to be stable in final volumes of surface and ground water samples over a time period of 5 days, when stored refrigerated and in the dark.
Reproducibility	Reproducibility of the method was determined within an independent laboratory validation study summarized in section CA 4.2 [see KCA 4.2/9 2015/7006199].

Conclusion

The method for analysis of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in water uses LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of BAS 750 F and its metabolites in surface and ground water.

Report: CA 4.1.2/4
Penning H. et al., 2013 a
Validation of analytical method L0199/01 for the determination of 1,2,4-Triazole (Reg.No. 87084) in water by LC-MS/MS
2012/1297158

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 850.7100

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: A 2 mL water sample is given on an SPE column. The column is washed with water and the received filtrate is evaporated to dryness in the nitrogen evaporator at 45°C. Subsequently, the residue is dissolved in 0.5 mL water. The concentration of 1,2,4-triazole is measured by HPLC-MS/MS.

Recovery findings: In surface and ground water, the mean recovery values for LC-MS/MS determination were between 70% and 120% for all fortification levels and all mass transitions. The detailed results are given in the table below.

Table 4.1.2-4: Validation results of method L0199/01: Determination of 1,2,4-triazole in surface and ground water

Test substance / Matrix	HPLC column	Fortification level (mg/kg)	No of tests	Average recovery (%)	Relative standard deviation (%)
Transition				**70 → 43**	**70 → 43**
1,2,4-triazole / surface water	Hypercarb	0.05	5	97.2	14
		0.5	5	90.7	3.2
	Aquasil C18	0.05	5	94.1	3.0
		0.5	5	95.5	1.1
1,2,4-triazole / ground water	Hypercarb	0.05	5	87.2	13
		0.5	5	95.0	4.0
	Aquasil C18	0.05	5	97.2	1.7
		0.5	5	97.2	2.6

Linearity: Good linearity ($r > 0.999$) was observed in the range of 0.05 ng/mL to 5 ng/mL for the measurements of 1,2,4-triazole on both HPLC columns. At least seven calibration points were determined. Calculation of results was based on peak area measurements using a calibration curve with internal standard (1,2,4- triazole, stable isotope labelled).

Specificity:	To confirm the determination of 1,2,4-triazole, the validation was successfully performed with two different chromatographic columns.
Matrix effects:	The method L0199/01 used an internal standard (stable isotope labelled 1,2,4-triazole) for quantitation. Any influence by the matrix carried with the samples affected the analyte as well as the internal standard in the same way. Therefore, the matrix does not influence the analytical results.
Interference:	The method determined 1,2,4-triazole in water. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions considered.
Limit of quantitation:	The limit of quantitation is defined as the lowest fortification level. The LOQ is 0.05 µg/kg, corresponding to a concentration of 0.2 ng/mL in the filtrates.
Limit of detection:	The limit of detection is 0.013 µg/kg, corresponding to a concentration of 0.05 ng/mL in the filtrates.
Repeatability:	The relative standard deviations (RSD, %) for all fortification levels were below 20%.The detailed values are shown in Table 4.1.2-4.
Reproducibility:	Reproducibility of the method was not determined within this validation study.
Standard stability:	Standard solutions of 1,2,4-triazole were found stable in water for at least 30 days (less than 10% decline), when stored refrigerated.
Extract stability:	The mean recovery values found during the experiment ranged between 97.5 and 100.5%. This demonstrates that 1,2,4-triazole was stable in the SPE-filtrates of ground water over the tested time period of 7 days.
Conclusion:	**It could be demonstrated that method L0199/01 fulfills the requirements with regard to specificity, linearity, repeatability, limit of quantitation and recoveries and is therefore applicable to correctly determine residues of 1,2,4-triazole in surface and ground water.**

Air

Report: CA 4.1.2/5
Obermann M.,Studenroth S., 2015 a
Validation of analytical method L0327/01, for the determination of BAS 750 F in air by LC-MS/MS
2015/1111330

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4, EPA 850.6100, OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: The test item BAS 750 F is spiked onto the front filter of an adsorbent tube (ORBO^{TM}), air is passed over the filter and the test item is extracted from the adsorbent material with acetonitrile. The residues are extracted and reconstituted in acetonitrile/water (70/30, v/v) before final determination.
The samples are analyzed by LC-MS/MS at mass transition 398 → 182 for quantification and 398 → 133 for confirmation (ESI, positive mode). Analysis was accomplished on an Aquasil C18 column applying an acetonitrile / water gradient using 0.1% formic acid as modifier.

Recovery findings: In the tested matrix air, the mean recovery values for BAS 750 F were between 70% and 110% for both fortification levels and mass transitions. The detailed results are given in the table below.

Table 4.1.2-5: Results of the method validation for the determination of BAS 750 F in air

Analyte	Matrix Air	m/z	Fortification level [ng/L air]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
BAS 750 F	Orbo^{TM} adsorbent	398 → 182	0.01 0.1	5 5	89 89	2.5 2.8	89	2.5
		398 → 133	0.01 0.1	5 5	88 90	2.1 2.2	89	2.5

RSD = Relative standard deviation

Linearity: Good linearity (r > 0.995) was observed in the range of 0.05 ng/mL to 5 ng/mL for the two mass transitions of BAS 750 F. At least seven calibration levels, prepared in acetonitrile/water (70/30, v/v), were injected.

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.

Matrix effects:	The matrix load in the tested matrix-matched standards had no influence on the analysis of BAS 750 F.
Interference:	Significant interferences (> 30% of LOQ) were not observed at the retention time and mass transitions of the analyte.
Limit of quantitation:	The method has a limit of quantitation (LOQ) of 0.01 ng/L air, corresponding to an amount of 5 ng active substance per 540 L air.
Limit of detection:	The limit of detection (LOD) for BAS 750 F is 0.002 ng/L air.
Repeatability:	The relative standard deviations (RSD, %) for all fortification levels were < 20%. The detailed values are shown in Table 4.1.2-5.
Reproducibility:	Reproducibility of the method was not tested within the validation study.
Stability of working solutions:	Stock and fortification solutions, prepared in acetonitrile, as well as calibration solutions, prepared in acetonitrile/water (70/30, v/v), of BAS 750 F were stable for at least 30 days, when stored refrigerated at 4°C.
Stability on Adsorber Material:	BAS 750 F was considered stable on the adsorber material over a time period of 7 days, when stored refrigerated at 4°C.
Conclusion:	**It could be demonstrated that the method L0327/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in air.**

(b) Methods in soil, water and any additional matrices used in support of efficacy studies

No stand-alone validation of analytical methods was required in support of efficacy studies.

(c) Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicological studies

Since BAS 750 F is not classified as toxic or very toxic, methods of analysis for parent or metabolites in human body tissues or fluids are not required according to SANCO/825/00 rev. 8.1. However, an analytical method for BAS 750 F in plasma is available. This method was validated and used in toxicological studies [see KCA 4.1.2/15, 2015/1186912] with a LOQ of 0.05 mg/L. For tissues, the analytical method L0272/01 can be used [see KCA 4.1.2/24 2015/1106707]. Methods for concentration control in feed or other matrices are reported in this section, when a stand-alone validation was carried out. If the method was used and validated within the study, then, this can be found along with the respective toxicological studies.

Report: CA 4.1.2/6
Baltussen E., 2013 a
Development and validation of an analytical method for the analysis of BAS 750 F in diet
2015/1189151

Guidelines: SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Ministry of Health, Welfare and Sport, The Hague, The Netherlands)

Principle of the method: 10 g of the blank powder diet are extracted 3 times by shaking at 225 rpm for 30 minutes with 30 mL 1% formic acid in acetonitrile. The solutions are filtered through a 589/1 black ribbon filter (Whatman, Dassel, Germany). The three extracts are combined into one volumetric flask of 100 mL per sample. The volumetric flasks are filled up to the mark with 1% formic acid in acetonitrile. The solutions are filtered through a 0.2 µm Spartan 30/0.45 RC filter (Whatman, Dassel, Germany) and diluted in a 1:1 (v:v) ratio with water and analysed with UPLC-UV at 210 nm. Analysis is accomplished on an Acquity UPLC BEH Shield RP-18 column applying an acetonitrile / water gradient using 0.1% trifluoroacetic acid as modifier.

Recovery findings: In rodent diet, the mean recovery values for UPLC-UV determination were between 80% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-6: Recovery results of BAS 750 F in rodent diet

Matrix	Approx. Fortification level[1] [mg/kg]	n	Recovery [%]	Average recovery [%]	RSD [%]
Ground Kliba rat/mouse maintenance diet "GLP"	100	5	87, 94, 97, 104, 106	98	7.8
	750	5	92, 92, 93, 80, 89	89	6.1
	750 (repeat)	5	82, 82, 92, 91, 92	88	6.2

Linearity: Good linearity ($r > 0.9992$) was observed in the range of 0.1 mg/L to 50 mg/L for BAS 750 F. At least five calibration levels, prepared in acetonitrile/water (50/50, v/v) with 0.5 % formic acid as modifier, were injected.

Specificity: The identification and quantitation were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in the rodent diet. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: No significant matrix interferences were observed in the investigated control extract at the expected retention time of the analytes of interest.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analytes of interest.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for standard powder rodent diet is 10 mg/kg for BAS 750 F, which corresponds to a concentration in the extract of 1 mg/L.

Limit of detection: The lowest calibration standard of 0.1 mg/L for BAS 750 F is defined as limits of detection of the analytical method, corresponding to 1 mg/kg.

Repeatability: The relative standard deviations (RSD, %) for rodent diet at all fortification levels were well below 20%. The detailed values are shown in Table 4.1.2-6.

Reproducibility: Reproducibility of the method was not tested within the validation study.

Stability of stock solutions: The coefficient of variation on the response factors of the calibration solutions prepared with fresh and stored stock solutions in acetonitrile was 1.3%. Since the value was ≤ 10% the stock solutions were stable when stored at room temperature for at least 6 days.

Extract stability: Since the mean recovery of the frozen samples was within 10% of the freshly analysed samples, the samples were stable when stored in the freezer for at least one day.

Table 4.1.2-7: Recovery results of BAS 750 F in rodent diet after storage of 1 day

Matrix	Approx. Fortification level[1] [mg/kg]	n	Recovery [%]	Average recovery [%]
Rodent diet	10	5	92, 96	94
	750	5	90, 96	93

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in rodent diet.**

Report:	CA 4.1.2/7 Baltussen E., 2016 a Report Amendment Number 1: Development and validation of an analytical method for the analysis of BAS 750 F in diet 2016/1041496
Guidelines:	SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)
GLP:	yes (certified by Ministry of Health, Welfare and Sport, The Hague, The Netherlands)

Additional recovery data for a fortification level of 10 ppm was added. The recovery results for BAS 750 F at a concentration of 10 ppm are summarized in the following table.

Table 4.1.2-8: Recovery results of BAS 750 F in rodent diet at LOQ (10 ppm)

Matrix	Approx. Fortification level[1] [mg/kg]	n	Recovery [%]	Average recovery [%]	RSD [%]
Ground Kliba rat/mouse maintenance diet "GLP"	10	12	86, 100, 94, 102, 90, 107, 96, 100, 87, 87, 88, 93	94	7.0

Report: CA 4.1.2/8
Becker M.,Kamp H., 2015 i
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in diet using HPLC-UV
2015/1174512
Guidelines: SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 10 g of the sample are extracted 3 times with 30 mL 1% formic acid in acetonitrile for 30 minutes using a laboratory shaker. After centrifugation at 4500 rpm for 5 min the supernatants are collected in a 100 mL volumetric flask and filled up to volume with extraction solution. The solutions are filtered through a 0.2 µm cellulose filter and analysed with HPLC-UV at 210 nm. Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile / water gradient using 0.1% trifluoroacetic acid as modifier.

Recovery findings: In rodent diet, the mean recovery values for UPLC-UV determination were between 90% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-9: Recovery results of BAS 750 F in rodent diet

Matrix	Approx. Fortification level[1] [mg/kg]	n	Recovery [%]	Average recovery [%]	RSD [%]
Rodent diet	30	5	94.0, 93.8, 94.0, 97.5, 97.9	95.4	2.2
	250	5	102, 103, 98.1, 101, 97.7	101	2.5
	6000	5	102, 103, 101, 101, 104	102	1.3

Linearity: Good linearity (r > 0.999) was observed in the range of 2 mg/L to 20 mg/ L for BAS 750 F. At least seven calibration levels, prepared in matrix solution (matrix extracted with 1 % formic acid in acetonitrile as described above), were injected.

Specificity: The identification and quantitation were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in the rodent diet. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: No significant matrix interferences were observed in the investigated control extract at the expected retention time of the analytes of interest.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analytes of interest.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for mouse/rat diet is 30 mg/kg for BAS 750 F, which corresponds to a concentration in the extract of 3 mg/L.

Limit of detection: The lowest calibration standard of 2 mg/L for BAS 750 F is defined as limits of detection of the analytical method, corresponding to 20 mg/kg.

Repeatability: The relative standard deviations (RSD, %) for rodent diet at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-9.

Reproducibility: Reproducibility of the method was not tested within the validation study.

Stability of stock solutions: No stability experiments were performed during this study. However in study [see KCA 4.1.2/1 2015/1039006] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were necessary during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in rodent diet.**

Report: CA 4.1.2/9
Becker M.,Kamp H., 2015 j
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in diet using HPLC-UV
2015/1175541
Guidelines: SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 10 g (for the concentration range of 607 – 6249 mg/kg and 7591 – 37955 mg/kg) and 5 g (for the concentration range of 6250 – 7590 mg/kg) of the sample are extracted 3 times with 30 mL 1 % formic acid in acetonitrile for 30 minutes using a lab shaker. After centrifugation at 4500 rpm for 5 min the supernatants are collected in a 100 mL volumetric flask. The combined extracts are diluted with 1 % formic acid in acetonitrile to 100 mL. The solutions are filtered through a 0.2 µm cellulose filter and analysed with HPLC-UV at 210 nm. Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile / water gradient using 0.1% trifluoroacetic acid as modifier.

Recovery findings: In diet, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-10: Recovery results of BAS 750 F in quail/duck diet

Matrix	Approx. Fortification level [ppm]	n	Recovery [%]	Average recovery [%]	RSD [%]
Ground Kliba diet quail/duck "GLP"	607	5	96.1, 101, 99.1, 99.3, 102	99.4	2.2
	1250	5	106, 108, 108, 108, 103	107	2.2
	9996	5	103, 95.7, 101, 106, 105	102	3.8

RSD = relative standard deviation

Linearity: Good linearity (r > 0.999) was observed in the range of 0.305 mg/100 mL to 7.635 mg/100 mL for BAS 750 F. At least five calibration levels, prepared in matrix solution (matrix extracted with 1% formic acid in acetonitrile as described above), were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in quail/duck diet. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 607 mg/kg.

Limit of detection: The lowest calibration standard of 0.305 mg/100 mL for BAS 750 F is defined as limits of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the diet at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-10.

Reproducibility: Reproducibility of the method was not tested within the validation study.

Stability of stock solutions: No stability experiments were performed during this study. However in study 2015/1039006 [see KCA 4.1.2/1] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in diet formulations with Ground Kliba maintenance diet quail/duck "GLP" meal.**

Report: CA 4.1.2/10
Becker M.,Kamp H., 2015 a
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in diet using LC-MS/MS
2015/1175542

Guidelines: SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 10 g of the sample are extracted 3 times with 30 mL 1 % formic acid in acetonitrile for 30 minutes using a lab shaker. After centrifugation at 4500 rpm for 5 min the supernatants are collected in a 100 mL volumetric flask. The combined extracts are diluted with 1 % formic acid in acetonitrile to 100 mL. The solutions are filtered through a 0.2 µm cellulose filter and analysed with HPLC-MS/MS. Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile / water gradient using 0.005% formic acid as modifier.

Recovery findings: In rodent diet, the mean recovery values for HPLC-MS/MS determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-11: Recovery results of BAS 750 F in rodent diet

Matrix	Approx. Fortification level [ppm]	n	Recovery [%]	Average recovery [%]	RSD [%]
Ground Kliba rat/mouse maintenance diet "GLP"	10	5	97.6, 106, 105, 102, 101	102	3.3
	20	5	94.8, 100, 101, 101, 103	99.9	3.0
	30	5	101, 99.7, 99.8, 99.3, 103	101	1.6

RSD = relative standard deviation

Linearity: Good linearity (r > 0.999) was observed in the range of 499.0 ng/mL to 2994.0 ng/mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution (matrix extracted with 1 % formic acid in acetonitrile as described above), were injected and evaluated.

Specificity: The identification and quantification were based on the selected mass and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in rodent diet. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 10 mg/kg in the diet.

Limit of detection: The lowest calibration standard of 499.0 ng/mL for BAS 750 F is defined as limits of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the diet at all fortification levels were well below 5%. The detailed values are shown in Table 4.1.2-11.

Reproducibility: Reproducibility of the method was not tested within the validation study.

Stability of stock solutions: No stability experiments were performed during this study. However in study 2015/1039006 [see KCA 4.1.2/1] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in Ground Kliba maintenance diet mouse/rat "GLP" meal.**

Report: CA 4.1.2/11
Becker M.,Kamp H., 2015b
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in aqueous Carboxymethylcellulose (CMC) using HPLC-UV
2015/1177605

Guidelines: SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are transferred into appropriate volumetric flasks using approx. 10 mL of a mixture of acetonitrile/water (1 + 1, v/v) and filled up to volume with acetonitrile. If required, all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions are filtered through a 0.2 µm cellulose filter and analysed with HPLC-UV at 230 nm. Analysis is accomplished on a Chromolith Performance RP-18 column applying an acetonitrile / water gradient using 0.1% formic acid as modifier.

Recovery findings: In aqueous carboxymethylcellulose (CMC), the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-12: Recovery results of BAS 750 F in aqueous carboxymethylcellulose (CMC)

Matrix	Approx. Fortification level [mg/100 mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
CMC	10	5	101, 99.1, 102, 102, 98.9	101	1.5
	250	5	87.4, 99.1, 96.6, 97.2, 98.5	95.8	5.0
	10000	5	105, 105, 105, 106, 105	105	0.3
	25000	5	99.4, 100, 99.7, 99.7, 99.7	99.7	0.2

RSD = relative standard deviation

Linearity: Good linearity (r > 0.999) was observed in the range of 0.502 mg/100 mL to 5.02 mg/100 mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in acetonitrile) diluted with matrix solution (aqueous formulation, 1% CMC, w/v)], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in aqueous carboxymethylcellulose (CMC). As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 10 mg/100 mL in aqueous CMC formulations.

Limit of detection: The lowest calibration standard of 0.502 mg/100 mL for BAS 750 F is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the CMC formulations at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-12.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However in study [see KCA 4.1.2/1 2015/1039006] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in aqueous carboxymethylcellulose (CMC) formulations.**

Report: CA 4.1.2/12
Becker M.,Kamp H., 2015 c
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in a mixture of Dimethyl Sulfoxide and corn oil using HPLC-UV
2015/1185311
Guidelines: EEC 91/414, SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with acetone using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required, all dilutions are sonicated. The solutions are analysed with HPLC-UV at 230 nm. Analysis is accomplished on a Kinetex C18 column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In a mixture of dimethyl sulfoxide (DMSO) and corn oil, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-13: Recovery results of BAS 750 F in DMSO and corn oil

Matrix	Approx. fortification level [mg/mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
DMSO / corn oil	20	5	98.9, 98.5, 99.4, 99.6, 99.3	99.1	0.5
	100	5	98.2, 98.5, 98.5, 98.2, 98.2	98.3	0.2
	200	5	100, 98.7, 99.5, 99.1, 97.8	99.0	0.9

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 1.014 mg/100 mL to 10.140 mg/100 mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in acetone) diluted with matrix solution (DMSO/corn oil, 2+3, v/v)], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in a mixture of DMSO and corn oil. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 20 mg/mL in a mixture of DMSO and corn oil.

Limit of detection: The lowest calibration standard of 1.014 mg/100 mL for BAS 750 F is defined as the limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the test item in a mixture of DMSO and corn oil at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-13.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in a mixture of DMSO and corn oil (2+3, v/v).**

Report: CA 4.1.2/13
Becker M.,Kamp H., 2015 d
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in corn oil using HPLC-UV
2015/1184812
Guidelines: EEC 91/414, SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with acetone using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required, all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions are analysed with HPLC-UV at 230 nm. Analysis is accomplished on a Kinetex C18 column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In the vehicle corn oil, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-14: Recovery results of BAS 750 F in corn oil

Matrix	Approx. fortification level [g/100 mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
Corn oil	10	5	97.3, 98.5, 97.4, 96.9, 98.6	97.7	0.8
	35	5	98.5, 98.3, 97.6, 99.1, 98.8	98.4	0.6
	70	5	97.7, 98.1, 98.2, 98.3, 97.0	97.9	0.6

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 0.519 mg/100 mL to 10.38 mg/100 mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in acetone) diluted with matrix solution (corn oil)], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in corn oil. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 10 g/100 mL in corn oil.

Limit of detection: The lowest calibration standard of 0.519 mg/100 mL for BAS 750 F is defined as limits of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the test item in corn oil at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-14.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in corn oil.**

Report: CA 4.1.2/14
Becker M.,Kamp H., 2015 e
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in Dimethyl sulfoxide using HPLC-UV
2015/1184813
Guidelines: EEC 91/414, SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with acetonitrile using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required, all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions are analysed with HPLC-UV at 230 nm. Analysis is accomplished on a Chromolith Performance RP 18e column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In dimethyl sulfoxide (DMSO), the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-15: Recovery results of BAS 750 F in dimethyl sulfoxide

Matrix	Approx. fortification level [mg/100 mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
DMSO	50	5	102, 101, 101, 101, 103	101	0.7
	100	5	99.2, 98.8, 98.7, 99.7, 98.7	99.0	0.4
	150	5	101, 99.1, 100, 99.5, 103	101	1.3

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 0.488 mg/100 mL to 4.88 mg/100 mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in acetonitrile) diluted with matrix solution (DMSO)], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in DMSO. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 50 g/100 mL in DMSO.

Limit of detection: The lowest calibration standard of 0.488 mg/100 mL for BAS 750 F is defined as limits of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the test item in DMSO at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-15.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However, in study 2015/1039006 [see KCA 4.1.2/1] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in dimethyl sulfoxide.**

Report: CA 4.1.2/15
Becker M.,Kamp H., 2015 f
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in plasma using LC-MS/MS
2015/1186912

Guidelines: SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 30 µL of the study plasma are mixed with 270 µL acetonitrile in a plastic micro centrifuge tube (1.5 mL). After vortex mixing and protein precipitation, the samples are centrifuged at 13000 rpm for 5 min. The clear supernatant is used directly for LC-MS measurements. Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile/water gradient using 0.005% formic acid as modifier followed by LC-MS analysis.

Recovery findings: In rat plasma, the mean recovery values for HPLC-MS determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-16: Recovery results of BAS 750 F in rat plasma

Matrix	Approx. fortification level [ng/mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
Rat plasma	50	5	115, 103, 106, 111, 107	108	4.5
	1000	5	108, 111, 108, 108, 108	109	0.9
	10000	5	105, 103, 104, 104, 103	104	1.0

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 4.01 ng/mL to 75.15 ng/mL (low range) and 100.2 ng/mL to 1202.4 ng/mL (high range) for BAS 750 F. For each calibration range, at least six calibration levels, prepared in matrix solution (analogously as described in the principle of the method for the sample solution preparation) were injected and evaluated.

Specificity: The identification and quantification were based on the selected mass transition and the retention time. There was no peak present in the chromatogram corresponding to the analysis of the blank accuracy sample at the retention time and mass transition of the analyte. Therefore, under the described conditions the method is specific for the determination of BAS 750 F in rat plasma.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ, as no signal was observed at elution times of the analyte of interest in the blank sample.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 50 ng/mL in plasma.

Limit of detection: The lowest calibration standard of 4.01 ng/mL for BAS 750 F is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the diet at all fortification levels were well below 5%. The detailed values are shown in Table 4.1.2-16.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However in study 2015/1039006 [see KCA 4.1.2/1] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in rat plasma.**

Report: CA 4.1.2/16
Becker M.,Kamp H., 2015 g
BAS 750 F - Validation of an analytical method for the analysis of BAS 750 F in Paraffinum subliquidum using HPLC-UV
2015/1186913
Guidelines: EEC 91/414, SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with tetrahydrofuran using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required, all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions were analysed with HPLC-UV at 230 nm. Analysis is accomplished on a Chromolith Performance RP 18e column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In paraffinum subliquidum, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-17: Recovery results of BAS 750 F in paraffinum subliquidum

Matrix	Approx. fortification level [mg/g]	n	Recovery [%]	Average recovery [%]	RSD [%]
paraffinum subliquidum	100	5	100, 99.4, 99.3, 99.4, 98.9	99.4	0.4
	500	5	98.3, 99.6, 98.0, 97.5, 99.0	98.5	0.9
	600	5	97.8, 98.2, 99.1, 98.5, 97.6	98.3	0.6

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 2.048 mg/100 mL to 10.24 mg/100 mL for BAS 750 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in tetrahydrofuran) diluted with matrix solution (paraffinum subliquidum)], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of BAS 750 F in paraffinum subliquidum. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 100 mg/g in paraffinum subliquidum.

Limit of detection: The lowest calibration standard of 2.048 mg/100 mL for BAS 750 F is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the test item in paraffinum subliquidum at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-17.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run is always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantification, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in paraffinum subliquidum.**

Report: CA 4.1.2/17
Becker M.,Kamp H., 2015 k
Reg.No. 6011210 - Validation of an analytical method for the analysis of Reg.No. 6011210 in diet using HPLC-UV
2015/1188594
Guidelines: EEC 91/414, SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 10 g of the sample are weighed into a 50 mL polypropylene centrifuge tube with a screw cap. The sample is extracted 3 times with 30 mL extraction solution (1000 mL acetonitrile mixed with 20 mL formic acid) for 30 minutes using a lab shaker. After centrifugation at 4500 rpm for 5 min the supernatants are collected in a 100 mL volumetric flask. The combined extracts are diluted with acetonitrile to 100 mL. The solutions are filtered through a 0.2 µm cellulose filter and analysed with HPLC-UV at 234 nm. Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile/water gradient using 0.1% trifluoroacetic acid as modifier.

Recovery findings: In rodent diet, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-18: Recovery results of Reg. No. 6011210 in mouse /rat diet

Matrix	Approx. fortification level [mg/kg]	n	Recovery [%]	Average recovery [%]	RSD [%]
Mouse/ rat diet	50	5	102, 108, 107, 106, 107	101	2.0
	500	5	98.4, 103, 102, 98.9, 101	101	2.0
	5000	5	101, 102, 100, 103, 101	101	0.9

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 0.3078 mg/100 mL to 5.13 mg/100 mL for Reg. No. 6011210. At least seven calibration levels, prepared in matrix solution (matrix extracted with 2 % formic acid in acetonitrile as described above), were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of Reg. No. 6011210 in rodent diet formulations. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases.

Limit of Quantitation: The limit of quantification (LOQ) is assessed and confirmed at 50 mg/kg in the rodent diet.

Limit of Detection: The lowest calibration standard of 0.3078 mg/100 mL for Reg. No. 6011210 is defined as limits of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the diet at all fortification levels were well below 5%. The detailed values are shown in Table 4.1.2-18.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However in study 2015/1039006 [see KCA 4.1.2/1] standard solution of BAS 750 F in acetonitrile was stable for 43 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantification, and recoveries and is therefore applicable and suitable to correctly determine residues of Reg. No. 6011210 in formulations with Ground Kliba maintenance diet mouse/rat "GLP" meal.**

Report: CA 4.1.2/18
Hedrich R., 2015 a
Validation of an analytical method for the analysis of Reg.No. 6011210 in corn oil using HPLC
2015/1189154

Guidelines: OECD-DOC ENV/MC/CHEM(98)17 Paris 1998, EEC 91/414, SANCO/3029/99 rev. 4

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: 1.0 mL (electronic positive displacement pipette) sample material is filled into a 100 mL volumetric flask. The flask is filled to volume with tetrahydrofuran (THF) and mixed thoroughly. 75 µL of the corresponding THF solution are filled into a 10 mL volumetric flask and filled to volume with acetonitrile. The resulting solution is directly analysed with HPLC-UV at 250 nm. Analysis is accomplished on a Kinetex C18 column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In corn oil, the mean recovery values for UHPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-19: Recovery results of Reg. No. 6011210 in corn oil

Matrix	Approx. fortification level [mg/L]	n	Recovery [%]	Average recovery [%]	RSD [%]
Corn oil	200[1]	5	98.0, 99.3, 96.6, 102, 100	99.2	2.0
	400[2]	5	98.6, 99.1, 100, 101, 98.6	99.4	1.0
	800[3]	5	104, 99.6, 102, 101, 103	102	1.8

[1] corresponds to a concentration of approx. 15 mg/L after dilution
[2] corresponds to a concentration of approx. 30 mg/L after dilution
[3] corresponds to a concentration of approx. 60 mg/L after dilution
RSD = relative standard deviation

Linearity: Good linearity (r>0.995) was observed in the range of 4.028 mg/L to 70.49 mg/L for Reg. No. 6011210. At least seven calibration levels, prepared with a certain amount of standard stock solution (500 mg/L in acetonitrile), 75 µL matrix solution (10 mL/L in THF) and filled up to volume with acetonitrile. The adjusted matrix concentration covers the required dilution (dilution factor: 13333.3) of a typical sample nominal concentration of 400 g/L. The calibration solutions were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of Reg. No. 6011210 in corn oil. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for corn oil is 200 mg/kg for Reg. No. 6011210, which corresponds to a concentration in the extract of 15 mg/L.

Limit of detection: The lowest calibration standard of 4.028 mg/L for Reg. No. 6011210 is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for corn oil at all fortification levels were well below 5%. The detailed values are shown in Table 4.1.2-19.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However, in study 2015/1106707 [see KCA 4.1.2/24] standard solution of Reg. No. 6011210 (M750F022) in acetonitrile was stable for 91 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of Reg. No. 6011210 in corn oil.**

Report: CA 4.1.2/19
Becker M.,Kamp H., 2015 l
Reg.No. 6011210 - Validation of an analytical method for the analysis of Reg.No. 6011210 in Dimethyl sulfoxide using HPLC-UV
2015/1188599
Guidelines: SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with acetonitrile using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required, all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions are analysed with HPLC-UV at 250 nm. Analysis is accomplished on a Kinetex C18 column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In DMSO, the mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-20: Recovery results of Reg. No. 6011210 in DMSO

Matrix	Approx. fortification level [mg/100 mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
DMSO	5	5	99.6, 102, 101, 101, 100	101	0.9
	10	5	101, 99.2, 101, 101, 101	101	0.8
	20	5	99.7, 101, 101, 101, 101	101	0.6

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 0.506 mg/100 mL to 5.06 mg/100 mL for Reg. No. 6011210. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in acetonitrile) diluted with matrix solution (DMSO], were injected and evaluated.

Specificity: The identification and quantification were based on the selected wavelength and the retention time. Under the described conditions the method is specific for the determination of Reg. No. 6011210 in DMSO. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 5 mg/100 mL in DMSO.

Limit of detection: The lowest calibration standard of 0.506 mg/100 mL for Reg. No. 6011210 is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for DMSO at all fortification levels were well below 5%. The detailed values are shown in Table 4.1.2-20.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study. However in study [see KCA 4.1.2/24 2015/1106707] standard solution of Reg. No. 6011210 (M750F022) in acetonitrile was stable for 91 days when stored at 4 ± 2°C.

Extract stability: No stability experiments were performed during this study. As the duration of a complete analytical run was always below 24 hours.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of Reg. No. 6011210 in dimethyl sulfoxide.**

Report: CA 4.1.2/20
Mallat E., 2015 a
The validation of the determination of Reg.No. 6011210 in mouse EDTA plasma samples using LC-MS/MS
2015/1186930
Guidelines: <none>
GLP: yes
(certified by Ministry of Health, Welfare and Sport, The Hague, The Netherlands)

Principle of the method: M750F022 is extracted from mouse K2-EDTA plasma by liquid-liquid extraction. The extract is then evaporated under a stream of nitrogen. After reconstitution, the sample is injected into the chromatographic system. Chromatographic separation is performed on an XBridge phenyl column using gradient elution (mobile phase: 5% methanol in pure water and 500 mM ammoniumformate in pure water). An API 4000 tandem mass spectrometer equipped with a TIS probe operated in the multiple reaction monitoring (MRM9 in positive mode is used for quantification.

Recovery findings: In mouse EDTA plasma, the mean recovery values for HPLC-MS/MS determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-21: Recovery results of Reg. No. 6011210 in mouse EDTA plasma

Matrix	Approx. fortification level [ng/mL]	n	Recovery [%]	Average recovery [%]	RSD [%]
Mouse EDTA plasma	30	6	87.8, 86.6, 86.4, 85.6, 89.2, 109	90.7	9.8
	200	6	82.6, 90.0, 88.5, 79.9, 85.9, 79.6	84.4	5.2
	1600	6	102, 94.8, 97.4, 87.8, 82.4, 80.1	90.8	9.6

RSD = relative standard deviation

Linearity: Good linearity (r>0.997) was observed in the range of 10.0 ng/mL to 2000 ng/mL for Reg.No. 6011210. For each calibration range, at least nine calibration levels, prepared in matrix solution, were injected and evaluated. Calculation of results was based on peak area measurements using a calibration curve with internal standard (BAS 750 F).

Specificity:	The mass transition chosen is specific for the analyte Reg. No. 6011210 (m/z 364-309). The identification and quantification were based on the selected mass and the retention time. Under the described conditions the method is specific for the determination of Reg. No. 6011210 in mouse EDTA plasma. The method selectivity was evaluated by analyzing six different lots of blank EDTA plasma samples. The mean peak area of the blank EDTA plasma samples was compared to the mean peak area of the LLOQ QC samples prepared from the same six different lots of EDTA plasma as blank samples. No substantial interferences or from endogenous EDTA plasma components or from IS were observed in the first five blank matrices.
Matrix effects:	The method used an internal standard (BAS 750 F) for quantitation. Any influence by the matrix carried with the samples affected the analyte as well as the internal standard in the same way. Therefore, the matrix does not influence the analytical results.
Interference:	No significant interferences observed and no mutual interference between Reg.No. 6011210 and the internal standard (in 5 blank matrices).
Limit of quantitation:	The limit of quantitation is defined as the lowest fortification level, hence 30 ng/mL.
Limit of detection:	The lowest calibration standard of 10.0 ng/mL for Reg.No. 6011210 is defined as limit of detection of the analytical method.
Repeatability:	The relative standard deviations (RSD, %) at all fortification levels were below 10 %. The detailed values are shown in Table 4.1.2-21.
Reproducibility:	Reproducibility was not tested for this method.
Stability of solutions:	Stock solutions of Reg.No. 6011210 in acetonitrile were stable for 32 days at ≤ -18°C and fortification solutions of Reg.No. 6011210 and the internal standard work solution, both in acetonitrile, were stable for 31 days at 2-8 °C.
Stability of final volume:	It was found that extracts were stable for at least 103 hours in an autosampler set at 2-8 °C.
Conclusion:	**It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of Reg. No. 6011210 in mouse EDTA plasma.**

Report: CA 4.1.2/21
Becker M.,Kamp H., 2015h
BAS 750 01 F - Validation of an analytical method for the analysis of BAS 750 01 F in a mixture of acetone and olive oil using HPLC-UV
2015/1188600

Guidelines: SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: Samples are diluted completely with isopropanol using appropriate volumetric flasks to obtain sample solutions with test substance concentrations that match the calibration range. If required all dilutions are sonicated for 5 minutes to ensure a complete dissolution of the test substance. The solutions are analysed with HPLC-UV at 230 nm. Analysis was accomplished on a Kinetex C18 column applying an acetonitrile/water gradient using 0.1% formic acid as modifier.

Recovery findings: In acetone and olive oil. The mean recovery values for HPLC-UV determination were between 70% and 110% for all fortification levels. The detailed results are given in the table below.

Table 4.1.2-22: Recovery results of BAS 750 01 F in acetone and olive oil

Matrix	Approx. fortification level [mg/g]	n	Recovery [%]	Average recovery [%]	RSD [%]
Acetone and olive oil	5	5	97.6, 94.8, 97.0, 99.0, 99.1	97.5	1.8
	20	5	98.9, 97.0, 99.4, 98.1, 97.5	98.2	1.0
	60	5	99.8, 99.5, 99.1, 98.9, 99.5	99.4	0.3

RSD = relative standard deviation

Linearity: Good linearity (r>0.999) was observed in the range of 1.046 mg/100 mL to 10.460 mg/100 mL for BAS 750 01 F. At least seven calibration levels, prepared in matrix solution [stock solution (50 mg/100 mL test item in isopropanol) diluted with matrix solution consisting of acetone/olive oil (4+1, v/v)], were injected and evaluated.

Specificity: Under the described conditions the method is specific for the determination of BAS 750 01 F in acetone and olive oil. As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.

Matrix effects: Matrix-matched standards were used for evaluation of the results.

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analyte of interest.

Limit of quantitation: The limit of quantitation (LOQ) is assessed and confirmed at 5 mg/g in a mixture of acetone and olive oil.

Limit of detection: The lowest calibration standard of 1.046 mg/100 mL for BAS 750 01 F is defined as limit of detection of the analytical method.

Repeatability: The relative standard deviations (RSD, %) for the test item in a mixture of acetone and olive oil at all fortification levels were well below 5 %. The detailed values are shown in Table 4.1.2-22.

Reproducibility: Reproducibility was not tested for this method.

Stability of stock solutions: No stability experiments were performed during this study.

Extract stability: No stability experiments were performed during this study.

Conclusion: **It could be demonstrated that this method fulfills the requirements with regard to linearity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 01 F in acetone and olive oil (4+1, v/v).**

(d) Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

No stand-alone validated analytical methods for the determination of BAS 750 F were required for exposure studies.

(e) Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

Plant matrices

BAS 750 F

Report:	CA 4.1.2/22 Paula Jose W.F. de, 2015 a Validation of BASF Method Number L0076/09 for the determination of BAS 750 F in citrus (whole fruit), coffee (grain), dry beans (seed), soybeans (grain), tomato (whole fruit), wheat (grain) and wheat (straw) using LC-MS/MS 2015/3001681
Guidelines:	Resolucao RDC No. 4 - ANVISA (18/01/2012)
GLP:	yes (certified by Instituto Nacional de Metrologia, Normalizacao e Qualidade Industrial - INMETRO, Rio de Janeiro, Brazil)

Principle of the method: Residues of BAS 750 F are extracted from crop matrices with a mixture of methanol, water and 2M hydrochloric acid (70/25/5, v/v/v). For BAS 750 F in wheat straw and straw like matrices the extraction is performed twice. The extract is centrifuged and an aliquot of extract is transferred to another tube containing a 0.2 M sodium hydroxide solution. Cyclohexane is added, the solution is then partitioned and centrifuged. An aliquot of the organic phase (upper phase) is evaporated to dryness and dissolved in methanol/ water (50/50, v/v). The final solution is filtered before injection. All samples are analyzed by LC-MS/MS at mass transition 398 → 182 for quantitation and 398 → 133 for confirmation (ESI+). Analysis is accomplished on a Thermo Scientific Betasil C18 column (used for HPLC) and a Waters Acquity UPLC BEH C18 column (used for UPLC) applying a methanol-pure water gradient using 0.1% formic acid as modifier.

Recovery findings: In all matrices tested, the mean recovery values for HPLC as well as for UPLC determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.1.2-23: Validation results of method L0076/09 (HPLC): BAS 750 F in plant matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				**398 → 182**	**398 → 133**	**398 → 182**	**398 → 133**
BAS 750 F	Citrus	0.01	6	75	78	4	5
		0.1	6	79	83	6	5
		1.0	5	80	80	6	7
	Coffee	0.01	6	100	100	4	6
		0.1	6	89	90	3	5
		1.0	6	90	91	2	3
	Dry Beans	0.01	6	85	83	3	6
		0.1	6	82	83	3	9
		1.0	6	78	81	5	4
	Soybeans	0.01	5	83	84	6	4
		0.1	6	89	89	3	6
		1.0	5	88	91	3	5
	Tomato	0.01	5	87	86	8	5
		0.1	6	79	79	6	5
		1.0	5	73	75	5	5
	Wheat grain	0.01	5	84	81	9	11
		0.1	6	85	82	3	3
		1.0	6	87	84	10	12
	Wheat straw	0.01	6	86	87	9	10
		0.1	6	80	81	6	5
		1.0	6	80	83	6	4

Table 4.1.2-24: Validation results of method L0076/09 (UPLC): BAS 750 F in plant matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 182	398 → 133	398 → 182	398 → 133
BAS 750 F	Citrus	0.01	6	78	81	5	4
		0.1	6	81	86	5	10
		1.0	5	79	78	12	9
	Coffee	0.01	6	83	81	4	7
		0.1	6	86	87	3	6
		1.0	6	88	85	2	5
	Dry Beans	0.01	6	91	91	6	10
		0.1	6	87	84	8	9
		1.0	6	83	83	5	8
	Soybeans	0.01	5	94	78	7	4
		0.1	6	86	82	1	9
		1.0	5	89	82	5	4
	Tomato	0.01	5	89	78	7	6
		0.1	6	81	79	8	9
		1.0	5	77	79	7	8
	Wheat grain	0.01	5	78	90	7	14
		0.1	6	82	95	9	8
		1.0	6	92	90	11	12
	Wheat straw	0.01	6	73	82	4	10
		0.1	6	78	84	6	6
		1.0	6	84	83	11	7

Linearity: Good linearity was observed over the concentration range tested for the HPLC-MS/MS and UPLC-MS/MS detectors. Linear correlations with coefficients >0.99 were obtained for BAS 750 F At least six calibration points distributed over a concentration range of 0.04 to 2.0 ng/mL were used. Standards were injected in triplicate and the response plotted against concentration. Calibration standards were prepared in a mixture of methanol and pure water (50/50 v/v).

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions, hence no additional confirmatory analytical technique is required.

Matrix effects: No significant matrix effects were observed (deviation of matrix matched standards from standards prepared in acetonitrile were <20%).

Interference: No significant interference was observed. At elution time of the signals of interest, interference was below the required limit of 30% LOQ in all cases, as no signal was observed at elution times of the analytes of interest.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for citrus, coffee, dry beans, soybeans, tomato, wheat grain and wheat straw is 0.01 mg/kg for BAS 750 F, which corresponds to a concentration in the extract of 0.2 ng/mL.

Limit of detection: The limit of detection (LOD) for BAS 750 F in plant commodities is set at 20% of the LOQ, or 0.002 mg/kg, which corresponds to 0.04 ng/mL.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were well below 20%, for HPLC as well as for UPLC. The detailed values are shown in Table 4.1.2-23 and Table 4.1.2-24.

Reproducibility: Reproducibility of the method was not determined within this validation study.

Standard stability: In this study, BAS 750 F was shown to be stable in methanol (stock solutions, fortification solutions) and in a methanol/Milli-Q water mixture (50/50 v/v) (calibration solutions) for up to 30 days, when stored refrigerated.

Extract stability: In this study, BAS 750 F was shown to be stable after extraction with a mixture consisting of methanol/Milli-Q water/2mol/L HCl solution (70/25/5, v/v/v) for a time interval of 0 to 7 days for coffee, citrus, dry beans, soybeans, tomato and wheat grain and of 0 to 8 days for wheat straw, when stored refrigerated.

In the final volume dissolved with a mixture of methanol/Milli-Q water (50/50, v/v) BAS 750 F was shown to be stable for up to 7 days.

Extractability: The extractability of BAS 750 F was tested with selected samples/matrices from the different metabolism studies [see KCA 6.2.1/4, 2014/1261057]. Results indicated that using analytical method L0076/09 good recoveries were obtained compared to the particular metabolism study. These ranged between 93.4% and 111%.

Conclusion: **It could be demonstrated that the method L0076/09 fulfills the requirements according to SANCO/825/00 rev.8.1, SANCO/3029/99 rev.4, OPPTS 860.1340, OECD ENV/JM/MONO(2007)17 with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in plant matrices.**

Triazole derivative metabolites (TDMs)

The triazole derivative metabolites 1,2,4 triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) are considered as relevant residue for dietary risk assessment in plant matrices. A full description of the relevant methods has been provided in a dossier submitted by the triazole derivative metabolite group (TDMG) to UK CRD acting as Rapporteur Member State for all related questions (CRD reference number COP 2011/00502). The CRD evaluation of the residue section is not yet finalized. The method cited below for food of plant origin has been used for the analysis of samples generated during supervised field trials. The relevant section of the TDMG dossier is available upon request.

Triazole derivative metabolites

The analytical method developed for the triazole derivative metabolites in food of animal origin were submitted by the Triazole Derivative Metabolite Group to CRD acting as Rapporteur Member State. The relevant information is available upon request. However, the BASF method No L0170/02 used for data generation is summarized below.

Report:	CA 4.1.2/23 Class T., 2011 a Modification M004 of BCS residue analytical method 01062 for the determination of 1,2,4-Triazole, Triazolylalanine, Triazole acetic acid and Triazole lactic acid by LC/DMS/MS/MS in plant materials 2012/1294644
Guidelines:	Guidance Document on Residue Analytical Methods (SANCO/3029/99 rev.4), OECD-ENV/JM/MONO/(2007)17 (OECD No. 39)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method

In method L0170/02 residues of 1,2,4-triazole (T), triazolylalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) are extracted in various plant materials using a mixture of methanol/water (4/1, v/v). An aliquot of the extract was filtered, concentrated, and cleaned-up using dispersive C18-SPE. The analytes are determined by LC-DMS/MS/MS, using two different HPLC columns/stationary phases (Thermo Aquasil C18 and Thermo Hypercarb) and an AB SCIEX QTRAP® 5500 LC-MS/MS instrument equipped with SelexIONTM ion mobility technology based on planar differential mobility spectrometry (DMS). Detection was accomplished by in positive mode at mass transition 70 m/z→43 m/z, 157 m/z →70 m/z, 128 m/z →70 m/z and 158 m/z →70 m/z for quantification of triazole, triazolylalanine, triazole acetic acid, and triazole lactic acid, respectively. The results are calculated by direct comparison of the sample peak responses of the internal standards.

Recovery findings

In all matrices successfully tested, the mean recovery values were between 70 and 110%. Few average recoveries were above 110% but < 120%, obviously caused by analyte(s) present in control samples requiring background correction and supported by acceptable relative standard deviations (RSDs) and thus considered acceptable. The detailed results are given in the table below.

Table 4.1.2-25: Recovery results from triazole derivative metabolites plant matrices

Crop	Fortification level (mg/kg)	Recovery (%)	Analyte[1]			
			T	TA	TAA	TLA
Tomato	0.01	Average	105	111	90	92
		RSD	10	14	5	13
		n	5	5	5	5
	1.0	Average	98	110	101	114
		RSD	5	12	5	5
		n	5	5	5	5
Cucumber	0.01	Average	90	111	100	100
		RSD	14	13	10	6
		n	3	3	3	3
	1.0	Average	100	109	105	108
		RSD	3	7	4	2
		n	3	3	3	3
Lettuce	0.01	Average	88	116	105	108
		RSD	8	6	5	6
		n	3	3	3	3
	1.0	Average	102	106	104	104
		RSD	1	9	5	7
		n	3	3	3	3
Cereal grain	0.01	Average	115	91	97	80
		RSD	4	12	9	3
		n	5	5	5	5
	1.0	Average	118	84	80	79
		RSD	5	6	5	5
		n	5	5	5	5
Cereal straw	0.01	Average	109	79	109	100
		RSD	17	19	17	6
		n	3	3	3	3
	1.0	Average	102	76	90	85
		RSD	19	1	9	12
		n	3	3	3	3
Cereal green plant	0.01	Average	109	108	103	89
		RSD	7	8	7	7
		n	3	3	3	3
	1.0	Average	116	100	102	98
		RSD	4	4	5	5
		n	3	3	3	3
Whole orange	0.01	Average	100	90	92	95
		RSD	10	6	3	6
		n	5	5	5	5
	1.0	Average	100	100	92	92
		RSD	2	3	3	7
		n	5	5	5	5

Table 4.1.2-25: Recovery results from triazole derivative metabolites plant matrices

Crop	Fortification level (mg/kg)	Recovery (%)	Analyte[1]			
			T	TA	TAA	TLA
Oilseed rape seed and sunflower seed (TA only)	0.01	Average	102	101	99	82
		RSD	7	25	13	10
		n	5	5	5	5
	1.0	Average	93	92	95	98
		RSD	6	5	4	3
		n	5	5	5	5
Melon peel	0.01	Average	94	97	92	105
		RSD	12	27	7	4
		n	3	3	3	3
	1.0	Average	108	96	96	93
		RSD	7	7	2	4
		n	3	3	3	3
Melon fruit	0.01	Average	98	101	97	106
		RSD	2	9	5	10
		n	3	3	3	3
	1.0	Average	100	107	110	109
		RSD	9	6	2	2
		n	3	3	3	3
Melon pulp	0.01	Average	97	77	99	103
		RSD	5	29	3	10
		n	3	3	3	3
	1.0	Average	110	110	105	108
		RSD	2	7	7	4
		n	3	3	3	3
Sweet pepper	0.01	Average	87	104	106	107
		RSD	11	21	1	9
		n	3	3	3	3
	1.0	Average	107	104	110	110
		RSD	9	9	1	1
		n	3	3	3	3
Carrot leaf	0.01	Average	112	118	106	118
		RSD	6	10	11	6
		n	3	3	3	3
	1.0	Average	97	110	108	102
		RSD	6	14	3	4
		n	3	3	3	3
Carrot root	0.01	Average	90	98	104	105
		RSD	5	9	9	5
		n	3	3	3	3
	1.0	Average	98	105	105	106
		RSD	6	1	4	4
		n	3	3	3	3
Dry bean seed	0.01	Average	104	88	103	91
		RSD	8	12	11	6
		n	5	5	5	5
	1.0	Average	96	81	72	94
		RSD	8	9	7	5
		n	5	5	5	5

1 T=1,2,4-triazole, TA=triazolylalanine, TAA=triazole acetic acid, TLA=triazole lactic acid
RSD Relative standard deviation
N Number of tests

Linearity	The linearity was tested using 4 standards at concentrations between 1 to 600 ng/mL. Standards dissolved in water were injected and the response was plotted against the concentration. Linear correlations with coefficients of r>0.99 were obtained for 1,2,4-triazole (T), triazolylalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA).
Specificity	LC-MS/MS method is considered a highly specific analytical technique, if monitoring two mass transitions are monitored. In this study only one mass transition per analyte was analyzed. However, different confirmatory method(s) are available. Monitoring a confirmatory mass transition (157→88 m/z, positive mode for triazolylalanine), a second LC (Hypercarb) column or monitoring in the negative ion modus (triazole acetic acid, triazole lactic acid), an additional stationary phase (e.g. Phenomenex Luna Synergi Polar-RP for 1,2,4-triazole) or multiple derivatisation with subsequent SPE clean-up were performed in former versions of the method (M001/M002 and M003). These versions were also submitted by the Triazole Derivative Metabolite Group to CRD. Therefore enough confirmatory methods are available.
Matrix Effects:	The internal standard procedure, using stable isotopically labelled internal standards, compensates for matrix effects.
Interference:	In general, no significant interference was observed at elution times of the analytes of interest (interference <30% LOQ). Triazole lactic acid (TLA) shows in dry bean seed only excessive matrix interference using the Aquasil C18 column, thus this analyte was evaluated using a Thermo Hypercarb column.
Limit of Quantitation	The limit of quantitation was defined by the lowest fortification level successfully tested and was 0.01 mg/kg for each analyte in all tested matrices.
Repeatability	Relative standard deviations were below 20% for all analytes and sample materials fortified at 0.01 mg/kg (LOQ), except for triazolylalanine (TA) in sunflower seed, melon peel and melon pulp (RSDs < 30%, caused by endogenous TA present in the untreated sample requiring background subtraction). Nevertheless, these results are considered acceptable.
Stability of Solutions:	The stability of the analytes and their internal standards in solution and extracts was not tested specifically. Acceptable recoveries (obtained with fortification and internal standard solutions dosed separately) obtained with calibration solutions (with both the analytes and their internal standards present) sufficiently demonstrate stability.

Extract Stability Extracts in methanol/water (4/1, v/v) stored frozen were found stable up to 3 weeks in the version number 01062/M003. Fortifications in the residue trials showed that the extracts were stable during these studies.

Conclusion **The method L0170/02 is considered suitable for the analysis of 1,2,4-triazole (T), triazolylalanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in/on five different crop types (full validation sets performed) and in many additional plants matrices (reduced validation sets performed).**

Animal matrices

Report: CA 4.1.2/24
Devine C., 2015 a
Validation of the BASF analytical method L0272/01 for BAS 750 F in animal matrices
2015/1106707

Guidelines: EPA 860.1340 (1996), SANCO/3029/99 rev. 4 (11 July 2000), SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

Principle of the method: For matrices containing fat, BAS 750 F is extracted with a mixture of acetonitrile and iso hexane. An aliquot of the extract is centrifuged and partitioned twice against iso hexane.
For matrices containing proteins BAS 750 F is extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract is centrifuged and partitioned twice against cyclohexane using alkaline conditions.
Final analysis of BAS 750 F is performed by LC-MS/MS.
The method has a limit of quantitation of 0.01 mg/kg in animal matrices for BAS 750 F.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.1.2-26: Validation results of method L0272/01: BAS 750 F in animal matrices

Test substance	Matrix	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 182	398 → 133	398 → 182	398 → 133
BAS 750 F	Bovine meat	0.010	5	85.0	93.0	4.4	5.8
		0.10	5	110	108	0.4	1.8
	Bovine milk	0.010	5	82.0	76.5	8.8	7.0
		0.10	5	85.8	86.3	1.4	2.9
	Bovine cream	0.010	5	72.6	72.1	3.2	3.8
		0.10	5	86.4	86.9	5.6	4.4
	Bovine fat	0.010	5	80.2	80.8	11	8.6
		0.10	5	104	104	4.9	4.7
	Bovine liver	0.010	5	87.5	88.3	5.2	3.9
		0.10	5	96.4	95.3	3.8	3.2
	Bovine kidney	0.010	5	96.2	95.9	2.3	2.1
		0.10	5	101	100	4.6	6.7
	Hen eggs	0.010	5	93.3	92.9	3.1	6.4
		0.10	4	105	110	2.0	0.5

Linearity:	Good linearity was observed over the concentration range tested for the LC-MS/MS detector. Linear correlations with coefficients >0.99 were obtained for BAS 750 F At least eight calibration points distributed over a concentration range of 0.04 to 10.0 ng/mL were used. Standards were injected in duplicate and the response plotted against concentration. Calibration standards were prepared in a mixture of methanol and pure water (50/50 v/v) or sample matrix.
Specificity:	LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.
Matrix Effects:	Matrix effects on the detection of BAS 750 F were found to be insignificant (< 20 %).
Interference:	No significant interference above 20 % of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.
Limit of Quantitation:	The limit of quantitation for animal matrices is 0.01 mg/kg for BAS 750 F.
Limit of Detection:	The limit of detection (LOD) for BAS 750 F in in animal matrices was set at 20% of the LOQ, or 0.002 mg/kg.
Repeatability:	The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.1.2-26.
Reproducibility:	An independent laboratory validation has been successfully conducted and is reported under 2015/1240005 [see KCA 4.2/4].
Standard stability:	In this study, BAS 750 F was shown to be stable in methanol (stock solutions) for up to 98 days and in a methanol / water mixture (50/50 v/v) (fortification and calibration solutions) for up to 7 days.
Extract stability:	Following first analysis, the final extracts of fortified samples at the LOQ level were stored refrigerated for 7 days. Mean recovery values of the re-analysed extracts were in the range of 70 – 110 % and the RSD was less than 20 %. Therefore, extracts are considered to be stable when stored for at least 7 days.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0272/01 [see KCA 6.2.2/2, 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.4 and 107% in the different matrices analyzed (mil, kidney, cream, fat, muscle and egg). Therefore, L0272/01 showed good extractability for BAS 750 F and can be used for residue analysis.

Conclusion: **It could be demonstrated that the analytical method L0272/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in animal matrices.**

Report:	CA 4.1.2/25 Heger N.,Taraschewski I., 2016 b Validation of the BASF analytical method L0309/01: For the determination of M750F022 (Reg.No. 6011210) in animal matrices 2015/1106706
Guidelines:	SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), SANCO/825/00 rev. 8.1 (16 November 2010), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: The animal commodities were analyzed for residues of M750F022 using method L0309/01.

A 5 g sample aliquot is extracted for fat contained matrices (milk, fat) by shaking or macerating with 50 mL acetonitrile and 20 mL iso-hexane. 20 mL of the acetonitrile extract is shaken again with 20 mL iso-hexane. An aliquot of the acetonitrile phase is dried and dissolved in MeOH/H_2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS. Prior to GC-MS analysis an Analyte Protectant (AP) Mix is added.

For protein contained matrices (egg, muscle, liver and kidney) a 5 g sample aliquot is extracted by macerating with 50 mL MeOH/H_2O/2N HCl (70/25/5). 20 mL of the extract is shaken with 20 mL 0.2N NaOH and 100 mL cyclohexane (muscle and liver) or dichloromethane (egg and kidney). An aliquot of the cyclohexane or dichloromethane phase is dried and dissolved in MeOH/H_2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS. Prior to GC-MS analysis an Analyte Protectant (AP) Mix is added.

Quantitation is achieved by gas chromatography using mass spectrometric detection (GC/MS), equipped with a RTX-5 Amine column (30 m x 0.25 mm x 0.25µm), monitoring one fragment ion for quantification and two for confirmation.

The method has a limit of quantitation of 0.01 mg/kg in each matrix for the analyte.

Recovery findings: In all matrices tested, the mean recovery values for GC-MS determination were between 70% and 110% for all fortification levels and all mass transitions in all matrices except for cow fat, where the recoveries ranged between 108% and 124%. The overall recoveries in cow fat were below 120%. The detailed results are given in the tables below.

Table 4.1.2-27: Validation results of method L0309/01: M750F022 in animal matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)			Relative standard deviation (%)		
Transition				**295 m/z**	**297 m/z**	**317 m/z**	**295 m/z**	**297 m/z**	**317 m/z**
M750F022	Cow (liver)	0.01	5	83.9	82.8	82.1	5.8	3.9	3.4
		0.10	5	71.0	70.8	71.2	8.6	8.1	7.0
		Overall	**10**	**77.5**	**76.8**	**76.7**	**11**	**10**	**9.0**
	Cow (kidney)	0.01	5	89.2	84.1	86.5	5.6	3.7	4.3
		0.10	5	75.6	73.4	77.3	4.6	3.5	5.0
		Overall	**10**	**82.4**	**78.7**	**81.9**	**10**	**8.0**	**7.3**
	Cow (muscle)	0.01	5	82.4	79.2	79.9	7.7	5.5	6.9
		0.10	5	79.5	80.4	79.0	7.9	9.9	9.4
		Overall	**10**	**81.0**	**79.8**	**79.4**	**7.6**	**7.6**	**7.8**
	Cow (fat)	0.01	5	124	121	114	3.4	5.1	6.6
		0.10	5	113	113	108	9.5	9.7	8.4
		Overall	**10**	**119**	**117**	**111**	**8.2**	**8.1**	**7.6**
	Cow (milk)	0.01	5	79.5	80.2	79.7	8.2	6.9	6.7
		0.10	5	76.8	76.6	76.9	7.1	7.7	4.6
		Overall	**10**	**78.2**	**78.4**	**78.3**	**7.4**	**7.3**	**5.8**
	Hen (egg)	0.01	5	85.6	84.1	81.1	7.8	7.1	6.3
		0.10	5	102	102	104	8.6	7.5	9.6
		Overall	**10**	**93.8**	**92.9**	**92.4**	**12**	**12**	**15**

Linearity: Good linearity was observed over the concentration range tested for the GC-MS detector. Linear correlations with coefficients > 0.993 were obtained for M750F022. At six calibration points distributed over a concentration range of 2.5 to 100 ng/mL. Duplicate standards were injected and the response plotted against concentration. Calibration standards were prepared acetonitrile.

Specificity: GC/MS, using three fragments, is a highly specific detection technique and a confirmatory technique is therefore not required.

Matrix effects: The matrix effect was tested for each matrix. Significant matrix effects (>20%) on GC-MS response were observed in nearly all matrices, except for hen egg and cow muscle (only for the quantitative fragment).

Interference: No significant interference above 30 % of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantification of the method is 0.01 mg/kg for the analyte M750F022.

Limit of detection: The limit of detection (LOD) for M750F022 in animal commodities is set at 25% of the LOQ, or 0.003 mg/kg.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.1.2-27.

Reproducibility: An independent laboratory validation has been successfully conducted and is reported under 2015/1240006 [see KCA 4.2/6].

Standard stability: During the stability tests, it was found that the mean recoveries (measured concentration at day 0 set to 100 %) were in an acceptable range between 100 and 111% (stock solutions), 100 and 104% (calibration solutions) and 94.9 to 101% (AP-mix solutions). A decline less than 10% was observed. This demonstrates that M750F022 was stable in stock, calibration and AP-mix solutions for at least 91, 29 or 30 days, respectively, when stored refrigerated at 4 °C in the dark.

Extract stability: The stability of extracts and final volumes was investigated after 7 days of storage at approximately 4°C for extracts and after 3 and 7 days of storage at approximately 4°C for final volumes. The mean corrected recoveries (day 0 set to 100%) found during the experiments ranged between 72.0 % and 103% (extracts) and 86.5% and 129% (final volumes). Only the analytical measurements of the extracts and final volumes of cow kidney could not be evaluated after 7 days. This demonstrates that M750F022 was stable in extracts and final volumes over the tested time period of 7 days, except for cow kidney, which is only stable for three days.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0309/01 [see KCA 6.2.2/2 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.5 and 108% for milk, kidney, cream, muscle and fat. Extraction efficiency was lower in egg yolk (56.7 and 49.5%). Although notable differences in the extractable radioactive residues were observed for egg yolk, it can be concluded that L0309/09 showed a good extractability of BAS 750 F.

Conclusion: **It could be demonstrated that the analytical method L0309/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of M750F022 in animal matrices.**

The following study is a combination of residue analytics and a validation study. BASF analytical method L0309/02 was validated for the determination of the fatty acid conjugates of M750F022. Therefore, the following summary is restricted to the validation part of the method. The summary and the data regarding the results of the residue analysis can be found in section KCA 6.4 [see KCA 6.4.2/2, 2016/1001326].

Report:	CA 4.1.2/26 Guedez Orozco A.A.,Heger N., 2016 a Determination of the fatty conjugates metabolites of M750F022 (Reg. No. 6011210) in animal matrices 2016/1001326
Guidelines:	SANCO/3029/99 rev. 4 (11 July 2000)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

BASF Method L0309/02 can be used for the determination of the total content of the fatty acid conjugates of M750F022 in animal matrices. Through an alkaline hydrolysis step, fatty acid conjugates are broken down and measured as M750F022 by GC/MS. Therefore, the LOQ and the LOD of the method is expressed as M750F022. However, as typical representative of this compound class (fat acid conjugates), M750F025 was used for the validation.

The conditions for the alkaline hydrolysis were tested with radiolabeled samples and reported in the hen metabolism study [see KCA 6.2.2/1 2015/1001001]. The results showed that under this conditions all fatty acid conjugates found in metabolism (including M750F025) were broken down into M750F022. Therefore, the method L0309/02 is suitable for the determination of the total residues of the fatty acid conjugates expressed as M750F022.

Principle of the method:

A 5 g sample aliquot is extracted for **fat contained matrices** (fat and skin) by macerating with 50 mL acetonitrile and 20 mL isohexane. The isohexane extract is shaken again with 20 mL acetonitrile, twice. The whole acetonitrile extract is evaporated until dryness and then dissolved in 5 mL THF. 20 mL of NaOH (10M) is added and shaken for 1 hour at room temperature. The extract is shaken with 20 mL THF and centrifuged. An aliquot of the THF phase is dried and dissolved in MeOH/H_20 (50/50). Then a SPE clean-up step is carried out for the preparation of the samples by GC/MS. The method has a limit of quantitation of 0.01 mg/kg in each matrix expressed as M750F022. The limit of detection expressed as M750F022 is 0.003 mg/kg (25% of LOQ).

For **protein contained** matrices (egg, muscle, liver) a 5g sample aliquot is extracted by macerate with 50 mL MeOH. 20 mL of the extract is evaporated until dryness and dissolved in 5 mL THF. 20 mL of NaOH (10 M) is added and shaken during 1 hour at room temperature. An aliquot of the THF phase is dried and dissolved in MeOH/H_20 (50/50). Then a SPE clean-up step is carried out for the preparation of the samples by GC/MS. The method has a limit of quantitation of 0.01 mg/kg in each matrix expressed as M750F022. The limit of detection expressed as M750F022 is 0.003 mg/kg (25% of LOQ).

Recovery findings:

The mean recovery values were between 70 % and 110 % of the nominal values except for fat (mean values were slightly below, between 67.9% and 68%). However, these recoveries values are acceptable, because the matrices are difficult to analyze and the analytical method used is complex including a cleavage step, a liquid-liquid partition and SPE clean-up step. Therefore, it can be concluded, that the method L0309/02 is suitable for the determination of the fatty acid conjugates of M750F022 in animal matrices. The results are summarized in the following table.

Table 4.1.2-28: Validation results of method L0309/02:M750F025 expressed as M750F022 in animal matrices

Matrix	Fragment	Fortification Level [mg/kg]	Number of Replicates	Mean [%]	RSD [%]	Overall Mean [%]	Overall RSD [%]
Hen Egg	295 m/z	0.01	5	86.7	6.6	**85.5**	**5.8**
		0.1	5	84.4	5.3		
	297 m/z	0.01	5	83.4	5.0	**83.8**	**5.1**
		0.1	5	84.2	5.8		
	317 m/z	0.01	5	82.3	7.1	**83.2**	**6.1**
		0.1	5	84.1	5.4		
Hen Liver	295 m/z	0.01	5	75.4	16	**82.7**	**13**
		0.1	5	90.0	3.4		
	297 m/z	0.01	5	75.3	12	**82.5**	**12**
		0.1	5	89.6	2.7		
	317 m/z	0.01	5	70.5	15	**79.3**	**16**
		0.1	5	88.1	8.1		
Hen Muscle	295 m/z	0.01	5	96.9	7.3	**93.8**	**7.4**
		0.1	5	90.7	6.6		
	297 m/z	0.01	5	94.5	8.4	**92.2**	**7.7**
		0.1	5	89.9	6.7		
	317 m/z	0.01	5	86.8	13	**82.2**	**11**
		0.1	5	77.5	4.8		

Matrix	Fragment	Fortification Level [mg/kg]	Number of Replicates	Mean [%]	RSD [%]	Overall Mean [%]	Overall RSD [%]
Hen Fat	295 m/z	0.01	5	70.8	4.1	**68.0**	**7.1**
		0.1	5	65.3	7.7		
	297 m/z	0.01	5	70.7	3.5	**67.9**	**7.0**
		0.1	5	65.0	7.6		
	317 m/z	0.01	5	73.7	4.3	**68.0**	**10**
		0.1	5	62.3	7.1		

Linearity: At six calibration points were distributed over a concentration range of 2.5 to 100 ng/mL. At least duplicate standards were injected and the response plotted against concentration (R<0.99). Calibration standards were prepared in acetonitrile.

Specificity: GC/MS, using three fragments, is a highly specific detection technique and a confirmatory technique is therefore not required.

Matrix effects: During the validation, quality standards samples were run together with the samples. The results showed, that the instrument recoveries were between 70% and 120%, therefore no matrix effects were observed.

Interference: Significant interferences (> 25 % of LOQ) were not observed at the retention time and fragments of M750F022.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantification of the method is 0.01 mg/kg expressed as M750F022.

Limit of detection: The limit of detection (LOD) in animal commodities is set at 25% of the LOQ, or 0.003 mg/kg expressed as M750F022.

Repeatability: The relative standard deviations for all fortification levels were ≤ 16%.

Reproducibility: Reproducibility of the method was not determined within this validation study.

Extract stability: The stability of the sample extracts have been demonstrated by the acceptable procedural recoveries obtained during routine analysis of residue samples.

Extractability: The extraction solvents used (MeOH for protein contained matrices / ACN and Iso-Hexane for fat contained matrices) are identical to the solvents used in the metabolism study. Therefore, no additional data or study is required.

Conclusion: It could be demonstrated that method L0309/02 fulfils the requirements with regard to recoveries, linearity, specificity, limit of quantification and repeatability according to SANCO 3029/99 rev. 4 and is therefore applicable to correctly determine the total residues of fatty acid conjugates of M750F022 (Reg. No. 6011210) in animal matrices.

Triazole derivative metabolites (TDMs)

The triazole derivative metabolites 1,2,4 triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) are considered as relevant residue for dietary risk assessment in animal matrices. A full description of the relevant methods has been provided in a dossier submitted by the triazole derivative metabolite group (TDMG) to UK CRD acting as Rapporteur Member State for all related questions (CRD reference number COP 2011/00502). The CRD evaluation of the residue section is not yet finalized. The method cited below for feed of animal origin has been used for the analysis of samples generated during feeding studies.

The relevant section of the TDMG dossier is available upon request.

Triazole derivative metabolites

The analytical method developed for the triazole derivative metabolites in food of animal origin were submitted by the Triazole Derivative Metabolite Group to CRD acting as Rapporteur Member State. The relevant information is available upon request. However, the BASF method No L0263/01 (Method 001132) used for data generation is summarized below.

Report:	CA 4.1.2/27 Billian P.,Druskus M., 2009 a Residue analytical method 01132 for the determination of 1,2,4-Triazole, Triazole Alanine, Triazole Acetic Acid and Triazole Lactic Acid in/on milk, egg, muscle, fat, liver and kidney by HPLC-MS/MS (including amendment No. 1) 2010/1230632
Guidelines:	EEC 91/414, EEC 96/68, EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), SANCO/3029/99, SANCO/825/00 rev. 7 (17 March 2004), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07
GLP:	yes (certified by Ministerium fuer Arbeit, Gesundheit und Soziales des Landes Nordrhein-Westfalen Duesseldorf)

Principle of the method

Analytical method 01132 was developed for the determination of residues of 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) in animal commodities.
The analytes are extracted from animal samples with methanol/water (4/1 v/v) using a high speed homogeniser. An aliquot of the raw extract is passed through a cartridge, eluted with methanol/water or water and evaporated to dryness. Extracts are fortified with stable isotope internal standards and analysed directly for triazole alanine, triazole acetic acid and triazole lactic acid. An aliquot of the extracts are derivatised with dansyl chloride and analysed for 1,2,4-triazole.

Analysis for 1,2,4-triazole, triazole alanine and triazole acetic acid is by LC-MS/MS using electrospray ionisation (ESI) operating in positive or negative ionisation mode and monitoring two different ion transitions. Analysis of triazole lactic acid is by LC-MS/MS using ESI operating in positive ionisation mode with one chromatographic column or negative ionisation mode with a different chromatographic column (or in the case of milk products using one chromatographic column and analysis using firstly positive ionisation mode and secondly negative ionisation mode).

Recovery Findings

Validation of method 01132 was conducted in milk, egg, muscle, fat, liver and kidney using fortification levels of 0.01 and 0.1 mg/kg. Results are summarised in Table 4.1.2-29 to Table 4.1.2-36.

Table 4.1.2-29: Recovery results obtained during validation/confirmation of method 01132 for 1,2,4-Triazole in animal tissues

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Whole Milk m/z 302.9 to m/z 170.0	0.01	5	105	4.7	97-109
	0.10	5	101	7.3	91-108
	Overall	**10**	**103**	**6.1**	**91-109**
Whole Milk m/z 302.9 to m/z 234.0	0.01	5	114	2.8	110-119
	0.10	5	100	6.2	91-107
	Overall	**10**	**107**	**8.1**	**91-119**
Skimmed Milk m/z 302.9 to m/z 170.0	0.01	3	105	3,4	101-108
	0.10	3	106	2.5	103-108
	Overall	**6**	**106**	**2.7**	**101-108**
Skimmed Milk m/z 302.9 to m/z 234.0	0.01	3	108	2.4	106-111
	0.10	3	109	2.4	107-112
	Overall	**6**	**109**	**2.2**	**106-112**
Cream m/z 302.9 to m/z 170.0	0.01	3	110	6.4	103-117
	0.10	3	110	6.3	106-118
	Overall	**6**	**110**	**5.7**	**103-118**
Cream m/z 302.9 to m/z 234.0	0.01	3	109	1.4	107-110
	0.10	3	113	2.7	110-116
	Overall	**6**	**111**	**2.9**	**107-116**
Meat m/z 302.9 to m/z 170.0	0.01	5	113	2.7	110-118
	0.10	5	104	3.1	99-107
	Overall	**10**	**109**	**5.0**	**99-118**
Meat m/z 302.9 to m/z 234.0	0.01	5	115	2.6	111-118
	0.10	5	107	4.2	99-110
	Overall	**10**	**111**	**5.2**	**99-118**
Liver m/z 302.9 to m/z 170.0	0.01	5	96	3.5	92-101
	0.10	5	100	3.3	97-105
	Overall	**10**	**98**	**3.8**	**92-105**
Liver m/z 302.9 to m/z 234.0	0.01	5	94	2.9	90-97
	0.10	5	91	3.5	87-95
	Overall	**10**	**93**	**3.6**	**87-97**
Fat m/z 302.9 to m/z 170.0	0.01	5	105	8.3	96-117
	0.10	5	100	5.1	96-108
	Overall	**10**	**103**	**7.0**	**96-117**
Fat m/z 302.9 to m/z 234.0	0.01	5	105	6.1	98-115
	0.10	5	104	2.6	100-107
	Overall	**10**	**105**	**4.4**	**98-115**

Table 4.1.2-30: Recovery results obtained during validation/confirmation of method 01132 for 1,2,4-Triazole in animal tissues

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Kidney m/z 302.9 to m/z 170.0	0.01	5	82	9.0	73-89
	0.10	5	98	0.9	96-98
	Overall	**10**	**90**	**10.7**	**73-98**
Kidney m/z 302.9 to m/z 234.0	0.01	5	93	6.0	87-100
	0.10	5	92	2.9	88-95
	Overall	**10**	**93**	**4.5**	**87-100**
Whole Egg m/z 302.9 to m/z 170.0	0.01	5	95	7.0	89-105
	0.10	5	98	4.1	92-102
	Overall	**10**	**97**	**5.6**	**89-105**
Whole Egg m/z 302.9 to m/z 234.0	0.01	5	100	4.2	95-105
	0.10	5	93	2.5	91-96
	Overall	**10**	**97**	**4.7**	**91-105**
Egg Yolk m/z 302.9 to m/z 170.0	0.01	3	100	2.6	98-103
	0.10	3	86	4.2	83-90
	Overall	**6**	**93**	**8.8**	**83-103**
Egg Yolk m/z 302.9 to m/z 234.0	0.01	3	107	4.7	102-112
	0.10	3	85	2.4	83-87
	Overall	**6**	**96**	**13.2**	**83-112**
Egg White m/z 302.9 to m/z 170.0	0.01	3	93	6.7	86-98
	0.10	3	98	5.8	92-103
	Overall	**6**	**96**	**6.4**	**86-103**
Egg White m/z 302.9 to m/z 234.0	0.01	3	96	10.3	85-104
	0.10	3	109	9.2	100-120
	Overall	**6**	**103**	**11.2**	**85-120**

Table 4.1.2-31: Recovery results obtained during validation/confirmation of method 01132 for Triazole Alanine in animal tissues

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Milk m/z 156.7 to m/z 70.1	0.01	5	83	7.7	74-92
	0.10	5	88	3.0	86-92
	Overall	**10**	**86**	**6.0**	**74-92**
Milk m/z 156.7 to m/z 88.0	0.01	5	77	8.1	71-87
	0.10	5	90	4.5	84-94
	Overall	**10**	**84**	**10.5**	**71-94**
Skimmed Milk m/z 156.7 to m/z 70.1	0.01	3	90	3.2	87-92
	0.10	3	96	3.9	92-99
	Overall	**6**	**93**	**4.8**	**87-99**
Skimmed Milk m/z 156.7 to m/z 88.0	0.01	3	106	8.2	96-112
	0.10	3	95	1.8	93-96
	Overall	**6**	**101**	**8.2**	**93-112**
Cream m/z 156.7 to m/z 70.1	0.01	3	94	12.9	81-105
	0.10	3	89	2.8	87-92
	Overall	**6**	**92**	**9.0**	**81-105**
Cream m/z 156.7 to m/z 88.0	0.01	3	103	13.6	92-119
	0.10	3	88	3.0	85-90
	Overall	**6**	**96**	**12.9**	**85-119**
Meat m/z 156.7 to m/z 70.1	0.01	5	87	7.2	80-95
	0.10	5	97	4.0	91-101
	Overall	**10**	**92**	**7.5**	**80-101**
Meat m/z 156.7 to m/z 88.0	0.01	5	80	5.4	75-85
	0.10	5	100	8.4	87-110
	Overall	**10**	**90**	**13.8**	**75-110**
Liver m/z 156.7 to m/z 70.1	0.01	5	84	14.0	66-97
	0.10	5	95	1.6	94-97
	Overall	**10**	**90**	**10.9**	**66-97**
Liver m/z 156.7 to m/z 88.0	0.01	5	77	13.5	62-91
	0.10	5	97	1.8	94-98
	Overall	**10**	**87**	**14.4**	**62-98**
Fat m/z 156.7 to m/z 70.1	0.01	5	99	3.6	95-105
	0.10	5	119	1.5	117-121
	Overall	**10**	**109**	**9.9**	**95-121**
Fat m/z 156.7 to m/z 88.0	0.01	5	97	5.8	90-104
	0.10	5	122	2.1	118-125
	Overall	**10**	**109**	**12.6**	**90-125**

Table 4.1.2-32: Recovery results obtained during validation/confirmation of method 01132 for Triazole Alanine in animal tissues (continued)

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Kidney m/z 156.7 to m/z 70.1	0.01	5	103	4.2	98-108
	0.10	5	108	1.8	106-111
	Overall	**10**	**105**	**4.5**	**98-111**
Kidney m/z 156.7 to m/z 88.0	0.01	5	100	6.2	92-107
	0.10	5	107	4.4	99-111
	Overall	**10**	**103**	**6.2**	**92-111**
Whole Egg m/z 156.7 to m/z 70.1	0.01	5	86	4.9	80-90
	0.10	5	95	2.5	92-98
	Overall	**10**	**91**	**6.5**	**80-98**
Whole Egg m/z 156.7 to m/z 88.0	0.01	5	86	9.0	75-93
	0.10	5	99	2.5	97-103
	Overall	**10**	**92**	**9.6**	**75-103**
Egg Yolk m/z 156.7 to m/z 70.1	0.01	3	108	7.0	100-115
	0.10	3	84	7.7	78-91
	Overall	**6**	**96**	**15.0**	**78-115**
Egg Yolk m/z 156.7 to m/z 88.0	0.01	3	99	12.9	85-110
	0.10	3	84	8.6	78-92
	Overall	**6**	**92**	**13.5**	**78-110**
Egg White m/z 156.7 to m/z 70.1	0.01	3	86	5.5	82-91
	0.10	3	92	11.5	82-103
	Overall	**6**	**89**	**9.1**	**82-103**
Egg White m/z 156.7 to m/z 88.0	0.01	3	77	3.3	75-80
	0.10	3	87	9.2	79-95
	Overall	**6**	**82**	**9.1**	**75-95**

Table 4.1.2-33: Recovery results obtained during validation/confirmation of method 01132 for Triazole Acetic Acid in animal tissues

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Milk m/z 127.5 to m/z 69.9	0.01	5	103	4.9	96-109
	0.10	5	88	6.1	80-94
	Overall	**10**	**96**	**9.7**	**80-109**
Milk m/z 126.0 to m/z 81.9	0.01	5	108	10.4	93-120
	0.10	5	97	6.1	89-105
	Overall	**10**	**103**	**9.9**	**89-120**
Skimmed Milk m/z 127.5 to m/z 69.9	0.01	3	89	7.9	83-97
	0.10	3	94	1.1	93-95
	Overall	**6**	**92**	**5.7**	**83-97**
Skimmed Milk m/z 126.0 to m/z 81.9	0.01	3	89	4.0	85-92
	0.10	3	104	2.0	102-106
	Overall	**6**	**97**	**9.3**	**85-106**
Cream m/z 127.5 to m/z 69.9	0.01	3	86	2.9	84-89
	0.10	3	94	1.8	93-96
	Overall	**6**	**90**	**5.1**	**84-96**
Cream m/z 126.0 to m/z 81.9	0.01	3	94	4.9	89-97
	0.10	3	104	4.0	101-109
	Overall	**6**	**99**	**6.8**	**89-109**
Meat m/z 127.5 to m/z 69.9	0.01	5	98	2.4	96-102
	0.10	5	98	1.3	96-99
	Overall	**10**	**98**	**1.8**	**96-102**
Meat m/z 126.0 to m/z 81.9	0.01	5	87	6.1	81-94
	0.10	5	87	1.9	86-90
	Overall	**10**	**87**	**4.3**	**81-94**
Liver m/z 127.5 to m/z 69.9	0.01	5	81	3.5	76-83
	0.10	5	97	4.0	93-103
	Overall	**10**	**89**	**10.2**	**76-103**
Liver m/z 126.0 to m/z 81.9	0.01	5	91	4.6	86-96
	0.10	5	98	6.2	92-105
	Overall	**10**	**95**	**6.4**	**86-105**
Fat m/z 127.5 to m/z 69.9	0.01	5	96	2.8	93-100
	0.10	5	97	1.6	95-99
	Overall	**10**	**97**	**2.2**	**93-100**
Fat m/z 126.0 to m/z 81.9	0.01	5	91	5.1	87-98
	0.10	5	93	5.4	88-99
	Overall	**10**	**92**	**5.1**	**87-99**

Table 4.1.2-34: Recovery results obtained during validation/confirmation of method 01132 for Triazole Acetic Acid in animal tissues (continued)

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Kidney m/z 127.5 to m/z 69.9	0.01	5	100	7.5	93-110
	0.10	5	102	2.7	98-105
	Overall	**10**	**101**	**5.4**	**93-110**
Kidney m/z 126.0 to m/z 81.9	0.01	5	92	16.0	67-104
	0.10	5	106	3.2	101-110
	Overall	**10**	**99**	**12.4**	**67-110**
Whole Egg m/z 127.5 to m/z 69.9	0.01	5	92	4.1	90-99
	0.10	5	96	1.7	93-97
	Overall	**10**	**94**	**3.5**	**90-99**
Whole Egg m/z 126.0 to m/z 81.9	0.01	5	98	3.2	95-103
	0.10	5	97	3.8	91-100
	Overall	**10**	**97**	**3.4**	**91-103**
Egg Yolk m/z 127.5 to m/z 69.9	0.01	3	111	1.9	109-113
	0.10	3	94	3.8	90-97
	Overall	**6**	**103**	**9.6**	**90-113**
Egg Yolk m/z 126.0 to m/z 81.9	0.01	3	102	1.5	101-104
	0.10	3	92	3.3	89-95
	Overall	**6**	**97**	**6.2**	**89-104**
Egg White m/z 127.5 to m/z 69.9	0.01	3	99	4.6	94-103
	0.10	3	123	1.9	120-124
	Overall	**6**	**111**	**12.1**	**94-124**
Egg White m/z 126.0 to m/z 81.9	0.01	3	97	1.8	96-99
	0.10	3	110	3.3	106-113
	Overall	**6**	**104**	**7.3**	**96-113**

Table 4.1.2-35: Recovery results obtained during validation/confirmation of method 01132 for Triazole Lactic Acid in animal tissues

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Milk m/z 157.8 to m/z 70.1 +ve ESI mode	0.01	5	99	5.3	94-107
	0.10	5	84	4.5	78-88
	Overall	**10**	**91**	**9.9**	**94-107**
Milk m/z 155.9 to m/z 68.0 -ve ESI mode	0.01	5	91	3.3	87-95
	0.10	5	100	8.1	88-109
	Overall	**10**	**95**	**7.6**	**87-109**
Skimmed Milk m/z 157.8 to m/z 70.1 +ve ESI mode	0.01	3	84	1.8	82-85
	0.10	3	86	1.3	85-87
	Overall	**6**	**85**	**1.9**	**82-87**
Skimmed Milk m/z 155.9 to m/z 68.0 -ve ESI mode	0.01	3	112	4.9	107-118
	0.10	3	108	1.1	107-109
	Overall	**6**	**110**	**4.0**	**107-118**
Cream m/z 157.8 to m/z 70.1 +ve ESI mode	0.01	3	77	3.4	75-80
	0.10	3	94	2.2	92-96
	Overall	**6**	**85**	**11.0**	**75-96**
Cream m/z 155.9 to m/z 68.0 -ve ESI mode	0.01	3	105	1.0	104-106
	0.10	3	104	4.4	99-108
	Overall	**6**	**105**	**2.9**	**99-108**
Meat m/z 157.8 to m/z 70.1 Hypercarb column	0.01	5	90	6.7	80-96
	0.10	5	95	2.0	93-97
	Overall	**10**	**92**	**5.5**	**80-97**
Meat m/z 155.9 to m/z 68.0 SCX column	0.01	5	107	4.4	103-115
	0.10	5	104	1.3	103-106
	Overall	**10**	**106**	**3.4**	**103-115**
Liver m/z 157.8 to m/z 70.1 Hypercarb column	0.01	5	84	2.7	82-88
	0.10	5	95	1.2	94-97
	Overall	**10**	**90**	**6.7**	**82-97**
Liver m/z 155.9 to m/z 68.0 SCX column	0.01	5	99	1.2	97-100
	0.10	5	99	3.1	95-102
	Overall	**10**	**99**	**2.2**	**95-102**
Fat m/z 157.8 to m/z 70.1 Hypercarb column	0.01	5	88	7.2	83-99
	0.10	5	101	1.7	100-104
	Overall	**10**	**95**	**8.5**	**83-104**
Fat m/z 155.9 to m/z 68.0 SCX column	0.01	5	96	1.0	95-97
	0.10	5	98	3.0	96-103
	Overall	**10**	**97**	**2.4**	**95-103**

Table 4.1.2-36: Recovery results obtained during validation/confirmation of method 01132 for Triazole Lactic Acid in animal tissues (continued)

Matrix	Fortification level (mg/kg)	Number of Analyses	Mean Recovery (%)	RSD (%)	Recovery Range (%)
Kidney m/z 157.8 to m/z 70.1 Hypercarb column	0.01	5	111	5.0	106-120
	0.10	5	92	2.9	89-96
	Overall	**10**	**101**	**10.7**	**89-120**
Kidney m/z 155.9 to m/z 68.0 SCX column	0.01	5	93	6.0	89-102
	0.10	5	93	1.8	90-94
	Overall	**10**	**93**	**4.2**	**89-102**
Whole Egg m/z 157.8 to m/z 70.1 Hypercarb column	0.01	5	94	3.4	90-98
	0.10	5	94	3.4	91-98
	Overall	**10**	**94**	**3.2**	**90-98**
Whole Egg m/z 155.9 to m/z 68.0 SCX column	0.01	5	85	2.6	82-88
	0.10	5	94	1.6	92-96
	Overall	**10**	**90**	**5.9**	**82-96**
Egg Yolk m/z 157.8 to m/z 70.1 Hypercarb column	0.01	3	88	3.0	85-90
	0.10	3	97	2.7	95-100
	Overall	**6**	**93**	**3.9**	**85-100**
Egg Yolk m/z 155.9 to m/z 68.0 SCX column	0.01	3	81	7.5	75-87
	0.10	3	81	1.2	80-82
	Overall	**6**	**81**	**4.8**	**75-87**
Egg White m/z 157.8 to m/z 70.1 Hypercarb column	0.01	3	99	2.7	96-101
	0.10	3	121	1.7	119-123
	Overall	**6**	**110**	**11.3**	**96-123**
Egg White m/z 155.9 to m/z 68.0 SCX column	0.01	3	95	6.9	89-102
	0.10	3	115	3.5	111-119
	Overall	**6**	**105**	**11.4**	**89-119**

Specificity

The method is suitable to determine 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in animal commodities. LC-MS/MS using two independent ion transitions, differing chromatographic columns or differing ionisation techniques for quantification and confirmation is considered a highly specific technique.

The unfortified sample used to validate the method was found to contain residues greater than 30% of the LOQ. Thus the blank value was subtracted for the calculation of the recoveries. The residues found in the unfortified samples were considered to be real and therefore did not indicate a lack of specificity of the method.

Linearity Linearity of the method for 1,2,4-triazole was in the range 0.00002 µg/mL to 0.02 µg/mL. Linearity of the method for triazole alanine, triazole acetic acid and triazole lactic acid was in the range 0.000125 – 0.025 µg/mL. The correlation coefficient of the calibration curves were > 0.9907.

Accuracy The mean recovery values at both fortification levels and overall for each animal commodity using the quantification and confirmation techniques were between 70% and 110%. This meets the requirements of EU guidance SANCO 3029/99 rev.4, demonstrating the method has satisfactory accuracy.

The only exceptions to this were in the case of:

- The second ion transition for 1,2,4-triazole in milk where the mean recovery at the lower fortification level was 114%.
- The second ion transitions for 1,2,4-triazole in cream where the mean recovery at the upper fortification level was 113% and the overall mean recovery for the second ion transition was 111%.
- The first and second ion transitions for 1,2,4-triazole in meat where the mean recoveries at the lower fortification levels were 113% and 115% respectively and the overall mean recovery for the second ion transition was 111%.
- The first and second ion transitions for triazole alanine in fat where the mean recoveries at the upper fortification levels were 119% and 122% respectively.
- The first ion transition for triazole acetic acid in egg yolk where the mean recovery at the lower fortification level was 111%.
- The first ion transitions for triazole acetic acid in egg white where the mean recovery at the upper fortification level was 123% and the overall mean recovery for the first ion transition was 111%.
- The second ion transition for triazole lactic acid in skimmed milk where the mean recovery at the lower fortification level was 112%.
- The first ion transition for triazole lactic acid in kidney where the mean recovery at the lower fortification level was 111%.
- The first and second ion transitions for triazole lactic acid in egg white where the mean recoveries at the upper fortification levels were 121% and 115% respectively.

These minor deviations from the values suggested in the EU guidance are not viewed to be significant and the method is considered to have acceptable accuracy in all animal commodities.

Repeatability	The mean RSDs at both fortification levels and overall for each animal commodity using quantification and confirmation techniques were below 20%. This meets the requirements of EU guidance SANCO 3029/99 rev.4, demonstrating the method has satisfactory repeatability.
Limit of Quantification	The LOQ of a method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and for which a mean recovery of 70-110% with a relative standard deviation (RSD) of < 20% has been obtained. The LOQ for 1,2,4-triazole, triazole alanine, triazole acetic acid and triazole lactic acid in animal commodities is 0.01 mg/kg.

(f)Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

No stand-alone analytical methods were validated in support of ecotoxicological studies. Methods for the determination of concentration, whenever necessary, are reported along with the respective ecotoxicological study.

(g)Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

Where necessary, these methods are reported along with the respective studies.

CA 4.2 Methods for post-approval control and monitoring purposes

(a) Methods for the analysis in food and feed of plant and animal origin

Plants

Report:	CA 4.2/1 Klimmek S. et al., 2015 a Validation of the multi-residue method QuEChERS, BASF method number L0295/01, for the determination of BAS 750 F in different matrices of plant origin 2015/1106708
Guidelines:	SANCO/3029/99 rev. 4 (11 July 2000), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07, EPA 860.1340 (1996), SANCO/825/00 rev. 8.1 (16 November 2010)
GLP:	yes (certified by Freie und Hansestadt Hamburg, Behoerde fuer Gesundheit und Verbraucherschutz, Hamburg, Germany)

Principle of the method: Residues of BAS 750 F are extracted from crop matrices (tomato, orange, wheat grain, dry bean seeds, soybean seeds) with acetonitrile after addition of water if needed (QuEChERS modules E1 and E5). A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate is added and the extract is shaken. After centrifugation an aliquot of the acetonitrile phase is cleaned by freezing out (except for tomato fruit) and by adding primary secondary amine (PSA) and C18 material (QuEChERS modules C1 and C4). All samples are analyzed by LC-MS/MS at mass transition 398 → 70 for quantitation and 398 → 182 for confirmation (ESI+). Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile-pure water gradient using 0.1% formic acid as modifier.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.2-1: Validation results of method L0295/01: BAS 750 F in plant matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 70	398 → 182	398 → 70	398 → 182
BAS 750 F	Tomato (fruit)	0.01	5	74.8	81.5	2.2	2.9
		0.10	5	81.5	80.0	3.8	1.9
	Orange (whole fruit)	0.01	5	84.3	83.8	6.2	3.5
		0.10	5	86.0	80.8	4.2	3.5
	Dry Beans (seeds)	0.01	5	91.1	91.6	4.4	3.7
		0.10	5	97.0	90.4	20	18
	Wheat (grain)	0.01	5	83.7	87.5	7.5	1.1
		0.10	5	91.9	92.7	4.3	4.5
	Dry Soybeans (seeds)	0.01	5	75.3	71.7	6.1	3.9
		0.10	5	71.8	70.2	5.1	8.0

Linearity: Good linearity was observed over the concentration range tested for the LC-MS/MS detector. Linear correlations with coefficients >0.99 were obtained for BAS 750 F. At seven calibration points distributed over a concentration range of 0.15 to 7.5 ng/mL, corresponding to 0.003 mg/kg to 0.15 mg/kg were used. Single standards were injected and the response plotted against concentration. Calibration standards were prepared in a mixture of acetonitrile and pure water (20/80 v/v).

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions, hence no additional confirmatory analytical technique is required.

Matrix effects: Matrix effects on the detection of BAS 750 F in extracts of tomato (fruit), orange (whole fruit), dry beans (seeds) and dry soybeans (seeds) were found to be insignificant (< 20 %). Therefore, solvent standards were used for quantification, respectively. No significant matrix effects (< 20 %) were found on the detection of BAS 750 F in extracts of wheat (grain), nevertheless matrix-matched standards were used for quantification of BAS 750 F in extracts of wheat (grain).

Interference: No significant interference above 30% of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for tomato fruit, orange fruit, dry beans seeds, wheat grain and dry soybeans seeds is 0.01 mg/kg for BAS 750 F.

Limit of detection: The limit of detection (LOD) for BAS 750 F in plant commodities is set at 30% of the LOQ, or 0.003 mg/kg, which corresponds to 0.15 ng/mL.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-1.

Reproducibility: An independent laboratory validation has been successfully conducted and is reported under [see KCA 4.2/2 2015/1240004]

Standard stability: In this study, BAS 750 F was shown to be stable in acetone (stock solutions) for up to 44 days and in an acetonitrile / water mixture (20/80 v/v) (calibration solutions) for up to 30 days, when stored at 5 °C ± 4 °C.

Extract stability: Following first analysis, the final extracts of fortified samples at 10xLOQ level together with one control specimen extract were stored at 5 °C ± 4 °C for 6 to 11 days. Mean recovery values of the re-analysed extracts were in the range of 70 – 120 % and within 20 % of the original result. Therefore, extracts are considered to be stable when stored at 1 °C to 10 °C for at least 6 days.

Extractability: The extractability of BAS 750 F residues from selected plant samples from metabolism studies was addressed in the BASF Study [see KCA 6.2.1/4 2014/1261057]. The extraction procedures of the plant/metabolism study and of different multi residue analytical methods (Quechers, S19, SweEt) were compared. In the study, Quechers showed the best extractabilities ranging between 80.3% and 98.7% for BAS 750 F (excepting for straw 59.2%). Therefore, Quechers is recommended as monitoring method.

Conclusion: **It could be demonstrated that the multi-residue method QuEChERS (BASF Method Number L0295/01) fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in plant matrices.**

Report: CA 4.2/2
Richter S.,Schmiedt S., 2015 a
Independent method validation (ILV) of the QuEChERS method for the determination of BAS 750 F in 5 plant matrices, using LC/MS/MS (BASF Method No. L0295/01)
2015/1240004

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340 (1996), OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method: The original method was validated at Eurofins Agroscience Services Chem. GmbH in study 2015/1106708 [see KCA 4.2/1].
The analytical method is derived from the QuEChERS multi-residue method. Homogenized specimens are extracted with acetonitrile. After addition of $MgSO_4$, NaCl and buffering citrate salts (pH 5-5.5), the mixture is shaken intensively and centrifuged for phase separation. The organic extract is cleaned-up by freezing out and dispersive solid phase extractions (SPE) with primary secondary amine (PSA), $MgSO_4$ and C18 material.
All samples are analyzed by LC-MS/MS at mass transition 398 → 70 for quantitation and 398 → 182 for confirmation (ESI+). Analysis is accomplished on an Ascentis Express C18 column applying an acetonitrile-pure water gradient using 0.1% formic acid as modifier.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.2-2: Validation results of method L0295/01: BAS 750 in plant matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 70	398 → 182	398 → 70	398 → 182
BAS 750 F	Tomato (fruit)	0.01	5	84.5	84.6	1.5	1.7
		0.10	5	85.4	84.8	10	9.6
	Wheat (grain)	0.01	5	110	107	4.2	6.1
		0.10	5	110	110	1.9	2.1
	Dried broad beans (seeds)	0.01	5	108	107	5.2	4.8
		0.10	5	105	105	2.9	2.2
	Dried soybeans (seeds)	0.01	4*	110	109	4.7	5.8
		0.10	5	110	110	2.9	2.6
	Whole orange (fruit)	0.01	5	109	110	1.7	1.1
		0.10	5	110	110	5.1	6.9

* One statistical outlier (Dixons's Q test) not considered

Linearity: Good linearity was observed over the concentration range tested for the LC-MS/MS detector. Linear correlations with coefficients >0.99 were obtained for BAS 750 F. At seven calibration points distributed over a concentration range of 0.15 to 7.5 ng/mL, corresponding to 0.003 mg/kg to 0.15 mg/kg were used. Single standards were injected and the response plotted against concentration. Calibration standards were prepared in a mixture of acetonitrile and pure water (20/80 v/v).

Specificity: Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique was not necessary. The quantification was based on the monitoring of two mass transitions.

Matrix effects: The matrix effect was tested for each matrix. No significant matrix effects on LC-MS/MS response were observed for any matrix. Nevertheless, for the evaluation of the results calibration standards in matrix were used.

Interference: No significant interference above 30% of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantitation for all matrices is 0.01 mg/kg for BAS 750 F.

Limit of detection: The limit of detection (LOD) for BAS 750 F in plant commodities is set at 30% of the LOQ, or 0.003 mg/kg, which corresponds to 0.15 ng/mL.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-2.

Reproducibility: The independent laboratory validation confirmed the good results of the validation study 2015/1106708 [see KCA 4.2/1].

Standard stability: The stability of stock solution (44 days of refrigerated storage) and calibration solutions (30 days of refrigerated storage) was already shown in the validation of the original method (2015/1106708).The stability of the analyte in fortification solutions (solvent: acetonitrile) was shown in the present study for 11 days when stored refrigerated.

Extract stability: The stability of extract solutions (for at least 6 days of refrigerated storage) was already shown in the validation of the original method.

Extractability: The extractability of BAS 750 F residues from selected plant samples from metabolism studies was addressed in the BASF study 2014/1261057 [see KCA 6.2.1/4]. The extraction procedures of the plant/metabolism study and of different multi residue analytical methods (Quechers, S19, SweEt) were compared. In the study, Quechers showed the best extractabilities ranging between 80.3% and 98.7% for BAS 750 F (excepting for straw 59.2%). Therefore, Quechers is recommended as monitoring method.

Conclusion: **It could be demonstrated that the multi-residue method QuEChERS (BASF Method Number L0295/01) fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in plant matrices.**

Animals

Report: CA 4.2/3
Devine C., 2015 a
Validation of the BASF analytical method L0272/01 for BAS 750 F in animal matrices
2015/1106707

Guidelines: EPA 860.1340 (1996), SANCO/3029/99 rev. 4 (11 July 2000), SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

Principle of the method: For matrices containing fat, BAS 750 F is extracted with a mixture of acetonitrile and iso hexane. An aliquot of the extract is centrifuged and partitioned twice against iso hexane.
For matrices containing proteins BAS 750 F is extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract is centrifuged and partitioned twice against cyclohexane using alkaline conditions.
Final analysis of BAS 750 F is performed by LC-MS/MS.
The method has a limit of quantitation of 0.01 mg/kg in animal matrices for BAS 750 F.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.2-3: Validation results of method L0272/01: BAS 750 F in animal matrices

Test substance	Matrix	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 182	398 → 133	398 → 182	398 → 133
BAS 750 F	Bovine meat	0.010	5	85.0	93.0	4.4	5.8
		0.10	5	110	108	0.4	1.8
	Bovine milk	0.010	5	82.0	76.5	8.8	7.0
		0.10	5	85.8	86.3	1.4	2.9
	Bovine cream	0.010	5	72.6	72.1	3.2	3.8
		0.10	5	86.4	86.9	5.6	4.4
	Bovine fat	0.010	5	80.2	80.8	11	8.6
		0.10	5	104	104	4.9	4.7
	Bovine liver	0.010	5	87.5	88.3	5.2	3.9
		0.10	5	96.4	95.3	3.8	3.2
	Bovine kidney	0.010	5	96.2	95.9	2.3	2.1
		0.10	5	101	100	4.6	6.7
	Hen eggs	0.010	5	93.3	92.9	3.1	6.4
		0.10	4	105	110	2.0	0.5

Linearity:	Good linearity was observed over the concentration range tested for the LC-MS/MS detector. Linear correlations with coefficients >0.99 were obtained for BAS 750 F At least eight calibration points distributed over a concentration range of 0.04 to 10.0 ng/mL were used. Standards were injected in duplicate and the response plotted against concentration. Calibration standards were prepared in a mixture of methanol and pure water (50/50 v/v) or sample matrix.
Specificity:	LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.
Matrix Effects:	Matrix effects on the detection of BAS 750 F were found to be insignificant (< 20%).
Interference:	No significant interference above 20% of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.
Limit of Quantitation:	The limit of quantitation for animal matrices is 0.01 mg/kg for BAS 750 F.
Limit of Detection:	The limit of detection (LOD) for BAS 750 F in in animal matrices was set at 20% of the LOQ, or 0.002 mg/kg.
Repeatability:	The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-3.
Reproducibility:	An independent laboratory validation has been successfully conducted and is reported under CA 4.2/9 (DocID 2015/1240005).
Standard stability:	In this study, BAS 750 F was shown to be stable in methanol (stock solutions) for up to 98 days and in a methanol / water mixture (50/50 v/v) (fortification and calibration solutions) for up to 7 days.
Extract stability:	Following first analysis, the final extracts of fortified samples at the LOQ level were stored refrigerated for 7 days. Mean recovery values of the re-analysed extracts were in the range of 70 – 110 % and the RSD was less than 20%. Therefore, extracts are considered to be stable when stored for at least 7 days.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0272/01 [see KCA 6.2.2/2, 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.4 and 107% in the different matrices analyzed (mil, kidney, cream, fat, muscle and egg). Therefore, L0272/01 showed good extractability for BAS 750 F and can be used for residue analysis and can be recommended as monitoring method.

Conclusion: **It could be demonstrated that the analytical method L0272/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in animal matrices.**

Report:	CA 4.2/4 Richter S.,Djedovic S., 2015 b Independent method validation (ILV) of a method for the determination of BAS 750 F in various foodstuffs of animal origin, using LC/MS/MS - (BASF Method No. L0272/01) 2015/1240005
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340 (1996), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Principle of the method: The original method was validated at BASF in study BASF Method No. L0272/01 [see KCA 4.2/8, 2015/1106707]. For matrices containing fat, BAS 750 F is extracted with a mixture of acetonitrile and iso hexane. An aliquot of the extract is centrifuged and partitioned twice against iso hexane.
For matrices containing proteins BAS 750 F is extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract is centrifuged and partitioned twice against cyclohexane using alkaline conditions. Final analysis of BAS 750 F is performed by LC-MS/MS.
The method has a limit of quantitation of 0.01 mg/kg in animal matrices for BAS 750 F.

The method procedures with appropriate extraction and clean-up modules were established and subsequently independently validated as given in the original method validation report.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the tables below.

Table 4.2-4: Independent validation results of method L0272/01: BAS 750 F in animal matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				398 → 182	398 → 133	398 → 182	398 → 133
BAS 750 F	Milk	0.010	5	90.5	92.5	3.1	3.5
		0.10	5	89.6	90.0	2.2	3.3
	Cream	0.010	5	85.2	82.7	4.4	3.7
		0.10	5	83.0	77.1	5.2	6.8
	Fat	0.010	5	79.2	83.9	5.9	9.2
		0.10	5	81.9	82.0	8.2	10
	Egg	0.010	5	76.7	80.9	8.0	9.7
		0.10	5	92.8	88.4	19	19
	Meat	0.010	5	79.8	83.0	19	19
		0.10	5	99.9	97.8	4.0	4.8
	Kidney	0.010	5	100	101	5.3	4.7
		0.10	5	92.4	91.5	15	15
	Liver	0.010	5	98.4	97.4	3.8	8.0
		0.10	5	100	98.9	4.8	4.4

Linearity: Linear calibration curves were calculated and plotted by regression analysis. Correlation coefficients (r) were always ≥0.99. At seven calibration points distributed over a concentration range of 0.10 to 10.0 ng/mL (for milk, cream and fat matrices) and 0.040 to 5.0 ng/mL (for egg, meat, kidney and liver) were used. Single standards were injected and the response plotted against concentration. Calibration standards were prepared in a mixture of methanol and water (50/50 v/v).

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.

Matrix effects: Matrix effects on the detection of BAS 750 F were found to be insignificant (< 20%). Therefore, solvent standards were used for quantification.

Interference: No significant interference above 20 % of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.

Limit of quantitation: The limit of quantitation for foodstuffs of animal origin is 0.01 mg/kg for BAS 750 F.

Limit of detection: The limit of detection (LOD) for BAS 750 F in foodstuffs of animal origin is set at 20% of the LOQ, or 0.002 mg/kg.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-4.

Reproducibility: The independent laboratory validation confirmed the good results of the validation study (see M-CA 4.2/8, DocID 2015/1106707).

Standard stability: Stock solutions prepared in methanol were tested in the original study. The stability of solution was shown for 30 days when stored refrigerated. The stability of fortification solutions (stored refrigerated) prepared in acetonitrile or methanol and of the calibration solutions prepared in methanol/water (1/1, v/v) was shown for 7 days in the original study.

Extract stability: The stability of BAS 750 F in extracts was already tested in the original method validation. The extracts were shown to be stable for a storage period of 7 days when stored refrigerated.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0272/01 [see KCA 6.2.2/2, 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.4 and 107% in the different matrices analyzed (mil, kidney, cream, fat, muscle and egg). Therefore, L0272/01 showed good extractability for BAS 750 F and can be used for residue analysis and can be recommended as monitoring method.

Conclusion: **As confirmed by an independent laboratory validation, analytical method L0272/01 is considered fully suitable for the analysis of BAS 750 F in different animal matrices for enforcement purposes.**

Report:	CA 4.2/5 Heger N.,Taraschewski I., 2016 b Validation of the BASF analytical method L0309/01: For the determination of M750F022 (Reg.No. 6011210) in animal matrices 2015/1106706
Guidelines:	SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), SANCO/825/00 rev. 8.1 (16 November 2010), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: The animal commodities were analyzed for residues of M750F022 using method L0309/01.

A 5 g sample aliquot is extracted for fat contained matrices (milk, fat, cream) by shaking or macerating with 50 mL acetonitrile and 20 mL iso-hexane. 20 mL of the acetonitrile extract is shaken again with 20 mL iso-hexane. An aliquot of the acetonitrile phase is dried and dissolved in MeOH/H_2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS. Prior to GC-MS analysis an Analyte Protectant (AP) Mix is added.

For protein contained matrices (egg, muscle, liver and kidney) a 5 g sample aliquot is extracted by macerating with 50 mL MeOH/H_2O/2N HCl (70/25/5). 20 mL of the extract is shaken with 20 mL 0.2N NaOH and 100 mL cyclohexane (muscle and liver) or dichloromethane (egg and kidney). An aliquot of the cyclohexane or dichloromethane phase is dried and dissolved in MeOH/H_2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS. Prior to GC-MS analysis an Analyte Protectant (AP) Mix is added.

Quantitation is achieved by gas chromatography using mass spectrometric detection (GC/MS), equipped with a RTX-5 Amine column (30 m x 0.25 mm x 0.25µm), monitoring one fragment ion for quantification and two for confirmation.

The method has a limit of quantitation of 0.01 mg/kg in each matrix for the analyte.

Recovery findings: In all matrices tested, the mean recovery values for GC-MS determination were between 70% and 110% for all fortification levels and all mass transitions in all matrices except for cow fat, where the recoveries ranged between 108% and 124%. The overall recoveries in cow fat were below 120%. The detailed results are given in the tables below.

Table 4.2-5: Validation results of method L0309/01: M750F022 in animal matrices

Test substance	Crop	Fortification level (mg/kg)	No of tests	Average recovery (%)			Relative standard deviation (%)		
Transition				**295 m/z**	**297 m/z**	**317 m/z**	**295 m/z**	**297 m/z**	**317 m/z**
M750F022	Cow (liver)	0.01	5	83.9	82.8	82.1	5.8	3.9	3.4
		0.10	5	71.0	70.8	71.2	8.6	8.1	7.0
		Overall	**10**	**77.5**	**76.8**	**76.7**	**11**	**10**	**9.0**
	Cow (kidney)	0.01	5	89.2	84.1	86.5	5.6	3.7	4.3
		0.10	5	75.6	73.4	77.3	4.6	3.5	5.0
		Overall	**10**	**82.4**	**78.7**	**81.9**	**10**	**8.0**	**7.3**
	Cow (muscle)	0.01	5	82.4	79.2	79.9	7.7	5.5	6.9
		0.10	5	79.5	80.4	79.0	7.9	9.9	9.4
		Overall	**10**	**81.0**	**79.8**	**79.4**	**7.6**	**7.6**	**7.8**
	Cow (fat)	0.01	5	124	121	114	3.4	5.1	6.6
		0.10	5	113	113	108	9.3	9.7	8.4
		Overall	**10**	**119**	**117**	**111**	**8.1**	**8.1**	**7.6**
	Cow (milk)	0.01	5	79.5	80.2	79.7	8.2	6.9	6.7
		0.10	5	76.8	76.6	76.9	7.1	7.7	4.6
		Overall	**10**	**78.2**	**78.4**	**78.3**	**7.4**	**7.3**	**5.8**
	Hen (egg)	0.01	5	85.6	84.1	81.1	7.8	7.1	6.3
		0.10	5	102	102	104	8.7	7.5	9.6
		Overall	**10**	**93.9**	**92.9**	**92.4**	**12**	**12**	**15**

Linearity: Good linearity was observed over the concentration range tested for the GC-MS detector. Linear correlations with coefficients >0.99 were obtained for M750F022. At six calibration points distributed over a concentration range of 2.5 to 100 ng/mL. Duplicate standards were injected and the response plotted against concentration. Calibration standards were prepared in acetonitrile.

Specificity: GC/MS, using three fragments, is a highly specific detection technique and a confirmatory technique is therefore not required.

Matrix effects: The matrix effect was tested for each matrix. Significant matrix effects (>20%) on GC-MS response were observed in nearly all matrices, except for hen egg and cow muscle (only for the quantitative fragment).

Interference: No significant interference above 30% of LOQ was detected in any of the control specimen extracts of each matrix or the reagent blanks, so that a high level of selectivity was demonstrated.

Limit of quantitation: Limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested. The limit of quantification of the method is 0.01 mg/kg for the analyte M750F022.

Limit of detection: The limit of detection (LOD) for M750F022 in animal commodities is set at 25% of the LOQ, or 0.003 mg/kg.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-5.

Reproducibility: An independent laboratory validation has been successfully conducted and is reported under CA 4.2/12, DocID 2015/1240006.

Standard stability: During the stability tests, it was found that the mean recoveries (measured concentration at day 0 set to 100%) were in an acceptable range between 100 and 111% (stock solutions), 100 and 104% (calibration solutions) and 94.9 to 101% (AP-mix solutions). A decline less than 10% was observed. This demonstrates that M750F022 was stable in stock, calibration and AP-mix solutions for at least 91, 29 or 30 days, respectively, when stored refrigerated at 4 °C in the dark.

Extract stability: The stability of extracts and final volumes was investigated after 7 days of storage at approximately 4°C for extracts and after 3 and 7 days of storage at approximately 4°C for final volumes. The mean corrected recoveries (day 0 set to 100%) found during the experiments ranged between 72.0 % and 103% (extracts) and 86.5% and 101% (final volumes). Only the analytical measurements of the extracts and final volumes of cow kidney could not be evaluated after 7 days. This demonstrates that M750F022 was stable in extracts and final volumes over the tested time period of 7 days, except for cow kidney, which is only stable for three days.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0309/01 [see KCA 6.2.2/2, 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.5 and 108% for milk, kidney, cream, muscle and fat. Extraction efficiency was lower in egg yolk (56.7 and 49.5%). Although notable differences in the extractable radioactive residues were observed for egg yolk, it can be concluded that L0309/09 showed a good extractability of BAS 750 F and can be also recommended as monitoring method.

Conclusion: **It could be demonstrated that the analytical method L0309/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of M750F022 in animal matrices.**

Report:	CA 4.2/6 Bendig P.,Wabbel C., 2015 a Independent method validation (ILV) of BASF method no. L0309/01 for the determination of the BAS 750 F diol metabolite in various foodstuffs of animal origin, using GC/MS 2015/1240006
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340 (1996), OECD-ENV/JM/MONO/(2007)17
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method: The original method was validated at BASF in study BASF Method No. L0309/01 [see KCA 4.2/11, 2015/1240006]. A 5 g sample aliquot is extracted for fat containing matrices (milk, fat) with 50 mL acetonitrile and 20 mL iso-hexane using an Ultra Turrax. 20 mL of the acetonitrile extract are shaken again with 20 mL iso-hexane. An aliquot of the acetonitrile phase is evaporated to dryness and redissolved in MeOH/$H_2$0 (50/50). Then a SPE clean-up step is carried out for the preparation of the samples for analysis by GC/MS. The SPE eluate is evaporated to dryness and redissolved in acetonitrile. Prior to GC/MS analysis an Analyte Protectant (AP) Mix is added.
For protein containing matrices (egg, muscle, liver and kidney) a 5 g sample aliquot is extracted with 50 mL MeOH/H_2O/2N HCl (70/25/5) using an Ultra Turrax. 20 mL of the extract are shaken with 20 mL 0.2N NaOH and 100 mL cyclohexane or dichloromethane. The cyclohexane or dichloromethane phase is evaporated to dryness and redissolved in MeOH/$H_2$0 (50/50). Then a SPE clean-up step is carried out for the preparation of the samples for analysis by GC/MS. The SPE eluate is evaporated to dryness and redissolved in acetonitrile. Prior to GC/MS analysis an Analyte Protectant (AP) Mix is added.
Quantitation is achieved by gas chromatography using mass spectrometric detection (GC/MS), equipped with a RTX-5 Amine column (30 m x 0.25 mm x 0.25µm), monitoring one fragment ion for quantification and two for confirmation.

The method procedures with appropriate extraction and clean-up modules were established and subsequently independently validated as given in the original method validation report.

Recovery findings: In all matrices tested, the mean recovery values for GC-MS determination were between 70% and 110% for all fortification levels and all mass transitions in all matrices. Detailed results are given in the tables below.

Table 4.2-6: Validation results of method L0309/01: M750F022 in animal matrices

Test substance	Matrix	Fortification level (mg/kg)	No of tests	Average recovery (%)			Relative standard deviation (%)		
Transition				295 m/z	297 m/z	317 m/z	295 m/z	295 m/z	295 m/z
M750F022	milk	0.01	5	79.3	80.3	91.8	6.1	4.5	4.4
		0.10	5	77.1	72.4	76.9	10	12	11
		overall		**78.2**	**76.3**	**84.4**	**7.8**	**10**	**12**
	fat	0.01	5	100	91.8	96.6	3.1	6.3	4.9
		0.10	5	82.3	88.6	99.4	11	12	15
		overall		**91.1**	**90.2**	**98.0**	**12**	**8.9**	**11**
	kidney	0.01	5	73.2	77.4	76.6	7.6	8.4	6.5
		0.10	5	83.2	84.6	84.6	16	18	15
		overall		**78.2**	**81.0**	**80.6**	**14**	**14**	**13**
	liver	0.01	5	89.4	88.3	89.7	5.6	4.8	10
		0.10	5	83.6	82.0	81.0	13	13	15
		overall		**86.5**	**85.1**	**85.4**	**10**	**10**	**13**
	egg	0.01	5	91.2	93.3	97.9	10	7.7	7.0
		0.10	5	99.4	98.4	101	5.8	6.3	5.7
		overall		**95.3**	**95.8**	**99.3**	**8.6**	**7.2**	**6.2**
	muscle	0.01	5	84.8	82.3	83.5	11	12	8.3
		0.10	5	80.6	82.6	79.1	14	14	14
		overall		**82.7**	**82.5**	**81.3**	**12**	**12**	**11**

Linearity: Good linearity ($R^2 > 0.99$) was observed in the range of 2.5 ng/mL to 125 ng/mL for the three fragment ions. Except for egg were good linearity ($R^2 > 0.99$) observed in the range of 2.5 ng/mL to 50 ng/mL and 10 ng/mL to 125 ng/mL for the three fragment ions. At least five calibration levels were injected.

Specificity: The highly selective and sensitive GC/MS method was used for the determination of M750F022 monitoring one characteristic fragment ion for quantification and two characteristic fragment ions for confirmation. A confirmatory technique is therefore not required.

Matrix effects: The matrix effect was tested for each matrix. No significant matrix effects (i.e. > 20% suppression or enhancement) on GC/MS response was observed for liver and muscle. Significant matrix effects (i.e. > 20% suppression or enhancement) on GC/MS response was observed for milk, egg, kidney and fat. For all matrices calibrations solutions in matrix were used for the evaluation of the results.

Interference: The interferences/residues of the analyte measured in the control samples were always below 30% of the limit of quantification (LOQ) for each matrix and each fragment ion.

Limit of quantitation: Limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested. The limit of quantification of the method is 0.01 mg/kg for the analyte M750F022, corresponding to a concentration in the extract of 10 ng/mL for all matrices.

Limit of detection: The limit of detection (LOD) for M750F022 in animal commodities is defined as 25% of the LOQ, or 0.0025 mg/kg, corresponding to a concentration in the extract of 2.5 ng/mL.

Repeatability: The relative standard deviations (RSD, %) for all commodities and all fortification levels were ≤ 20%. The detailed values are shown in Table 4.2-6.

Reproducibility: This independent method validation study confirmed the validity of the BASF method No. L0309/01 as shown in study M-CA 4.2/10, DocID 2015/1106706.

Standard stability: The stability of stock solutions (91 days of refrigerated storage in the dark), calibrations solutions (29 days of refrigerated storage in the dark) and AP-Mix solutions (at least 30 days of refrigerated storage in the dark) was shown in the validation of the original BASF method L0309/01.

Extract stability: The stability of M750F022 in extracts and final volumes (at least 7 days of refrigerated storage, except cow kidney, which was stable for at least 3 days) was already shown in the validation of the original BASF method L0309/01.

Extractability: Selected samples from the animal metabolism studies were extracted and analyzed using the analytical method L0309/01 [see KCA 6.2.2/2, 2015/1161960]. The extraction efficiency compared to the metabolism studies ranged between 62.5 and 108% for milk, kidney, cream, muscle and fat. Extraction efficiency was lower in egg yolk (56.7 and 49.5%). Although notable differences in the extractable radioactive residues were observed for egg yolk, it can be concluded that L0309/09 showed a good extractability of BAS 750 F and can be also recommended as monitoring method.

Conclusion: **As confirmed by an independent laboratory validation, BASF method L0309/01 fulfills the requirements with regard to linearity, specificity, repeatability, reproducibility, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of M7520F022 in animal matrices.**

(b) Methods for the analysis in soil and water

Soil

Report:	CA 4.2/7 Studenroth S.,Lueer D., 2015 b Validation of analytical method L0214/01 for the determination of BAS No. 750 F (Reg.No. 5834378) and metabolites of Reg.No. 5924326 and 1,2,4-Triazole (Reg.No. 87084) in soil by LC-MS/MS 2015/1039006
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 850.7100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: A 5 g soil sample is extracted with a mixture of acetonitrile/water (70/30, v/v) for 30 min on a mechanical shaker at 225 rpm. After centrifugation (10 min at 4000 rpm) an aliquot of 10 mL is taken (extract 1) and the remaining supernatant is decanted and discarded. The same extraction procedure is repeated once and after centrifugation a second aliquot of 10 mL (extract 2) is combined with extract 1 and thoroughly mixed. Residues of Reg.No. 5834378 (BAS 750 F) and metabolite Reg.No. 5924326 (M750F003) are directly analysed by LC-MS/MS. For analysis of Reg.No. 87084 (1,2,4-Triazole), 5 mL of the combined extracts 1 and 2 are transferred into a tared glass tube and the volume is reduced in a nitrogen evaporator to a volume less than 1 mL (confirmation by weighing, assuming a density of 1 g/cm^3). The concentrated extract is filled up to a volume of 1 mL with ultra-pure water and analysed by LC-MS/MS. For the investigation of Reg.No. 5834378 (BAS 750 F) and metabolite Reg.No. 5924326(M750F003) an Aquasil C-18 column is used. Reg. No. 87084 is investigated on two different columns, a Hypercarb and a Synergy Hydro-RP column. For all anaylses a water/acetonitrile gradient is used with 0.1% formic acid as modifier.

Recovery findings: In all matrices tested, the mean recovery values for LC-MS/MS determination were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the table below.

Table 4.2-7: Validation results of method L0214/01: Determination of Reg. No. 5834378 (BAS 750 F), Reg. No. 5924326 (M750F003) and Reg. No. 87084 (1,2,4-Triazole) in soil

Test substance	Soil type	Fortification level (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Transition				**398 → 182**	**398 → 133**	**398 → 182**	**398 → 133**
Reg. No. 5834378 (BAS 750 F)	Lufa 2.2	0.002	5	102	98	1.6	2.1
		0.02	5	102	102	1.4	1.0
	Lufa 2.3	0.002	5	109	107	2.4	3.2
		0.02	5	96	96	12.1	15.0
Transition				**288 → 159**	**288 → 103**	**288 → 159**	**288 → 103**
Reg. No. 5924326 (M750F003)	Lufa 2.2	0.002	5	101	98	2.3	3.7
		0.02	5	102	103	1.6	2.7
	Lufa 2.3	0.002	5	99	99	11.2	7.3
		0.02	5	82	82	2.7	4.1
Transition				**70 → 43 (Hypercarb)**	**70 → 43 (Synergy)**	**70 → 43 (Hypercarb)**	**70 → 43 (Synergy)**
Reg. No. 87084 (1,2,4-triazole)	Lufa 2.2	0.002	5	102	93	3.1	0.5
		0.02	5	88	97	7.5	2.5
	Lufa 2.3	0.002	5	88	95	8.4	5.6
		0.02	5	85	96	3.7	5.6

Linearity: Good linearity (r > 0.995) was observed in the range of 0.025 ng/mL to 3.0 ng/mL for the two mass transitions of Reg.No. 5834378 and metabolite Reg.No. 5924326 and in the range of 0.125 ng/mL to 15 ng/mL for the one mass transition of metabolite Reg. No. 87084. At least seven calibration points were determined.

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.

Matrix effects: It was demonstrated that the matrix-load in the tested matrix-matched standards of Reg.No. 5834378 had an influence on the detection of Reg. No. 5834378 (BAS 750 F) which could not be neglected (up to 21 %). No matrix effect was observed for both metabolites Reg. No. 5924326 and Reg. No. 87084.

Interference: The method determined residues of Reg.No. 5834378 and its metabolites Reg.No. 5924326 and Reg.No. 87084 in soil. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions considered.

Limit of quantitation: The limit of quantitation is defined by the lowest fortification level successfully tested, hence 0.002 mg/kg, corresponding to a concentration of 0.125 ng/mL Reg. No. 5834378 and Reg. No. 5924326 and 0.625 ng/mL Reg. No. 87084 in the soil extract.

Limit of detection: The limit of detection is estimated as 20% of the limit of quantitation, equivalent to 0.0004 mg/kg, corresponding to a concentration of 0.025 ng/mL Reg. No. 5834378 and Reg. No. 5924326 and 0.125 ng/mL Reg. No. 87084 in the soil extract.

Repeatability: The relative standard deviations (RSD, %) for all fortification levels were below 20%.The detailed values are shown in Table 4.2-7.

Reproducibility: Reproducibility of the method was not determined within this validation study.

Standard stability: Standard solutions of Reg.No. 5834378 and Reg.No. 5924326 were stable (≤ 10% decline) for 43 days when stored at 4 ± 2°C. Standard solutions of Reg.No. 87084 were stable (≤ 10% decline) for 31 days when stored at 4 ± 2°C.

Extract stability: The mean recovery values of Reg.No. 5834378 found during experiments ranged between 90% and 107%. The corrected mean recovery values (time zero recovery values were set as 100% reference value) of the metabolites Reg.No. 5924326 and Reg.No. 87084 found during the experiments ranged between 91% to 111% (uncorrected mean values ranged between 89% and 106%). This demonstrates that Reg.No. 5834378 and its metabolites Reg.No. 5924326 and Reg. No. 87084 were stable in the soil extracts over the tested time period of at least 7 days.

Extractability The extractability of BAS 750 F residues from selected soil samples from metabolism studies was addressed in the BASF Study [see KCA 7.1.2.2.1/5 2015/1182724]. The extraction procedures of the soil/metabolism study and of the residue analytical method were compared. Results showed that the extractability was similar for both extraction schemes ranging between 91.4% and 108%.

Conclusion: **It could be demonstrated that method L0214/01 fulfills the requirements with regard to specificity, linearity, repeatability, limit of quantitation and recoveries and is therefore applicable to correctly determine residues of Reg.No. 5834378 (BAS 750 F) and its metabolites Reg.No. 5924326 and Reg.No. 87084 (1,2,4-Triazole) in both soil types.**

Water

Report:	CA 4.2/8 Malinsky D.S., 2016 a Validation of analytical method D1506/01: Method for the determination of Mefentrifluconazole (BAS 750 F, Reg. No.5834378) and its metabolites M750F003 (Reg. No.5924326), M750F005 (Reg. No.6003433), M750006 (Reg. No.5863469), M750F007 (Reg. No.6003432) and M750F008 (Reg. No.6010286) in surface and drinking water by LC-MS/MS 2015/7001125
Guidelines:	EPA 850.6100, SANCO/3029/99 rev. 4 (11 July 2000), SANCO/825/00 rev. 8.1 (16 November 2010)
GLP:	yes (certified by United States Environmental Protection Agency)

Principle of the method

The analytical method D1506/01 is developed for the determination of BAS 750 F and its metabolites M750F003 (Reg. No. 5924326), M750F005 (Reg. No. 6003433), M750F006 (Reg. No. 5863469), M750F007 (Reg. No. 6003432) and M750F008 (Reg. No. 6010286) in surface and drinking water by LC-MS/MS.
The water samples are diluted with acetonitrile, mixed, filtered and then analyzed by direct injection onto a high performance liquid chromatography (HPLC) column with detection by positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring the following ion transitions: m/z 398→70 and 400→70 for the parent compound BAS 750 F; m/z 288→70 for M750F003; m/z 380→70 for M750F005; m/z 356→259 and 356→217 for M750F006; m/z 338→241 for M750F007 and m/z 356→259 and 356→241 for M750F008. In lieu of secondary (alternate) ion transitions for M750F003, M750F005 and M750F007, confirmatory analysis is performed using a different LC-MS/MS column (C18 and phenyl column options are available). The results are calculated by direct comparison of the sample peak responses to those of external standards.

Recovery findings

The results show that the method is suitable to determine residues of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in water. Samples spiked with the analytes at the limit of quantification of 30 ng/L and ten times higher (300 ng/L) have overall recovery values (mean of five replicates per fortification level, matrix and analyte) between 70% and 120%, except for except for the analyte M750F005 with the mass transition 380→70 in drinking water. The detailed results are given in the table below (Table 4.2-8).

Table 4.2-8: Results of the method validation for the determination of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in surface and drinking water

Matrix	Analyte	m/z	Fortification level [ng/L]	Number of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	Overall RSD [%]
Surface water	BAS 750 F	398→70	30	10	93	18	94	15
			300	10	95	12		
		400→70	30	10	77	13	83	14
			300	10	89	11		
	M750F003	288→70	30	10	93	7	99	15
			300	10	105	18		
		288→70	30	10	98	7	99	7
			300	10	101	6		
	M750F005	380→70	30	10	104	15	108	15
			300	10	112	15		
		380→70	30	10	100	6	104	9
			300	10	108	11		
	M750F006	356→259	30	10	98	9	102	8
			300	10	105	7		
		356→217	30	10	90	15	94	14
			300	10	98	12		
	M750F007	338→241	30	10	112	10	115	13
			300	10	117	16		
		338→241	30	10	102	7	103	7
			300	10	105	7		
	M750F008	356→259	30	15	92	13	97	18
			300	15	103	19		
		356→241	30	15	92	19	94	17
			300	15	95	15		
Drinking water	BAS 750 F	398→70	30	10	91	9	93	13
			300	10	96	15		
		400→70	30	10	93	18	95	15
			300	10	98	12		
	M750F003	288→70	30	9	107	16	102	17
			300	10	99	19		
		288→70	30	10	97	7	96	9
			300	10	95	10		
	M750F005	380→70	30	5	76	21	83	18
			300	5	89	15		
		380→70	30	15	98	18	108*	18*
			300	10	123	7		
	M750F006	356→259	30	10	102	8	103	9
			300	10	105	10		
		356→217	30	10	91	15	95	14
			300	10	99	13		
	M750F007	338→241	30	10	75	14	80	19
			300	10	86	20		
		338→241	30	15	99	13	106*	13*
			300	10	116	5		
	M750F008	356→259	30	10	97	16	95	16
			300	10	94	16		
		356→241	30	10	90	15	98	17
			300	10	105	15		

RSD = Relative standard deviation

*Matrix-Matched Standards were used.

Linearity	Acceptable linearity of $r \geq 0.9876$ was observed in the range of 0.006 ng/mL to 0.3 ng/mL.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the analytes in water. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions of the analytes. According to the method, at least one mass transition was used and confirmatory analysis was performed using a different LC-MS/MS column (C18 and phenyl column options are available).
Matrix effects	Matrix effects were tested preparing matrix-matched standards for each matrix and analyte. It was shown that the matrix-load in the tested matrix-matched standards of M750F005 and M750F007 in both matrix types had an influence on the detection. Therefore, matrix-matched standards are needed for further experiments.
Limit of quantification	The method has a limit of quantification (LOQ) of 30 ng/L per analyte, corresponding to the lowest fortification level.
Limit of detection	The limit of detection (LOD) is 6 ng/L for each analyte, corresponding to 20% of the LOQ.
Repeatability	The overall relative standard deviations (RSD) for all matrices, analytes and fortification levels were ≤ 20%, except for the analyte M750F005 with the mass transition 338->70 in drinking water (see Table 4.2-8).
Standard stability	The stability of the analytes in standard solutions has been determined. The storage stability data indicate that stock and fortification solutions of each analyte prepared in acetonitrile are stable for at least 3 months when held under refrigeration and in the dark, and that calibration standards prepared in acetonitrile / water (20/80, v/v) are stable for at least 1 month when held under refrigeration and in the dark.
Final volume stability	All analytes were considered to be stable in final volumes of surface and ground water samples over a time period of 5 days, when stored refrigerated and in the dark.
Reproducibility	Reproducibility of the method was determined within an independent laboratory validation study summarized in section CA 4.2/9 [BASF DocID 2015/7006199].

Conclusion

The method for analysis of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in water uses LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of BAS 750 F and its metabolites in surface and drinking water.

Report:	CA 4.2/9 Gu G. et al., 2016 a Independent laboratory validation of BASF analytical method D1506/01: Method for the determination of BAS 750 F (Reg. No. 5834378) and its metabolites M750F003 (Reg. No. 5924326), M750F005 (Reg. No. 6003433), M750F006 (Reg. No. 5863469), M750F007 (Reg. No. 6003432) and M750F008 (Reg. No. 6010286) in surface and drinking water by LC-MS/MS 2015/7006199
Guidelines:	EPA 850.6100, SANCO/825/00 rev. 8.1 (16 November 2010)
GLP:	yes (certified by United States Environmental Protection Agency)

Remark: The objective of this study was to independently validate the determination of BAS 750 F (Reg. No. 5834378) and five of its metabolites M750F003 (Reg. No. 5924326), M750F005 (Reg. No. 6003433), M750F006 (Reg. No. 5863469), M750F007 (Reg. No. 6003432) and M750F008 (Reg. No. 6010286) by means of LC-MS/MS in surface and drinking water. BASF method no. D1506/01 (DocID 2015/7006199) was independently validated in this study.

Principle of the method Residues of BAS 750 F and its metabolites were determined from surface and drinking water by dilution with acetonitrile. Subsequently, the samples were processed for final analysis using LC-MS/MS in the positive ion mode. The samples were injected twice, once with an XBridge BEH C18 column and again with an XBridge BEH Phenyl column for the confirmatory analysis of the metabolites M750F003, M750F005 and M750F007.

Recovery findings The method proved to be suitable to determine BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in surface water and drinking water. Samples were spiked at the limit of quantification of 30 ng/L and ten times higher (300 ng/L). The overall recovery values were between 70% and 110% for all matrices and analytes. The detailed results are given in Table 4.2-9.

Table 4.2-9: Results of the method validation for the determination of BAS 750 F and its metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in surface and drinking water

Matrix	Analyte	m/z	Fortification level [ng/L]	Number of replicates	Mean recovery [%]	RSD [%]	Overall recovery [%]	Overall RSD [%]
Surface water	BAS 750 F	398→70	30	5	89.3	23	104	20
			300	5	119	4.8		
		400→70	30	5	92.0	15	105	17
			300	5	119	7.2		
	M750F003	288→70	30	5	90.0	4.1	89.3	4.9
			300	5	88.6	6.0		
		288→70	30	5	115	3.5	106	10
			300	5	96.6	4.4		
	M750F005	380→70	30	5	91.7	3.2	88.4	4.7
			300	5	85.1	1.9		
		380→70	30	5	95.6	5.6	91.7	6.5
			300	5	87.7	4.1		
	M750F006	356→259	30	5	94.2	7.2	89.7	8.2
			300	5	85.2	6.0		
		356→217	30	5	91.8	4.3	88.8	6.0
			300	5	85.8	5.9		
	M750F007	338→241	30	5	117	4.4	101	17
			300	5	85.0	3.1		
		338→241	30	5	118	7.4	104	15
			300	5	90.2	4.2		
	M750F008	356→259	30	5	91.6	9.3	86.2	11
			300	5	80.9	8.9		
		356→241	30	5	92.9	5.9	85.7	9.9
			300	5	78.5	2.1		
Drinking water	BAS 750 F	398→70	30	5	104	4.2	103	3.6
			300	5	102	2.8		
		400→70	30	5	102	2.7	104	4.2
			300	5	106	4.7		
	M750F003	288→70	30	5	94.1	2.7	92.5	3.5
			300	5	91.0	3.7		
		288→70	30	5	99.5	1.9	98.4	3.0
			300	5	97.3	3.6		
	M750F005	380→70	30	5	95.6	3.5	95.3	3.2
			300	5	94.9	3.3		
		380→70	30	5	102	2.4	101	2.6
			300	5	99.4	2.5		
	M750F006	356→259	30	5	104	4.8	104	4.3
			300	5	105	4.2		
		356→217	30	5	105	4.2	104	4.3
			300	5	103	4.6		
	M750F007	338→241	30	5	102	4.2	101	4.1
			300	5	99.8	4.1		
		338→241	30	5	101	8.4	100	6.2
			300	5	99.6	3.6		
	M750F008	356→259	30	5	102	11	103	7.7
			300	5	103	4.5		
		356→241	30	5	107	6.4	104	4.9
			300	5	102	1.6		

RSD = Relative standard deviation

Linearity	For all analytes, standards were prepared covering the range of 6 ng/L to 200 ng/L. Good linearity (coefficient of determination [r^2] ≥ 0.98) was observed over this range.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions (at least one mass transition available and a different LC-MS/MS column (C18 and phenyl column options are available)), the method is specific for the determination of all six analytes in surface and drinking water.
Matrix effects	Matrix-matched standards for BAS 750 F in surface water were required due to significant matrix effects (≥ 20%). No significant matrix effects (< 20%) during the analyses of BAS 750 F in drinking water and the analyses of the metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 in both surface and drinking water were observed.
Interference	No significant interferences (> 30% LOQ) at the retention times and mass transitions of the analytes were observed.
Limit of quantification	The limit of quantification (LOQ) was defined as the lowest fortification level successfully tested. For this method, the LOQ was 30 ng/L for all analytes in both surface and drinking water.
Limit of detection	The limit of detection (LOD) was set at the lowest calibration standard, which was 6 ng/L (20% of LOQ).
Repeatability	The overall relative standard deviations (RSD) for all matrices, analytes and fortification levels were ≤ 20%.
Reproducibility	These results of the independent laboratory validation confirm the results of the validation study which is reported in section [see KCA 4.1.2/3 2015/7001125].
Conclusion	**The described method D1506/01 is considered suitable for the quantitative analysis and was successful independent validated for BAS 750 F and its metabolites M750F003 (Reg. No. 5924326), M750F005 (Reg. No. 6003433), M750F006 (Reg. No. 5863469), M750F007 (Reg. No. 6003432) and M750F008 (Reg. No. 6010286) in surface and drinking water.**

(c) Methods for the analysis in air

Report: CA 4.2/10
Obermann M.,Studenroth S., 2015 b
Validation of analytical method L0327/01, for the determination of BAS 750 F in air by LC-MS/MS
2015/1111330

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4, EPA 850.6100, OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method: The test item BAS 750 F is spiked onto the front filter of an adsorbent tube (ORBO™), air is passed over the filter and the test item is extracted from the adsorbent material with acetonitrile. The residues are extracted and reconstituted in acetonitrile/water (70/30, v/v) before final determination.
The samples are analyzed by LC-MS/MS at mass transition 398 → 182 for quantification and 398 → 133 for confirmation (ESI, positive mode). Analysis was accomplished on an Aquasil C18 column applying an acetonitrile / water gradient using 0.1% formic acid as modifier.

Recovery findings: In the tested matrix air, the mean recovery values for BAS 750 F were between 70% and 110% for both fortification levels and mass transitions. The detailed results are given in the table below.

Table 4.2-10: Results of the method validation for the determination of BAS 750 F in air

Analyte	Matrix Air	m/z	Fortification level [ng/L air]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
BAS 750 F	Orbo™ adsorbent	398 → 182	0.01	5	89	2.5	89	2.5
			0.1	5	89	2.8		
		398 → 133	0.01	5	88	2.1	89	2.5
			0.1	5	90	2.2		

RSD = Relative standard deviation

Linearity: Good linearity (r > 0.995) was observed in the range of 0.05 ng/mL to 5 ng/mL for the two mass transitions of BAS 750 F. At least seven calibration levels, prepared in acetonitrile/water (70/30, v/v), were injected.

Specificity: LC-MS/MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible at two different mass transitions.

Matrix Effects:	The matrix load in the tested matrix-matched standards had no influence on the analysis of BAS 750 F.
Interference:	Significant interferences (> 30% of LOQ) were not observed at the retention time and mass transitions of the analyte.
Limit of quantitation:	The method has a limit of quantitation (LOQ) of 0.01 ng/L air, corresponding to an amount of 5 ng active substance per 540 L air.
Limit of detection:	The limit of detection (LOD) for BAS 750 F is 0.002 ng/L air.
Repeatability:	The relative standard deviations (RSD, %) for all fortification levels were < 20%.
Reproducibility:	Reproducibility of the method was not tested within the validation study.
Stability of working solutions:	Stock and fortification solutions, prepared in acetonitrile, as well as calibration solutions, prepared in acetonitrile/water (70/30, v/v), of BAS 750 F were stable for at least 30 days, when stored refrigerated at 4°C.
Stability on adsorber material:	BAS 750 F was considered stable on the adsorber material over a time period of 7 days, when stored refrigerated at 4°C.
Conclusion:	**It could be demonstrated that the method L0327/01 fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of BAS 750 F in air.**

(d) Methods for the analysis in body fluids and tissues

Since BAS 750 F is not classified as toxic or very toxic, methods of analysis for parent or metabolites in human body fluids are not required according to SANCO/825/00 rev. 8.1. However, an analytical method for BAS 750 F in plasma is available. This method was validated and used in toxicological studies [see KCA 4.1.2/15, 2015/1186912] with a LOQ of 0.05 mg/L. Analytical methods for body tissues for BAS 750 F and M750F022 can be found in KCA 4.2/4 [2015/1240005] and KCA 4.2/6 [2015/1240006].

We create chemistry

BAS 750 F

Document M-CA, Section 5

TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Compiled by:

[redacted]

Telephone: [redacted]
E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
21/Mar/2016	MCA 5.2 (Acute toxicity) Clarification / justification for performing in vivo studies to assess the acute dermal toxicity, the skin irritation and eye irritation potential of BAS 750 F, and the rationale for performing the Guinea Pig Maximization test for assessing the skin sensitisation potential of BAS 750 F.	Document MCA Section 5 Version 1 BASF DocID 2016/1000814 replaced by revised Version BASF DocID 2016/1103852

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

The absorption, distribution, excretion and metabolism of BAS 750 F in mammals was investigated using the active substance radiolabeled either in the chlorophenyl ring (C-label), in the trifluoromethylphenyl ring (TFMP-label) or in the triazole moiety (T-label).
For all labels, a mixture of ^{14}C-radiolabeled, ^{13}C-labeled BAS 750 F and non-labeled BAS 750 F was used. The molecular structures and the positions of the labels are shown below:

BAS 750 F
= Reg. No. 5834378
= CAS No. 1417782-03-6

Figure 5.1/1 [Chlorophenyl-U-C14-]-BAS 750 F

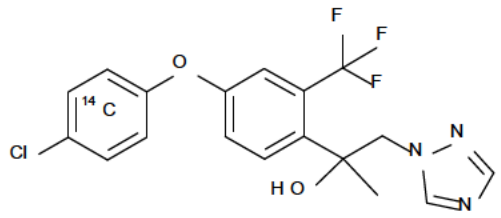


Figure 5.1/2 [Trifluoromethyl-ring-U-C14-]-BAS 750 F

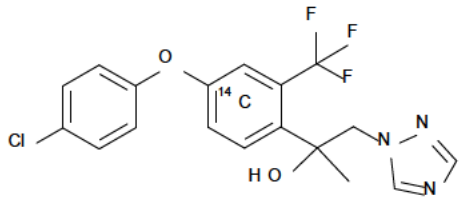


Figure 5.1/3 [Triazole-3(5)-C14-]-BAS 750 F

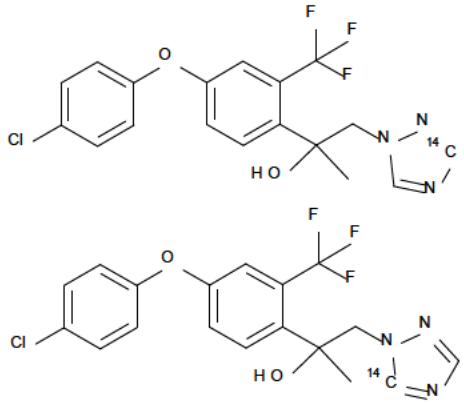


Figure 5.1/4 [Chlorophenyl-1-C13-]-BAS 750 F

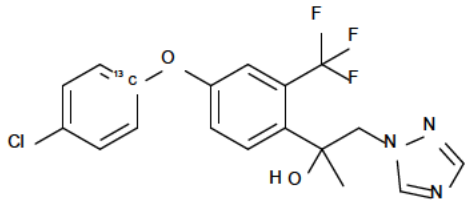


Figure 5.1/5 BAS 750 F, unlabeled

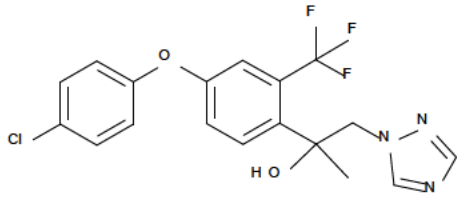
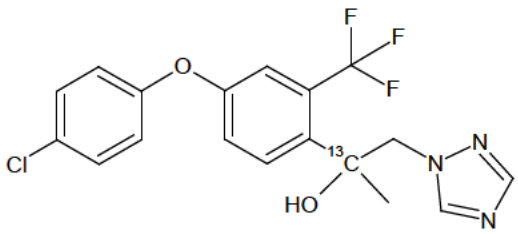


Figure 5.1/6: [Trifluoromethylphenyl-^{13}C]-BAS 750 F



CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Report:	CA 5.1.1/1 [redacted] 2015 a 14C-BAS 750 F (14C-Chlorophenyl and Trifluoromethylring-U-14C labels): Study on kinetics and excretion in Wistar rats after single and repeated oral administration 2015/1208128
Guidelines:	OECD 417, EPA 870.7485, EPA 860.1000, MAFF Testing Guidelines for Toxicology Studies: Metabolism Animals (Japan)
GLP:	yes (certified by Ministry of Health, Welfare and Sport, The Hague, The Netherlands)
Report:	CA 5.1.1/2 [redacted] 2016 a 14C-BAS 750 F (triazole-3(5)-C14) - Study on the biokinetics in rats 2015/1078847
Guidelines:	OECD 417, EPA 870.7485, (EC) No 440/2008 of 30 May 2008 - Part B No. L 142, JMAFF Guidelines on the Compiling of Test Results on Toxicity - Tests on In Vivo Fate in Animals (2001)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Report:	CA 5.1.1/3 [redacted] 2016 a Excretion and metabolism of 14C-BAS 750 F (Reg.No. 5834378) after oral administration in rats 2015/1107610
Guidelines:	EPA 870.7485, EPA 860.1000, OECD 417, EEC 87/302 B, JMAFF
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Absorption, distribution, metabolism, elimination and plasma kinetics of BAS 750 F (Reg. No. 5834378), was investigated in male and female rats. Three radiolabels were used in the course of the investigations (C-, TFMP- and T-label). The study was conducted according to OECD 417 and was divided into three separate studies. The biokinetics parts were performed separately for the C- / TFMP-label and the T-label, while the metabolism part was performed for all three labels (C- / TFMP- and T-label).

Biokinetics study (C- / TFMP-label)

In the **biokinetics study (C- / TFMP-label, DocID 2015/1208128)**, the report outlines the results of an investigation on the absorption, distribution, elimination and plasma kinetics of ^{14}C-BAS 750 F, labeled at the chlorophenyl or the trifluoromethyl moiety, in male and female Wistar rats.

Plasma kinetics were investigated after single oral (p.o.) administration. Target dose levels were 5 and 180 mg/kg bw. Both dose levels were also tested for mass balance, biliary excretion and tissue distribution experiments.

Taken together, BAS 750 F (either dosed as C-labeled or TFMP-labeled compound), was rapidly absorbed from the gastrointestinal tract after oral administration to male and female rats and was fast excreted via urine and feces. Excretion was more pronounced via feces. After multiple administration of the test substance (14 oral administrations with unlabeled BAS 750 F at 180 mg/kg bw and one oral administration with labeled BAS 750 F at 180 mg/kg bw, C-label), urinary excretion was comparable to single dosing.

Investigations on plasma kinetics demonstrate an internal exposure clearly related to the dosing regimen of BAS 750 F. In addition, male rats showed roughly a twice higher internal dose compared to female rats.

Based on bile excretion experiments, absorption of ^{14}C- BAS 750 F in male and female rats after single oral dosing was comparable for both dose levels and both labels and was calculated to be about 78% and 85% of dose for males and females at a dose level of 5 mg/kg bw C-label, about 67% and 64% of dose for males and females at a dose level of 180 mg/kg bw C-label and 71% of dose for both sexes at a dose level of 180 mg/kg bw TFMP-label.

Tissue distribution experiments showed for male and female animals that radioactive residue concentrations generally declined in organs and tissues parallel to the radioactive residues in plasma for low and high dose levels.

Biokinetics study (T-label)
In the **biokinetics study (T-label, DocID 2015/1078847)**, the report outlines the results of an investigation on the absorption, distribution, elimination and plasma kinetics of ^{14}C-BAS 750 F, labeled at the triazole moiety, in male and female Wistar rats.

Plasma kinetics was investigated after oral (p.o.) and intravenous (i.v.) administration. At the start of the study, kinetic parameters were investigated in order to see whether kinetic parameters related on the dose level. Target dose levels were 360, 120, 40 and 5 mg/kg bw (p.o.) and 0.4 mg/kg bw (i.v.).

For mass balance, biliary excretion and tissue distribution experiments, dose levels of 5 and 180 mg/kg bw were tested based on results of plasmakinetics and toxicological studies with single and repeated administrations.

^{14}C-BAS 750 F was rapidly absorbed from the gastrointestinal tract. Calculations based on the bile excretion experiments showed that about 85% of the administered dose were absorbed at a dose level of 5.0 mg/kg bw for both sexes whereas about 50% and 58% of the administered dose were absorbed for males and females at a dose level of 180 mg/kg bw. The excretion of radioactivity occurred mainly within three days after dosing with a lower excretion in urine than in feces, especially at the higher dose levels.

Plasma kinetics confirmed high absorptions and demonstrated potential enterohepatic recirculation, fast excretion and a more or less linear correlation of the internal exposure to the oral dose. Tissue distribution experiments showed a sublinear correlation between the radioactive residues in organs and tissues and the external dose.

Metabolism study

In the **metabolism study**, the excretion and metabolism of BAS 750 F was investigated in male and female rats after oral administration of a single dose of 5 and 180 mg/kg body weight (bw) and after oral administration of multiple dose (14 + 1) x 180 mg/kg body weight (bw) BAS 750 F. The test item was ^{14}C-radiolabeled either in in the chlorophenyl ring (C-label), in the trifluoromethylphenyl ring (TFMP-label) or at the triazole moiety (T-label).

In the metabolism part **(BASF DocID 2015/1107610)**, urine, faeces, tissue and plasma samples were collected from male and female rats of dose groups DX (180 mg/kg bw), V (5 mg/kg bw) and W (180 mg/kg bw) of the C- and T-label, respectively. In addition, urine, faeces and bile samples from the two biokinetics studies (BASF DocID 2015/1208128, C- and TFMP-label and BASF DocID 2015/1078847, T-label) after oral administration of BAS 750 F were examined.

For dose groups DXM and DXF, urine was collected after 6, 12, 24, 48, 72, 96, 120, 146 and 168 hours (± 3) hours. Faeces of these dose groups were collected initially after 12 and 24 hours and subsequently in intervals of 24 hours (± 2 hours) up to 168 hours (± 2 hours). Bile was collected in time intervals of 3 hours for up to 168 hours. All animals of dose groups VM, VF, WM and WF (both labels) were sampled one hour after dosing and the respective samples were taken. Sampling for the other dose groups is described in the corresponding biokinetics studies.

Identification of metabolites

The parent compound BAS 750 F and 68 biotransformation products were identified by HPLC-MS/MS and NMR analysis for structure elucidation. Confirmatory HPLC-MS analysis was used as second system for peak assignment. Additional, co-chromatography experiments and comparison of peak patterns were performed. For structure elucidation using HPLC-MS/MS, samples were chosen that exhibited representative peak patterns, include male and female dose groups and represented all labels. High dose groups were chosen to ensure sufficient amounts of radioactivity for the detection of metabolites and representative samples of the three most important matrices to provide a comprehensive view on the identified metabolites and potential specificity. Time intervals of the samples represented high amounts of radioactivity.

Excretion of radioactive residues

In the metabolism part, the excretion of radioactive residues *via* urine and faeces was rapid and comparable for both sexes. The major excretion of radioactive residues was observed within 48 hours. The majority of the radioactive residues (59 - 90% of the dose) were excreted *via* faeces. Smaller portions were excreted *via* bile (34 - 76% of the dose) or urine (4 - 41% of the dose).

Metabolite patterns in urine
For the C-label, comparable metabolite pattern for the dose groups D and DX were observed for male and female rats, respectively. For the dose groups DM and DXM, the main common metabolites were detected in comparable abundances of up to 1% of the dose, respectively: M750F049 - 19.0 min, M750F050 and M750F058 / M750F081 - 34.4 min. These metabolites were either abundant or not present in female rats. For female rats a broader spectrum of assigned metabolites was detected.
For the TFMP-label and the T-label, the metabolite pattern were largely comparable. For the TFMP-label, the main components in DM and DF were M750F054 and M750F049 - 19.0 min / M750F003.
Despite the variety of metabolites detected in female rats (C-label), the overall metabolic pathway of the two genders is comparable for urine for all three labels.

For the investigated dose groups of the T-label, the main compound detected within the periods of investigation was M750F001, with the minor exception of dose group BF, where the abundance of M750F001 ranged slightly below M750F049 - 19.0 min. Usually, the abundance of M750F001 was higher in male rats than in female rats.
For the T-label and comparable dose groups DM and DXM, the main common metabolites were detected in highly similar concentrations: M750F001 (9.6% and 10.5% of the dose, respectively), M750F071 and M750F054 (<5% of the dose, respectively). This was also the case for dose groups DF and DXF and the metabolites M750F001 and M750F054 (<5% of the dose, respectively). Additional metabolites were detected in both groups, DF and DXF, albeit in varying metabolite combinations at smaller concentrations.

Metabolite patterns in faeces
All dose groups of all labels showed a comparable metabolite pattern with M750F015 (ranging from 10 - 41% of the dose), M750F016 / M750F017 (ranging from 15 - 32% of the dose) and the parent compound BAS 750 F (ranging from 3 - 35% of the dose) as the major components. Metabolite M750F003 was detected at lower amounts in all dose groups of the TFMP- and the T-label. Except for dose group BM and BF, the parent compound was the most abundant component for T-label. For the C- and TFMP-label, M750F015 and / or M750F016 / M750F017 were usually more abundant than the parent compound. Besides the parent compound, only metabolites that have been once subjected to hydroxylation or which results from cleavage of the parent compound were excreted. Overall the metabolic pathway of the two genders and all labels is highly comparable in faeces.

Metabolite patterns in bile
Metabolites M750F035, M750F044, M750F045, M750F049 (including isomers) and M750F087 constituted the main portions of radioactive residues in bile samples of all labels (ranging in combination from 22 - 53% of the dose). These metabolites are either once or twice hydroxylated parent compounds, which have been subsequently subjected to glucuronidation. For all labels, these compounds were usually detected in large amounts within 0 - 6 hours. No hydroxylated parent compound or sulfate conjugates except in minor traces (up to 0.7% of the dose) for dose group RM, SM and SF1 (C-label) and SM (TFMP-label) were detected. Overall, the metabolic pathway of the two genders and all labels is highly comparable in bile.

Metabolite patterns in tissues and plasma
In general, the amount of radioactive residues was highest in liver (up to 9.5% of the dose) and <1% of the dose for other tissues and plasma (C- and T-label). The major part of metabolites were detected as hydroxylated parent compounds or as unchanged parent compound. Minor amounts were detected as glucuronide or sulfate conjugates of dihydroxylated parent compound. No gender specific differences were observed in the residue composition of tissues and plasma.

Proposed metabolic pathway
The identified metabolites comprise the following phase I conversions of the parent compound: mono, di- and trihydroxylation including CL-shift, methylation, and cleavage of the ether group or of the triazole ring from the parent compound. For phase II metabolism, phase I metabolites are either subjected to sulfation, glucuronidation and / or GSH adduction, which also includes corresponding decomposition products.

Enantiomer ratio of BAS 750 F
The relative amounts of the isomers were approximately 1:1 in the application formulation and remained highly similar in the methanol extracts of faeces. In the methanol extracts of liver and kidney as well as in plasma, the ratio between S- and R-enantiomer shifted towards a higher relative amount of the R-enantiomer.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F
Chemical Name: (2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Batch/purity #: see Table 5.1.1-1

Table 5.1.1-1: Test substance characteristics

Test item	Batch-No.	Radiochem. purity [%]	Chemical purity [%]	Specific radioactivity (MBq/mg)	Used in experiment / test group
chlorophenyl-U-C14	CFQ41561	98.9	99.1	7.9	Metabolism C-label
chlorophenyl-U-C14	1075-1001	99.3	-	8.1	Biokinetics C-Label
trifluoromethylring-U-C14	CFQ42039	98.3	-	8.3	Biokinetics TFMP-Label
trifluoromethylring-U-C14	CFQ42195	97.9	-	7.6	Biokinetics TFMP-Label
triazole-3(5)-C14	1062-1101	>98	-	5.4	Biokinetics T-Label
triazole-3(5)-C14	1062-2001	98.8	98.9	5.5	Biokinetics T-Label, Metabolism T-label
triazole-3(5)-C13	1077-1001	-	97.1	-	Biokinetics T-Label, Metabolism T-label
chlorophenyl-1-C13	RS4-2012-173A2	-	97.7	-	Biokinetics C-Label, Metabolism C-label
propyl-2-C13	1126-1006	-	99.5	-	Biokinetics TFMP-Label
unlabeled BAS 750 F	COD-001662	-	95.5	-	Biokinetics T-Label (only plasmakinetics)
unlabeled BAS 750 F	COD-001740	-	98.8	-	Biokinetics C-Label, Biokinetics TFMP-Label, Metabolism C-label, Metabolism T-label

Stability of test compound:

For the biokinetics study (C- and TFMP-label), the stability of the test substance in the test substance preparations over the test period was verified by HPLC analyses. Concentrations of the test substance in the test substance preparations were in close agreement with the target and nominal concentrations according to radioactivity analysis.

For the biokinetics study (T-label), the stability of the non-labeled test item was guaranteed for the duration of the study.
The stability, homogeneity and correctness of the concentrations of the test item in the test-substance preparations were confirmed by analyses.

The analytical investigations performed within the metabolism study demonstrated the specific radioactivity, purity, stability and homogeneity of ^{14}C- BAS 750 F in the vehicle for the performed experiments.

2. Vehicle: 0.5% Carboxymethylcellulose (CMC)

3. Test animals:

Species: Rat

Strain: Wistar / Crl:WI (Han)

Sex: male and female

Age:

Biokinetics study (C- and TFMP-label)

9-15 weeks

Biokinetics study (T-label)

at least 7 weeks at start of acclimatization period

Metabolism study

8 weeks at start of acclimatization period

Weight on day 0:

Biokinetics study (C- and TFMP-label)

Animals were at least 200 gram in case of blood sampling or bile collection.

♂: ca. 267 - 299 g; ♀: ca. 178 - 208 g

♂: ca. 216 - 414 g; ♀: ca. 175 - 255 g

Biokinetics study (T-label)

about 169 – 373 g

♂: ca. 246 - 373 g; ♀: ca. 169 - 297 g

	Metabolism study about 171 - 314 g ♂: ca. 233-314 g; ♀: ca. 171-225 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimatization period:	At least 5 days (Biokinetics study, C and TFMP- label). At least 7 days (Biokinetics study, T-label, and metabolism study).
Diet:	**Biokinetics study (C- and TFMP-label)** Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiaeten GmbH, Soest, Germany). **Biokinetics study (T-label)** Kliba maintenance diet for mouse/rats "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum **Metabolism study** Kliba 3433, "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Husbandry conditions during the in-life phase performed in the Netherlands were performed in an AAALAC-approved laboratory in accordance with the Dutch Animal Welfare Act and the effective European Council Directive (Biokinetics study, C- and TFMP-label). Husbandry conditions during the in-life phases performed in Germany were in accordance with the German Animal Welfare Act (Biokinetics study, T-label, and metabolism study). **Biokinetics study (C- and TFMP-label)** Group housing (up to 4 animals during acclimatization) in Macrolon cages (type MIV) containing sterilized sawdust bedding (Lignocel S 8-15, JRS GmbH + CO. KG, Germany). Paper was provided as nest material (Envirodri, Wm. Lilico & Son, United Kingdom). Single housing in metabolism cages or Macrolon cages during the experiment. Rats of ADME groups (Group 3/10, 4, 7, 11/14) were individually housed in stainless steel metabolism cages (LxWxH = 18.5x19x20 cm) with a grid. Two males of Group 3/10, 4 and 11/14 were individually housed in stainless steel metabolism cages (LxWxH = 18.5x19x20 cm) with a plexiglas lid.

Biokinetics study (T-label)

Group housing (5 animals/cage) in polysulfonate cages (H-Temp, PSU, 2065 cm², TECNIPLAST, Germany) Enrichment: Play tunnel (Plexx b.v., The Netherlands); wooden gnawing blocks (Type NGM E-022, Austria)

Biliary excretion experiment: After surgery until administration individual housing in polycarbonate type M III cages (floor area of about 800 cm², Becker & Co., Germany)

Plasmakinetic and tissue-distribution experiments: group housing in in steel wire mesh cages

Balance and bile excretion experiment after dosing: individual housing in all-glass metabolism cages (type Metabowl, Jencons Leighton Buzzard, U.K.)

Metabolism study

Acclimatization and prior to dosing: During acclimatization, animals were held in macrolon cages (type 4).

During the experiments of the dose groups DX the rats were placed individually in plexiglas metabolism cages and for dose groups V and W in macrolon cages (type 3).

Environmental conditions:

Biokinetics study (C- and TFMP-label)

Temperature:	18 – 24°C
Humidity:	40 – 70%
Air changes:	Fully air-conditioned rooms, at least 10 air changes/hour
Photoperiod:	Alternating 12-hour light and dark cycles, light from 7 am to 7 pm

Biokinetics study (T-label)

Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photoperiod:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

Metabolism study

Temperature:	19 – 24°C
Humidity:	31 – 72%
Air changes:	air-conditioning
Photoperiod:	Alternating 12-hour light and dark cycles

4. Preparations of dosing solutions:

a. Biokinetics study (C- and TFMP-label)

Appropriate amounts of the radiolabeled test substance solution were taken and the organic solvent acetonitrile (ACN) was evaporated to dryness when the labeled test substance was added as a solution (the trifluoromethylring-U-^{14}C- BAS 750 F batch CFQ42039 was delivered as a solid). Unlabeled and stable isotope (if required) material was evaporated to dryness when added as a solution (for low dose groups; stock solutions of unlabeled BAS 750 F were prepared separately). The aqueous vehicle (0.5% carboxymethylcellulose (CMC) in milli-Q water) was added to the test substance(s).

For the C-label, the blending ratio of labeled, stable isotope and unlabeled compound was 1+1+1 (Group 10 and 5), 1+8+16 (Group 4, 6, 13 and 7), 1+0+5 (Group 1 and 8) and 1+0+99 (Group 2 and 9). For the TFMP-label, the blending ratio of labeled, stable isotope and unlabeled compound was 2+49+49 (Group 12 and 14).

Preparations were homogenized by ultra-turrax, ultra-sonication, vortexing and continuous stirring. At least before start and at the end of the administration, samples were taken to determine the amount of radioactivity in the preparations to demonstrate the correct concentration of the test substance, its homogeneity and the radiochemical purity of prepared ^{14}C- BAS 750 F.

b. Biokinetics study (T-label)

Test-substance preparations were prepared with two different batches of the radio-labeled test substance, for the plasmakinetic experiments and the bile excretion, balance and tissue distribution experiments, respectively. If needed, test substance solutions in acetonitrile were prepared and were used for further preparation steps.

In order to achieve the required specific activity, appropriate amounts of the radio-labeled, non-labeled and, if foreseen ^{13}C-labeled test substance were taken (if added as solution the organic solvent acetonitrile was evaporated to dryness before further processing) and the aqueous vehicle (0.5% carboxymethylcellulose (CMC) in tap water) was added.

Due to the possibility to facilitate structure elucidation of formed metabolites in balance and bile excretion experiments for the high dose group, ^{13}C- BAS 750 F was mixed with non-labeled test substance in a ratio of 1:2 (w:w). In order to achieve the required specific activity of 0.3 MBq/mg, respective amounts of radio-labeled test substance were added. For the low dose group appropriate amounts of radio-labeled, ^{13}C-labeled and unlabeled test substance were mixed in a ratio of about 1:1:1 (w:w:w). For calculations a density of 1 g/mL is assumed for the preparations in 0.5% CMC in drinking water and rat plasma.

The preparations were homogenized by continuous stirring and, if necessary by additional ultra-sonication. The stability, homogeneity and correctness of the concentrations of the test item in the test substance preparations were confirmed by analysis.

c. Metabolism study
Details of the preparation procedure for dose groups B, D and C as well as R and S are described in the biokinetics study parts.

For dose groups DX, V and W and each label, appropriate weights of labeled (^{14}C- and ^{13}C-) and unlabeled BAS 750 F were mixed in order to achieve the required specific activity. 0.5% aqueous CMC were added to generate the application formulation.
The single oral doses were weighed into syringes and administered orally by gavage. The amount of the administered application formulation was calculated by weighing the syringes before and after application.

Samples were taken to check the amount of radioactivity in the preparation and to demonstrate the stability, homogeneity and the correctness of the concentration of the test substance in the preparation. For the confirmation of the purity of the test items, HPLC analysis was performed for each label, respectively. In order to confirm the identity and isotope pattern of the test item, aliquots of the application formulations were subjected to MS analysis.

B. STUDY DESIGN AND METHODS

1. Dates of experimental work:

Biokinetics study (C- and TFMP-label):	24-Jun-2014 to 19-May-2015
Biokinetics study (T-label):	02-Oct-2012 to 01-Mar-2014
Metabolism study (C-, TFMP- and T-label)	16-Apr-2013 to 16-Nov-2015

2. Study Design:
Three separate studies have been performed to investigate the biokinetics and metabolism of BAS 750 F. Samples of five dose groups of the two biokinetics studies were also used for the metabolism study.

In a biokinetics study (C- and TFMP-label), a series of experiments were carried out with a total of 12 test groups (up to group 14, including two replacements of test groups) to investigate plasmakinetics, bioavailability, distribution and excretion after oral gavage administration of radiolabeled BAS 750 F.
For oral dosing (plasmakinetics), the dosing was performed at dose levels of 5 and 180 mg/kg bw. For balance, bile and tissue distribution experiments, the dosing was also performed at dose levels of 5 mg/kg bw (C-label) and 180 mg/kg bw (both labels).
The study design for this biokinetics study is summarized in Table 5.1.1-2, Table 5.1.1-3, Table 5.1.1-4 and Table 5.1.1-5.

In a separate biokinetics study (T-label), a series of experiments were carried out with a total of 12 test groups (experiments 1 - 12) to investigate plasmakinetics, bioavailability, distribution and excretion after oral gavage administration of radiolabeled BAS 750 F.
For intravenous dosing (plasmakinetics), the dosing was performed at a dose level of 0.4 mg/g in rat plasma. For oral dosing, the dosing was performed at dose levels of 360, 120, 40 and 5 mg/kg bw.

For balance experiments, the dosing was performed at dose levels of 180 mg/kg bw and 5 mg/kg bw. For bile experiments, the dosing was performed at a dose level of 180 mg/kg bw. For the tissue distribution experiment, the dosing was performed at dose levels of 180 mg/kg bw and 5 mg/kg bw.
The study design for this biokinetics study is summarized in Table 5.1.1-6, Table 5.1.1-7, Table 5.1.1-8 and Table 5.1.1-9.

In a metabolism study, the nature of the biotransformation products of BAS 750 F in excreta, bile, liver, kidney, fat and plasma of rats after oral administration of the test substance at two dose levels was performed (5 and 180 mg/kg bw). Urine and faeces samples (dose groups B, C and D) and bile samples (dose groups R and S) originating from the biokinetics studies were used for the determination of the metabolic patterns. Three additional groups of animals (designated DX, V, and W) were dosed specifically for the metabolism study and urine, faeces, plasma, liver, fat and kidney samples were generated for isolation and identification of metabolites. The experiments were performed with ^{14}C-BAS 750 F labeled either in the chlorophenyl or the triazole moiety.
The study design for the metabolism study is summarized in Table 5.1.1-10.

a. Biokinetics study (C- and TFMP-label)

Blood/Plasma level
Blood was collected from all rats at the following time points: 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours after dosing.

The animals were transferred to an incubator set at 40°C approximately half an hour prior to blood sampling. Water was available in the incubator. The rats were maintained in restrainers during all blood sampling times. All blood samples were placed on melting ice immediately after sampling. A sub sample was removed for total radioactivity analysis. The remaining blood was centrifuged within 1 hour to obtain the plasma.

Table 5.1.1-2: Design of blood / plasma level study (C-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Group 1	oral, 1x	1.46 - 2.35	5	3 ♂+ 3 ♀	Analysis of total radioactivity in blood/plasma
Group 2	oral, 1x	2.98 - 5.37	180	3 ♂+ 3 ♀	

Dose volume oral: 10 mL/kg bw

Balance/Excretion experiments

Urine and feces were collected over the following time intervals: pre-dose (-24-0 h; not group 7 and 14), 0-6, 6-12, 12-24 and in 24-hour intervals thereafter, until max 168 hours.

In the balance experiment of groups 10, 4 and 14, the first two male animals were placed in closed metabolism cages in order to collect exhaled air for at least 48 h. Since <0.2% of the total radioactive dose was detected in exhaled air, all experiments were carried out in open systems. After 168 h, animals were sacrificed.
Each animal was deeply anaesthetized using isoflurane. By means of an aorta punction the maximum possible amount of blood was withdrawn, causing the animals to be exsanguinated.
Sampled blood was transferred into tarred tubes containing Li-heparin. A weighed subsample of the heparinized blood was removed for total ^{14}C analysis. The remaining blood was centrifuged to obtain the plasma.

Table 5.1.1-3: Design of balance / excretion experiments (C- and TFMP-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
C-label					
Group 10 (Dose group B)	oral, 1x	2.96 - 4.96	5	4 ♂+ 4 ♀	Analysis of total radioactivity to determine mass balance
Group 4 (Dose group D)	oral, 1x	10.75 - 19.22	180	4 ♂+ 4 ♀	
Group 7 (Dose group C)	oral, 14 + 1 x	11.60 - 19.37	180	4 ♂+ 4 ♀	
TFMP-label					
Group 14 (Dose group D)	oral, 1x	4.07 - 6.36	180	4 ♂+ 4 ♀	Analysis of total radioactivity to determine mass balance

Dose volume oral: 10 mL/kg bw; radioactivity per animal

The following organs / tissues were checked for remaining radioactivity:

heart	Residual carcass	adipose tissue
liver	muscle	stomach and stomach contents
spleen	kidney	thyroid gland
bone	testes	adrenal glands
skin	brain	blood cells and plasma
lung	pancreas	gut and gut contents
ovaries	uterus	bone marrow

For balance estimates, the cage wash was also checked for radioactivity.

Samples from muscle and bone were collected from the left hind leg.

Tissue distribution

Three animals were sacrificed at 4 defined time points after dosing. These animals were selected based on the results of the blood and plasma kinetics experiments. The time points selected correspond to the following time points in plasma kinetics: maximum plasma concentration MPC, 1/2 MPC, 1/4 MPC and 1/8 MPC at the high and low dose level.

Each animal was deeply anaesthetized using isoflurane. By means of an aorta punction the maximum possible amount of blood was withdrawn, causing the animals to be exsanguinated. Sampled blood was transferred into tarred tubes containing Li-heparin. A subsample of the heparinized blood was removed for total ^{14}C analysis. The remaining blood was centrifuged to obtain the plasma.

Table 5.1.1-4: Tissue distribution experiments (C-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Group 8	oral, 1x	1.33 - 2.51	5	12 ♂+ 12 ♀	Three animals were sacrificed at 4 defined time points after dosing, which correspond to the following time points in plasma kinetics: maximum plasma concentration MPC, 1/2 MPC, 1/4 MPC and 1/8 MPC at the high and low dose level.
Group 9	oral, 1x	2.88 - 5.05	180	12 ♂+ 12 ♀	

Dose volume oral: 10 mL/kg bw

The following organs / tissues were checked for remaining radioactivity:

heart	Residual carcass	adipose tissue
liver	muscle	stomach and stomach contents
spleen	kidney	thyroid gland
bone	testes	adrenal glands
skin	brain	blood cells and plasma
lung	pancreas	gut and gut contents
ovaries	uterus	bone marrow

Samples from muscle and bone were collected from the left hind leg.

Excretion via bile

Bile-duct catheterized animals were ordered from Charles River. They were equipped with the Dual VAH™ system (Instech Solomon, Plymouth Meeting, PA). Bile replacement fluid (18 mg/mL sodium cholate hydrate and 1.3 mg/mL of sodium bicarbonate, pH between 7.4 and 7.8, Sigma Aldrich Chemie GmbH, Germany) was infused into the duodenum during the experiment at a rate of 0.9 – 1.25 mL/hour.

Bile, urine and feces were collected over the following time intervals:
pre-dose (-24-0 h; not for Group 5 and 12), 0-3, 3-6, 6-9, 9-12, 12-18, 18-24 hours, and in 6-hour intervals thereafter, until at least 72 hours after dosing. From 72 hours onwards, urine, feces and bile were collected in 12-hour intervals until max. 168 hours after dosing, depending on the health state of the animals and the excretion rate.

After 168 h (or earlier, depending on health status of the animal), animals were sacrificed using isoflurane anesthesia. The following tissues and organs were harvested:
residual carcass
stomach and stomach contents
gut and gut contents

For balance estimates, the cage wash was also checked for radioactivity.

Table 5.1.1-5: Bile excretion experiments (C- and TFMP-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
C-label					
Group 5 (Dose group S)	oral, 1x	2.89 - 3.50	5	4 ♂+ 4 ♀	Analysis of total radioactivity to determine mass balance. Animals of dose Group 13 were dosed to complete the high dose group (Group 6).
Group 6 (Dose group R)	oral, 1x	12.7 - 15.0	180	2 ♂+ 2 ♀	
Group 13 (Dose group R)	oral, 1x	11.9 - 13.4	180	3 ♂+ 3 ♀	
TFMP-label					
Group 12 (Dose group S)	oral, 1x	6.92 - 8.75	180	4 ♂+ 4 ♀	Analysis of total radioactivity to determine mass balance

Dose volume oral: 10 mL/kg bw; radioactivity per animal

b. Biokinetics study (T-label)

Plasmakinetics (blood/plasma level)

Plasma kinetics was investigated after oral (p.o.) and intravenous (i.v.) administration. It was of special interest to evaluate kinetic parameters as a function of dose, which may become relevant for dose setting in kinetics and toxicological studies. Target dose levels were 360, 120, 40 and 5 mg/kg bw (p.o.) and 0.4 mg/kg bw (i.v.). Four and six animals per gender and dose group were treated for p.o. and i.v. administration, respectively, and blood samples (100 - 200 µL) were taken under isoflurane anesthesia from the retro-orbital sinus directly after administration and 0.5 hours (intravenous dosing) and 1, 2, 4, 8, 24 hours as well as daily until 168 hours after intravenous and oral dosing. The concentrations of the radioactive residues in plasma and blood cells were analyzed by LSC.

Table 5.1.1-6: Design of blood / plasma concentration / plasmakinetic study (T-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Exp. 1	oral, 1x	0.7 - 1.3	360	4 ♂+ 4 ♀	Blood samples (100 - 200 µl) were taken from the retro-orbital sinus at the following time points after dosing or by exsanguination at the last time point: directly after treatment and 0.5 (exp. 4 only) and 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours (all dose groups)
Exp. 2	oral, 1x	0.9 – 1.4	120	4 ♂+ 4 ♀	
Exp. 3	oral, 1x	0.9 – 1.7	40	4 ♂+ 4 ♀	
Exp. 5	oral, 1x	1.1 – 1.3	5	4 ♂+ 4 ♀	
Exp. 4	i.v., 1x	0.4 – 0.7	0.4	6 ♂+ 6 ♀	

Dose volume oral: 10 mL/kg bw; intravenous (i.v.): 1 mL/kg bw

Balance/Excretion experiments

Four animals per gender were tested at dose levels of 5 or 180 mg/kg bw. In experiment 8, rats were treated daily for 14 days with non-radiolabeled BAS 750 F at a dose of 180 mg/kg bw followed by a single treatment of radiolabeled BAS 750 F on day 15.

Urine was collected after 6, 12 and 24 hours and subsequently in 24 hour time intervals up to 168 hours and feces in 24 hour time intervals up to 168 hours. In the balance experiment of the low dose, the first two male animals were placed in closed metabolism cages in order to additionally collect exhaled air for up to 48 h. Since less than 2% of the total radioactive dose were detected in exhaled air, all experiments were carried out in open systems.

Table 5.1.1-7: Design of balance / excretion experiments (T-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Exp. 7 (Dose group B)	oral, 1x	2.3 - 2.8	5	4 ♂+ 4 ♀	Metabolic cages were used to sample urine only at 6 and 12 h, and both urine and feces at 24-h intervals up to 7 days (168 h) after radioactivity dosing. Expired air was collected from two males for 48 hours. At sacrifice the remaining radioactivity was determined in cage wash, selected organs and in the remaining carcass.
Exp. 6 (Dose group D)	oral, 1x	16.4 - 17.9	180	4 ♂+ 4 ♀	
Exp. 8 (Dose group C)	oral, 14 + 1 x	16.0 - 25.1	180	4 ♂+ 4 ♀	

Dose volume oral: 10 mL/kg bw; radioactivity per animal

After 168 hours following treatment with radiolabel, animals from all groups were sacrificed and the following tissues were checked for remaining radioactivity:

heart	carcass	adipose tissue
liver	muscle	stomach & stomach contents
spleen	kidney	thyroid gland
bone	testes	adrenal glands
skin	brain	blood cells and plasma
lung	pancreas	gut and gut contents
ovaries	uterus	bone marrow

For balance estimates the cage wash was also checked for radioactivity

Tissue distribution

Three animals per gender at dose levels of 5 or 180 mg/kg bw were sacrificed at four different time points after oral dosing to achieve information of time dependent decrease of radioactivity in blood, organs and tissues. The time points were selected according to the results of the plasmakinetic experiments, and corresponded to the time point of maximum plasma concentration (MPC), 1/2 MPC, 1/4 MPC and 1/8 MPC. After sacrifice remaining radioactivity was measured in the following organs and tissues:

heart	carcass	adipose tissue
liver	muscle	stomach & stomach contents
spleen	kidney	thyroid gland
bone	testes	adrenal glands
skin	brain	blood cells and plasma
lung	pancreas	gut and gut contents
ovaries	uterus	bone marrow

Table 5.1.1-8: Tissue distribution experiments (T-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Exp. 12	oral, 1x	1.2 - 2.0	5	12 ♂+ 12 ♀	Groups of three rats/sex/dose were sacrificed at time points that correspond to max. plasma concentration C_{max}, and the subsequent decline: $C_{max}/2$, $C_{max}/4$ or $C_{max}/8$. Concentrations in blood and selected tissues were subsequently determined
Exp. 11	oral, 1x	1.5 - 2.1	180	12 ♂+ 12 ♀	

Dose volume oral: 10 mL/kg bw

Excretion via bile

At least six animals per gender were tested at dose levels of 5 or 180 mg/kg bw. Following cannulation of bile ducts, the animals were dosed, placed in metabolism cages and bile was collected in 3 hour intervals as well as urine and feces in 24 hour time intervals up to 72 hours, depending on the health state of the animals and the excretion rate.

Table 5.1.1-9: Bile excretion experiment (T-label)

Test group	Route, no. of doses	MBq / rat	Dose (mg/kg bw)	Number of rats / gender	Remarks
Exp. 10 (Dose group R)	oral, 1x	2.0 - 3.3	5	6 ♂+ 10 ♀	Via surgery, bile ducts of rats were cannulated and the duodenum were catheterized to allow for supplementation of bile acid. Following oral treatment, bile was sampled at 3 hour intervals, and urine and feces at 24 hour intervals. After 72 hours, animals were sacrificed, residual radio–activity was determined in the cage wash, stomach and stomach contents, gut and gut contents, and carcass.
Exp. 9 (Dose group S)	oral, 1x	15.9 - 21.7	180	11 ♂+ 6 ♀	

Dose volume oral: 10 mL/kg bw

c. Metabolism study (C-, TFMP- and T-label)

Table 5.1.1-10: Dose groups within the metabolism study with ^{14}C-BAS 750 F

Dose Group	Origin	Nominal Dose [mg/kg bw]	Use	Comments
BM, BF	GB/T / WIL Research	5	- Metabolite patterns in urine and faeces extracts (single low dose)	Male and female (n=4) C-label, T-label
DM [1], DF [1]	GB/T / WIL Research	180	- Metabolite patterns in urine and faeces extracts (single high dose)	Male and female (n=4) C-label, TFMP-label, T-label
DXM [2] , DXF [2]	APD/EC	180	- Metabolite patterns in urine and faeces extracts (single high dose)	Male and female (n=10) C-label, T-label
CM [1], CF [1]	GB/T / WIL Research	(14 + 1) x 180	- Balance and excretion, metabolite patterns in urine and faeces extracts (multiple dose)	Male and female (n=4) C-label, T-label
RM, RF [1]	GB/T / WIL Research	5	- Metabolite patterns in bile (single low dose)	Male and female C-label (n=4), T-label (n=6)
SM [1, 3], SF [3]	GB/T / WIL Research	180	- Metabolite patterns in bile and / or urine (single high dose)	Male, C-label (n=4), female, C-label (n=2+3) Male and female TFMP-label (n=4), T-label (n=6)
VM, VF	APD/EC	5	- Metabolite patterns in liver extracts, kidney and plasma (single low dose)	Male and female (n=4) C-label, T-label
WM, WF	APD/EC	180	- Metabolite patterns in liver extracts, kidney and plasma (single high dose)	Male and female (n=4) C-label, T-label

Sample generation for biokinetic investigations for the T-label was performed at GB/T (formerly named GV/T); sample generation for the C-label and the TFMP-label at WIL Research. Sample generation for the metabolism investigations (T- and C-label) were performed at APD/EC as described within the metabolism study.

[1] For the T-label, the experiments for dose groups DM, DF, CM, CF, RF and SM were repeated. The resulting samples replace those of the first experiment. Samples of the first experiment were consequently not analysed.
[2] Dose groups DM and DF were additionally covered by dose groups DXM and DXF, due to higher amounts of sample material and more detailed elucidation of metabolites
[3] For the C-label, the experiments for dose group SF were repeated with three additional animals.
n: number of animals

Material from the animal groups described in Table 5.1.1-10 was collected. Groups B, C, D, R and S were dosed in the two separate biokinetics studies.

Sampling and storage
All samples, which were generated at other locations than Limburgerhof, were stored in a freezer and were transferred in frozen condition to the BASF Agricultural Center, Limburgerhof. At the Agricultural Center, samples that were either generated at other locations or within the metabolism study were stored at -10 °C or below prior to analysis.

Urine and faeces from dose group DX (C- and T-label) were collected after 6, 12, and 24 hours and thereafter in intervals of 24 hours for up to 171 hours (± 3 hours). Faeces of these dose groups were collected initially after 12 and 24 hours and subsequently in intervals of 24 hours (± 2 hours) up to 168 -171 hours.

Determination of radioactivity
Appropriate aliquots of liquid samples were mixed with scintillator (Lumasafe Plus, IRGA-Safe Plus and Hionic Fluor, all Perkin Elmer) and measured by liquid scintillation counting (LSC). For solid samples, appropriate aliquots were homogenized and combusted using an automated sample oxidizer. The $^{14}CO_2$ formed during combustion was trapped by an absorption liquid and the collected radioactivity was measured by LSC.

HPLC analysis
For the determination of the metabolic patterns, metabolite identification, and quantitation of metabolites different HPLC methods were applied. Different stationary phases with gradient elution were used for metabolite profiling and quantitation of metabolites. For identification and structure elucidation of metabolites, HPLC-MS/MS was performed. For confirmation purposes, confirmatory HPLC-MS analysis was used as second system for peak assignment.
Radioactivity data were recorded with a chromatography software (Chromeleon version 6.80) and transferred to a LIMS (Agilent) for detailed evaluation.

Metabolite analysis by HPLC-MS/MS, NMR and confirmatory HPLC-MS
The parent compound BAS 750 F and 68 biotransformation products were identified by HPLC-MS/MS and NMR analysis for structure elucidation. For structure elucidation using HPLC-MS/MS, the investigated samples exhibited representative peak patterns, include male and female dose groups and represented all labels. High dose groups were investigated to ensure sufficient amounts of radioactivity for the detection of metabolites and representative samples of the three most important matrices to provide a comprehensive view on the identified metabolites and potential specificity. Time intervals of the samples represented high amounts of radioactivity.

Confirmatory HPLC-MS analysis was used as second system for peak assignment of selected samples (urine, bile and methanol extracts of faeces samples of C-, TFMP- and T-label, respectively). For every dose group either pooled (urine samples and methanol extracts of faeces) or up to four defined time intervals of bile samples of male and female rats with representative amount of radioactive residues were subjected to confirmatory HPLC-MS analysis.

For polar compounds, purifications using solid phase extraction were performed as well as HPLC analyses using two specific methods and co-chromatographies. Furthermore, comparisons of peak patterns were performed.

Sample preparation for analysis
For generation of metabolite patterns, pooled urine and bile samples were directly subjected to radio-HPLC without any further workup.

For faeces, liver, fat and kidney, aliquots were extracted usually three times with methanol. After centrifugation aliquots of combined or individually extracts of faeces, liver and kidney were analyzed by LSC and subjected to radio-HPLC. Additional extraction with water was performed only initially once or twice for faeces and generally once for liver and kidney, respectively. After centrifugation aliquots of combined or individually extracts of faeces, liver and kidney were analyzed by LSC and, in case of sufficient amounts of radioactivity, subjected to radio-HPLC.
Solid residues of extractions were dried and radioactivity was determined by LSC after combustion.

For faeces, all residues of pooled samples with radioactive residues above 5% of the dose or above 10% TRR were subjected to pancreatin solubilization (Sigma P7545, from porcine pancreas; contains enzymatic components including trypsin, amylase, lipase, ribonuclease and protease) in order to analyse the bound residues under physiological conditions. The residues were mixed with a sufficient amount of artificial intestinal fluid and the mixture was incubated at 37 °C overnight. The supernatant after centrifugation was analysed using liquid scintillation counting. The released radioactivity is defined as bioavailable.
Thereafter, samples with residues above 5% of the applied dose or above 10% TRR were subjected to an additional solubilization step in order to release the radioactivity by an artificial method. Therefore, the residues were mixed with 2N sodium hydroxide and the mixture was boiled for one hour under reflux in a solid-liquid extractor (FexIKA, IKA). The sample was centrifuged and decanted from the residue. The residue was dried and combusted.

For fat, aliquots were extracted twice with a sufficient volume of acetonitrile : isohexane (1 : 1; v : v) using a dispersing instrument. After each extraction step, the extract was separated from the solid by centrifugation, and the acetonitrile and the isohexane phases were separated using a separatory funnel. The solid residue was subjected to the next extraction step.
The isohexane phases were extracted three times with sufficient volumes of acetonitrile. The acetonitrile phases and extracts were combined. Aliquots were radioassayed and in case of sufficient amounts of radioactivity analysed using HPLC. The isohexane phases were adjusted to volume using isohexane and radioassayed.

For plasma, samples were purified using ice-cold acetonitrile for protein precipitation. After addition of acetonitrile, the individual sample was intensely mixed followed by centrifugation. The supernatant was analyzed by LSC and radio-HPLC.

For enantiomer-specific investigations, representative samples were concentrated to dryness using a rotary evaporator. All samples were either diluted or taken up in adequate solvents containing ammonium formate buffer and subsequently fractionated using HPLC. The fraction containing the parent compound was purified by SPE, additionally concentrated, redissolved in adequate solvents and analysed using an enantiomer-specific HPLC method.

Determination of the total radioactive residue (TRR)
The TRR of urine, bile, plasma and cage wash was determined by direct liquid scintillation counting for all dose groups.
In the case of dose group DX, aliquots of faeces, liver and kidney were homogenised using an analytical mill, a tube mill or a scalpel and were combusted for the determination of the radioactive residues.

The TRR was used as basis for calculation of extractability of faeces, liver, kidney and fat samples. For dose groups B, D, C, R and S of the C- and the TFMP-label, the referring % dose values were taken from the biokinetics study for calculating the metabolite quantities, with the exception of pooled samples, where the TRR was determined within the metabolism study.

For faeces of the T-label and dose groups B, D, C, R and S, the TRR was determined by direct combustion within the metabolism study. Similarly, all liquid pool samples were subjected to LSC measurement within the metabolism study. For the remaining samples of these dose groups of the T-label, the TRR was determined within the biokinetics study.

II. RESULTS AND DISCUSSION

a. Biokinetics study (C- and TFMP-label)

1. Plasma and blood levels of ^{14}C-BAS 750 F

Plasma kinetics (Groups 1 and 2) were investigated after single oral (p.o.) administration. Target dose levels were 5 and 180 mg/kg bw.

Table 5.1.1-11: Mean plasma concentration of radioactivity (in µg Eq/g plasma, oral administration, C-label)

Label	**[^{14}C] chlorophenyl -BAS 750 F**			
Administration frequency	**Single**		**single**	
Time	**5 mg/kg bw**		**180 mg/kg bw**	
[h]	**male**	**female**	**male**	**female**
Plasma 0.5	1.47	1.67	49.9	49.3
Plasma 1	1.99	1.25	46.1	46.2
Plasma 2	1.96	0.949	52.4	34.9
Plasma 4	1.39	0.544	50.8	24.5
Plasma 8	0.943	0.514	50.4	21.6
Plasma 24	0.311	0.129	21.3	6.45 [1)]
Plasma 48	0.138	0.052	7.49	4.56
Plasma 72	0.094	0.028	4.10	2.21
Plasma 96	0.068	0.020	2.53	1.50
Plasma 120	0.052	0.012	1.75	1.96
Plasma 144	0.044	0.012	1.45	1.49
Plasma 168	0.037	0.010	1.25	1.03

1) Animal 54 excluded from mean of the 24-h plasma time point, due to measurement error.

Table 5.1.1-12: Mean blood concentration of radioactivity (in µg Eq/g blood, oral administration, C-label)

Label	[^{14}C] chlorophenyl -BAS 750 F			
Administration frequency	Single		single	
Time	5 mg/kg bw		180 mg/kg bw	
[h]	male	female	male	female
Blood 0.5	0.722	0.868	22.4	26.0
Blood 1	1.02	0.652	23.1	23.5
Blood 2	1.04	0.512	27.0	18.9
Blood 4	0.682	0.285	25.8	13.9
Blood 8	0.534	0.278	25.0	13.1
Blood 24	0.170	0.074	11.2	3.96
Blood 48	0.081	0.026	4.00	2.65
Blood 72	0.051	0.015	2.12	1.27
Blood 96	0.034	0.010	1.39	0.897
Blood 120	0.027	0.007	1.00	1.09
Blood 144	0.022	0.006	0.815	0.838
Blood 168	0.018	0.005	0.661	0.615

The parameters derived from the plasma kinetics are presented in the following table:

Table 5.1.1-13: Plasmakinetic parameters (C- and TFMP-label)

-	Dose [mg/kg bw]	cmax [µg Eq/g]	Tmax [h]	Tlast [h]	initial half-life [h]	terminal half-life [h]	AUC0→168 [µg Eq*h/g]	AUC0→∞ [µg Eq*h/g]
male	5, p.o.	2.04	1.2	168	7.68	85.7 1)	34.9	39.6 1)
	180, p.o.	62.5	5.5	168	12.9	87.7 1)	1650	1810 1)
female	5, p.o.	1.67	0.5	168	2.56 1)	62.1 1), 2)	15.7	15.3 1), 2)
	180, p.o.	49.9	0.7	168	3.99 1)	78.3 1), 3)	845	807 1), 3)

1) approximation
2) n=1
3) n=2

In plasma kinetics, AUC values indicate an internal exposure that is clearly correlated to the dosing regimen of ^{14}C-BAS 750 F. After a single oral dose of 5 as well as 180 mg/kg bw of ^{14}C-BAS 750 F, the internal dose (indicated as AUC0→∞) is approximately twice as high for males compared to females. Based on these data, the intergender factor of the internal dose is around 2 at both the low and high dose of 5 and 180 mg/kg bw, respectively.

2. Balance and excretion pattern of ^{14}C-BAS 750 F

The balance data demonstrate that major excretion of ^{14}C-BAS 750 F (C-label) dosed orally by gavage to Wistar rats occurred via the feces with mean values of 87% and 86%, as well as 86% and 80% of dose for male and female animals of the single high as well as the multiple (14+1) high dose groups, respectively. The mean total amount of radioactivity excreted via feces was found to be 89% and 88% of dose for male and female rats after single oral dosing of 5 mg/kg bw ^{14}C- BAS 750 F, respectively.

Urinary excretion was a minor excretion pathway with mean values of 6% and 8%, as well as 5% and 11% of dose for male and female animals of the single high as well as the multiple (14+1) high dose groups. The mean total amount of radioactivity excreted via urine was found to be 9 and 12% of dose for male and female rats after single oral dosing of 5 mg/kg bw ^{14}C-BAS 750 F.

The pattern of excretion after repeated oral administration (14 oral administrations with unlabeled BAS 750 F at 180 mg/kg bw and one oral administration with labeled ^{14}C-BAS 750 F at 180 mg/kg bw) showed similar amounts of radioactivity excreted in urine compared to the single dose experiment, giving comparable kinetics after single and multiple dosing. Excretion was fast and occurred to a major extent already within 2-3 days after dosing.

The balance data demonstrate that major excretion of BAS 750 F (TFMP-label) dosed orally by gavage to Wistar rats occurred via the feces with mean values of 76% and 84% of dose for male and female animals of the single high dose. Urinary excretion was a minor excretion pathway with mean values of 8% for male and female animals of the single high dose group. The excretion data obtained with the TFMP label were very similar to those observed after dosing C-labeled test substance.

Table 5.1.1-14: Excretion balance (% of administered radioactivity, C-label)

Label	**[^{14}C] chlorophenyl -BAS 750 F**					
Administration frequency	**Single**		**Single**		**15-days repeated**	
Balance / excretion	**5 mg/kg bw**		**180 mg/kg bw**		**180 mg/kg bw**	
Time interval [h]	**male**	**female**	**male**	**female**	**male**	**female**
Urine 0-6	1.63	2.01	0.407	0.305	0.580	2.40
Urine 6-12	1.78	2.58	0.779	0.622	0.911	1.38
Urine 12-24	2.89	2.58	1.82	2.03	1.61	2.45
Urine 24-48	1.74	2.44	1.85	2.25	1.04	2.36
Urine 48-72	0.550	1.64	0.808	1.24	0.617	1.10
Urine 72-96	0.167	0.476	0.364	0.949	0.129	0.444
Urine 96-120	0.076	0.199	0.101	0.383	0.069	0.221
Urine 120-144	0.073	0.166	0.051	0.150	0.058	0.115
Urine 144-168	0.053	0.142	0.036	0.131	0.030	0.083
Subtotal Urine	**8.95**	**12.2**	**6.22**	**8.06**	**5.05**	**10.6**
Feces 0-6	0.002	1.16	0.050	0.004	0.699	2.12
Feces 6-12	1.09	2.66	0.576	0.246	11.2	1.56
Feces 12-24	26.2	41.4	31.0	32.0	40.5	34.1
Feces 24-48	49.4	32.4	34.2	26.9	28.2	41.0
Feces 48-72	9.90	7.92	16.0	16.8	4.72	7.32
Feces 72-96	2.04	2.07	4.01	7.63	0.934	1.94
Feces 96-120	0.600	0.820	0.953	1.20	0.277	0.529
Feces 120-144	0.176	0.193	0.294	0.480	0.098	0.173
Feces 144-168	0.097	0.105	0.126	0.312	0.071	0.127
Subtotal Feces	**88.9**	**87.7**	**86.9**	**85.5**	**86.3**	**80.4**
Cage wash	0.320	0.674	0.432	0.924	0.280	0.629
Plasma	0.013	0.003	0.011	0.003	0.013	0.003
Blood	0.018	0.005	0.017	0.005	0.020	0.005
Heart	0.001	0.000	0.001	0.000	0.001	0.000
Lung	0.001	0.000	0.001	0.000	0.001	0.000
Spleen	0.000	0.000	0.000	0.000	0.000	0.000
Uterus	n.a.	0.000	n.a.	0.000	n.a.	0.000
Pancreas	0.000	0.000	0.001	0.000	0.000	0.000
Adipose tissue	0.002	0.001	0.010	0.002	0.006	0.004
Muscle	0.001	0.000	0.000	0.000	0.000	0.000
Testes / Ovaries	0.002	0.000	0.002	0.000	0.002	0.000
Adrenals	0.000	0.000	0.000	0.000	0.000	0.000
Thyroid	0.000	0.000	0.000	0.000	0.000	0.000
Bone marrow	0.000	0.000	0.000	0.000	0.000	0.000
Stomach	0.001	0.000	0.000	0.000	0.001	0.000
Liver	0.165	0.067	0.049	0.035	0.040	0.027
Brain	0.000	0.000	0.001	0.000	0.000	0.000
Kidney	0.008	0.007	0.007	0.004	0.005	0.003
Carcass	0.161	0.119	0.118	0.180	0.073	0.075
Skin	0.004	0.000	0.000	0.000	0.000	0.000
Gut	0.004	0.004	0.004	0.003	0.002	0.002
Bone	0.000	0.000	0.000	0.000	0.000	0.000
Stomach content	0.003	0.001	0.001	0.002	0.002	0.001
Gut content	0.037	0.043	0.054	0.046	0.025	0.023
Subtotal	**0.741**	**0.928**	**0.702**	**1.20**	**0.472**	**0.774**
Subtotal volatiles	**0.128**	**n.a.**	**0.057**	**n.a.**	**n.a.**	**n.a.**
Total	**98.7**	**100.9**	**93.8**	**94.8**	**91.8**	**91.7**

n.a. = not applied

Table 5.1.1-15: Excretion balance (% of administered radioactivity, TFMP-label)

Label	**trifluormethylring-U-C^{14} -BAS 750 F**	
Administration frequency	**Single**	
Balance / excretion	**180 mg/kg bw**	
Time interval [h]	**male**	**female**
Urine 0-6	0.671	0.866
Urine 6-12	1.47	1.25
Urine 12-24	3.06	3.01
Urine 24-48	3.04	3.21
Urine 48-72	0.913	1.07
Urine 72-96	0.244	0.320
Urine 96-120	0.101	0.200
Urine 120-144	0.064	0.145
Urine 144-168	0.042	0.100
Subtotal Urine	**9.61**	**10.2**
Feces 0-6	n.a.	0.840
Feces 6-12	4.18	4.30
Feces 12-24	37.4	50.2
Feces 24-48	26.3	23.4
Feces 48-72	6.16	4.49
Feces 72-96	1.03	0.842
Feces 96-120	0.309	0.194
Feces 120-144	0.123	0.137
Feces 144-168	0.154	0.052
Subtotal Feces	**75.7**	**84.5**
Cage wash	1.07	1.60
Plasma	0.008	0.004
Blood	0.013	0.006
Heart	0.000	0.000
Lung	0.001	0.001
Spleen	0.000	0.000
Uterus	n.a.	0.000
Pancreas	0.000	0.000
Adipose tissue	0.005	0.002
Muscle	0.000	0.000
Testes / Ovaries	0.002	0.000
Adrenals	0.000	0.000
Thyroid	0.000	0.000
Bone marrow	0.000	0.000
Stomach	0.000	0.000
Liver	0.023	0.031
Brain	0.000	0.000
Kidney	0.003	0.001
Carcass	0.107	0.096
Skin	0.000	0.000
Gut	0.003	0.003
Bone	0.000	0.000
Stomach content	0.003	0.001
Gut content	0.024	0.025
Subtotal	**1.26**	**1.77**
Total	**86.5**	**96.4**

n.a. = not applied

3. Biliary excretion

For the C-label, based on the amounts of radioactivity excreted via bile and urine, as well as the radioactive residues found in cage wash and carcass, oral absorption of ^{14}C-BAS 750 F in rats was dose dependent with lower bioavailability at the higher dose level and was calculated to be about 78% and 85% of the administered dose for males and females at a dose level of 5 mg/kg bw and about 67% and 64% of the applied dose for males and females 2 at a dose level of 180 mg/kg bw. After dosing of 5 mg/kg bw, most of the administered radiolabel was excreted within the first 24 hours in both males (9% via the urine and 66% via bile) and females (21% via the urine and 61% via the bile).

For the TFMP-label, within 18 h after administration of BAS 750 F at a dose level of 180 mg/kg bw, excretion via bile was found to be 56% and 55% of the administered radioactivity in males and females. Total excretion of radioactivity via bile until 168 h post dosing was 59% and 60% of dose for males and females respectively.

A slight difference was observed in oral absorption at 180 mg/kg bw between the C-label and the TFMP-label, which is likely due to experimental variability (formulation and inter-animal differences) rather than label-related.

Table 5.1.1-16: Bile excretion balance (% of administered radioactivity, C-label)

Label	**[^{14}C] chlorophenyl -BAS 750 F**				
Administration frequency	**Single 5 mg/kg bw**		**single 180 mg/kg bw**		
Time interval [h]	**male**	**female**	**male [1)]**	**female 1 [2)]**	**female 2 [3)]**
Urine 0-3	2.29	7.78	3.03	2.64	3.30
Urine 3-6	3.12	6.67	11.1	7.29	5.34
Urine 6-9	1.06	2.93	7.91	2.95	1.92
Urine 9-12	0.672	1.49	5.25	0.976	0.965
Urine 12-18	1.05	1.43	6.08	1.10	0.705
Urine 18-24	0.471	0.403	1.54	0.478	0.169
Urine 24-30	0.227	0.282	1.16	0.273	0.113
Urine 30-36	0.242	0.242	0.879	0.172	0.065
Urine 36-42	0.214	0.362	0.377	0.174	0.109
Urine 42-48	0.110	0.079	0.121	0.116	0.064
Urine 48-54	0.056	0.080	0.122	0.113	0.049
Urine 54-60	0.086	0.087	0.100	0.028	0.060
Urine 60-66	0.067	0.056	0.089	0.082	0.039
Urine 66-72	0.056	0.028	0.087	0.087	0.020
Urine 72-84	0.059	0.049	0.071	0.129	0.034
Urine 84-96	0.084	0.088	0.095	0.089	0.058
Urine 96-108	0.061	0.089	0.040	0.050	0.032
Urine 108-120	0.078	0.067	0.030	0.028	0.026
Urine 120-132	0.016	0.024	0.018	0.014	0.021
Urine 132-144	0.055	0.026	0.016	0.011	0.009
Urine 144-156	0.009	0.014	0.010	0.010	0.009
Urine 156-168	0.007	0.013	0.012	0.009	0.012
Subtotal Urine	**10.1**	**18.4**	**34.4**	**15.5**	**10.2**
Feces 0-3	0.002	0.002	0.002	0.000	0.000
Feces 3-6	0.006	0.204	0.003	0.001	n.a.
Feces 6-9	0.304	0.007	0.004	0.003	0.180
Feces 9-12	1.85	0.677	1.09	0.118	3.86
Feces 12-18	2.61	2.29	4.37	0.543	9.37
Feces 18-24	0.753	0.825	1.99	2.60	2.40
Feces 24-30	0.630	0.952	0.947	0.541	1.53
Feces 30-36	0.453	1.04	3.04	0.272	0.546
Feces 36-42	0.291	0.179	0.794	0.116	0.101
Feces 42-48	0.067	0.310	0.146	0.024	0.033
Feces 48-54	0.049	0.125	0.617	0.019	0.012

Label	[^{14}C] chlorophenyl -BAS 750 F				
Administration frequency	Single 5 mg/kg bw		single 180 mg/kg bw		
Time interval [h]	male	female	male [1)]	female 1 [2)]	female 2 [3)]
Feces 54-60	0.111	0.029	0.395	0.014	0.006
Feces 60-66	0.026	0.020	0.002	0.021	0.005
Feces 66-72	0.006	0.016	1.03	0.025	0.003
Feces 72-84	0.008	0.022	0.024	0.018	0.003
Feces 84-96	0.013	0.014	0.013	0.034	0.003
Feces 96-108	0.005	0.035	0.004	0.005	0.003
Feces 108-120	0.003	0.015	0.011	0.006	0.003
Feces 120-132	0.003	0.108	0.004	0.007	0.001
Feces 132-144	0.003	0.000	0.007	0.005	0.001
Feces 144-156	0.002	0.000	0.002	0.009	0.001
Feces 156-168	0.004	0.000	0.000	0.008	0.002
Subtotal Feces	**6.83**	**6.43**	**11.4**	**4.39**	**16.7**
Cage wash	0.220	0.287	0.492	0.246	0.191
Stomach content	0.000	0.000	0.002	0.000	0.000
Stomach	0.001	0.001	0.001	0.000	0.000
Gut content	0.001	0.004	0.204	0.002	0.001
Gut	0.003	0.002	0.007	0.001	0.002
Carcass	0.191	0.155	0.169	0.053	0.072
Subtotal	**0.416**	**0.449**	**0.875**	**0.302**	**0.267**
Subtotal Bile	**67.0**	**61.4**	**31.9**	**40.0**	**53.6**
Total	**84.3**	**86.6**	**78.7**	**60.2**	**80.8**
Bioavailability	**77.5**	**85.2**	**67.1**	**55.8**	**64.1**

1) Animal nos.19, 21, 126 and 127; animals 126 and 127 had a recovery of radioactivity <80% (72 and 74% respectively)
2) Animal nos. 69 and 71; both animals showed low recovery of radioactivity, possibly caused by problems with bile cannula
3) Animal nos. 128, 129 and 130

Table 5.1.1-17: Bile excretion pattern (% of administered radioactivity, C-label)

Label	[^{14}C] chlorophenyl -BAS 750 F				
Administration frequency	single 5 mg/kg bw		single 180 mg/kg bw		
Time interval [h]	male	female	male [1)]	female 1 [2)]	female 2 [3)]
Bile 0-3	33.3	32.7	8.44	6.80	21.7
Bile 3-6	22.4	19.0	6.81	7.02	14.4
Bile 6-9	5.63	4.49	4.45	6.65	7.08
Bile 9-12	2.40	1.69	3.68	7.17	3.87
Bile 12-18	1.76	2.28	7.16	9.54	4.30
Bile 18-24	0.540	0.532	3.18	2.14	1.60
Bile 24-30	0.271	0.175	0.775	0.280	0.387
Bile 30-36	0.239	0.115	0.685	0.108	0.116
Bile 36-42	0.167	0.085	0.164	0.081	0.047
Bile 42-48	0.070	0.058	0.167	0.049	0.021
Bile 48-54	0.035	0.038	0.079	0.028	0.013
Bile 54-60	0.027	0.018	0.057	0.027	0.011
Bile 60-66	0.023	0.032	0.056	0.018	0.012
Bile 66-72	0.017	0.021	0.048	0.012	0.009
Bile 72-84	0.024	0.026	0.028	0.029	0.012
Bile 84-96	0.019	0.020	0.022	0.021	0.009
Bile 96-108	0.014	0.010	0.008	0.010	0.006
Bile 108-120	0.010	0.008	0.013	0.016	0.008
Bile 120-132	0.007	0.004	0.008	0.012	0.005
Bile 132-144	0.004	0.003	0.009	0.006	0.004
Bile 144-156	0.004	0.002	0.007	0.005	0.002
Bile 156-168	0.005	0.006	0.005	0.004	0.002
Total	**67.0**	**61.4**	**31.9**	**40.0**	**53.6**

1) Animal nos.19, 21, 126 and 127
2) Animal nos. 69 and 71
3) Animal nos. 128, 129 and 130

Table 5.1.1-18: Bile excretion balance (% of administered radioactivity, TFMP-label)

Label	**trifluormethylring-U-C^{14} -BAS 750 F**	
Administration frequency	**single**	
Dose level	**180 mg/kg bw**	
Time interval [h]	**male**	**female**
Urine 0-3	0.109	0.980
Urine 3-6	1.86	2 97
Urine 6-9	2.61	2 15
Urine 9-12	0.789	1 28
Urine 12-18	1.98	1 12
Urine 18-24	1.10	0.377
Urine 24-30	0.562	0.158
Urine 30-36	0.339	0.270
Urine 36-42	0.277	0.200
Urine 42-48	0.475	0.165
Urine 48-54	0.220	0.076
Urine 54-60	0.308	0.061
Urine 60-66	0.150	0.282
Urine 66-72	0.099	0.068
Urine 72-84	0.119	0.146
Urine 84-96	0.125	0.095
Urine 96-108	0.039	0.171
Urine 108-120	0.033	0.055
Urine 120-132	0.069	0.063
Urine 132-144	0.027	0.015
Urine 144-156	0.014	0.005
Urine 156-168	0.008	0.005
Subtotal Urine	**11.3**	**10.7**
Feces 0-3	0.003	0.001
Feces 3-6	0.011	0.014
Feces 6-9	3.20	0.007
Feces 9-12	6.86	7.73
Feces 12-18	12.6	13.7
Feces 18-24	3.47	1 53
Feces 24-30	1.47	3 29
Feces 30-36	1.13	1 58
Feces 36-42	0.865	0.593
Feces 42-48	0.526	0.474
Feces 48-54	0.017	1.74
Feces 54-60	0.252	0.143
Feces 60-66	0.104	0.082
Feces 66-72	0.021	1 32
Feces 72-84	0.022	0.678
Feces 84-96	0.012	0.067
Feces 96-108	0.009	0.002
Feces 108-120	0.008	0.002
Feces 120-132	0.012	0.002
Feces 132-144	0.010	0.003
Feces 144-156	0.009	0.001
Feces 156-168	0.045	0.001
Subtotal Feces	**30.7**	**26.7**
Cage wash	0.575	0.250
Stomach content	0.000	0.000
Stomach	0.000	0.000
Gut content	0.002	0.003
Gut	0.001	0.001
Carcass	0.148	0.077
Subtotal	**0.726**	**0.331**
Subtotal Bile	**58.6**	**59.6**
Total	**101.3**	**97.3**
Bioavailability	**70.7**	**70.7**

Table 5.1.1-19: Bile excretion pattern (% of administered radioactivity, TFMP-label)

Label	**trifluormethylring-U-C^{14} -BAS 750 F**	
Administration frequency	**single**	
Dose level	**180 mg/kg bw**	
Time interval [h]	**male**	**female**
Bile 0-3	18.5	16.6
Bile 3-6	14.6	13.8
Bile 6-9	11.1	10.9
Bile 9-12	6.49	7.70
Bile 12-18	5.00	6.46
Bile 18-24	1.37	1.80
Bile 24-30	0.544	0.722
Bile 30-36	0.407	0.475
Bile 36-42	0.249	0.459
Bile 42-48	0.098	0.247
Bile 48-54	0.059	0.124
Bile 54-60	0.047	0.104
Bile 60-66	0.028	0.104
Bile 66-72	0.016	0.072
Bile 72-84	0.024	0.031
Bile 84-96	0.030	0.024
Bile 96-108	0.014	0.009
Bile 108-120	0.014	0.011
Bile 120-132	0.005	0.010
Bile 132-144	0.005	0.008
Bile 144-156	0.006	0.003
Bile 156-168	0.005	0.005
Total	**58.6**	**59.6**

4. Tissue distribution

In male and female animals, radioactive residue concentrations generally declined in organs and tissues parallel to the radioactive residues in plasma.

Table 5.1.1-20: Mean tissue concentration of radioactivity (in µg Eq/g tissue) after single oral administration of ^{14}C-BAS 750 F at a dose level of 5 mg/kg bw (C-label)

Label	**[^{14}C] chlorophenyl -BAS 750 F**							
Administration frequency	**single**				**single**			
Dose level	**5 mg/kg bw**				**5 mg/kg bw**			
	male animals				**female animals**			
Time after administration [h]	**T=1**	**T=7**	**T=20**	**T=34 1)**	**T=0.5**	**T=3**	**T=12**	**T=24**
Plasma	2.00	0.969	0.574	0.241	1.40	0.577	0.239	0.155
Blood	1.21	0.549	0.317	0.134	0.948	0.336	0.147	0.096
Heart	0.607	0.185	0.154	0.050	1.04	0.214	0.078	0.031
Lung	0.632	0.293	0.145	0.059	1.07	0.275	0.099	0.054
Spleen	0.329	0.105	0.054	0.026	0.630	0.138	0.040	0.021
Uterus	n.a.	n.a.	n.a.	n.a.	0.596	0.271	0.113	0.068
Pancreas	0.615	0.185	0.080	0.030	0.986	0.293	0.080	0.045
Adipose tissue	0.267	0.221	0.118	0.042	0.373	0.417	0.165	0.059
Muscle	0.169	0.068	0.033	0.015	0.307	0.086	0.024	0.016
Testes / Ovaries	0.232	0.193	0.118	0.041	1.04	0.287	0.106	0.052
Adrenals	1.31	0.298	0.120	0.041	3.32	0.535	0.155	0.057
Thyroid	0.349	0.154	0.065	0.031	0.674	0.109	0.040	0.024
Bone marrow	0.310	0.097	0.047	0.022	0.383	0.060	0.020	0.012
Stomach	22.4	1.83	0.347	0.041	15.6	4.46	0.313	0.132
Liver	12.7	5.60	2.47	1.03	9.72	4.16	2.11	1.29
Brain	0.256	0.042	0.012	0.005	0.777	0.090	0.014	0.006
Kidney	1.52	1.10	0.433	0.189	1.93	0.683	0.399	0.234
Carcass	0.192	0.118	0.056	0.025	0.284	0.139	0.053	0.044
Skin	0.124	0.116	0.064	0.028	0.153	0.104	0.036	0.021
Gut	4.80	4.61	1.84	0.626	7.39	4.71	2.45	1.83
Bone	0.102	0.051	0.028	0.014	0.118	0.049	0.020	0.011
Stomach content	413.9	18.8	0.707	0.128	164.8	49.6	3.03	3.23
Gut content	26.1	99.6	53.3	15.2	26.6	68.0	83.5	46.0

1) Animal 38 excluded from mean (too little formulation dosed)

Table 5.1.1-21: Mean tissue concentration of radioactivity (in µg Eq/g tissue) after single oral administration of ^{14}C-BAS 750 F at a dose level of 180 mg/kg bw (C-label)

Label	[^{14}C] chlorophenyl -BAS 750 F							
Administration frequency	single				single			
Dose level	180 mg/kg bw male animals				180 mg/kg bw female animals			
Time after administration [h]	T=2	T=22	T=38	T=53	T=0.5	T=4	T=17	T=24
Plasma	58.8	22.1	11.2	11.2	57.0	23.1	7.71	5.50
Blood	42.7	12.9	5.88	6.94	35.1	12.1	4.54	3.20
Heart	42.0	6.39	2.64	2.78	47.7	18.3	3.05	1.69
Lung	41.2	5.49	2.40	2.73	44.0	18.1	2.98	1.95
Spleen	23.9	2.63	1.02	1.12	27.9	11.2	1.24	0.840
Uterus	n.a.	n.a.	n.a.	n.a.	23.0	13.2	3.36	2.32
Pancreas	61.6	5.60	2.12	1.81	64.0	29.5	3.04	1.70
Adipose tissue	19.0	6.24	0.570	0.702	20.5	66.6	6.09	3.66
Muscle	64.9	16.6	2.64	4.35	17.8	8.82	0.795	0.573
Testes / Ovaries	23.3	5.19	2.15	1.90	46.3	21.6	3.97	2.24
Adrenals	99.0	9.32	2.41	2.68	136.0	52.1	6.59	3.59
Thyroid	40.9	9.03	3.16	2.92	50.0	16.8	20.0	2.86
Bone marrow	26.3	4.15	1.31	1.60	29.6	10.7	3.15	0.818
Stomach	322.8	5.72	9.08	4.86	683.3 [1)]	385.7	18.1	20.4
Liver	207.2	93.1	16.1	21.0	212.4	84.8	39.4	30.1
Brain	29.7	0.976	0.229	0.235	39.6	13.5	0.638	0.277
Kidney	51.7	12.1	5.96	6.31	62.8	25.4	6.78	4.99
Carcass	12.0	2.50	0.873	1.41	8.87	4.79	1.71	1.21
Skin	15.0	2.62	0.882	1.27	5.58	3.18	1.12	0.836
Gut	178.2	83.7	17.8	24.6	119.8	123.4	115.6	78.7
Bone	8.70	1.39	0.565	0.784	7.93	3.33	1.41	0.864
Stomach content	1940.8	33.6	9.24	14.8	10657.4	1678.3	61.1	30.7
Gut content	2020.1	1243.1	346.5	330.4	1535.2	1744.6	1340.1	1134.5

1) Animal 89 excluded from mean (stomach weight unrealistically low)

b. Biokinetics study (T-label)

1. Plasmakinetics and bioavailability of ^{14}C-BAS 750 F)

Maximum plasma concentrations occurred directly after intravenous administration and 1 to 24 hours post dosing after oral administration (Table 5.1.1-22 and Table 5.1.1-23).

Table 5.1.1-22: Plasmakinetics (intravenous application, T-label)

-	0 h	0.5 h	1 h	2 h	4 h	8 h	24 h	48 h	72 h	96 h
0.4 mg/kg bw(i.v.)	(µg Eq/g plasma)									
Males	1.35	0.36	0.25	0.15	0.11	0.10	0.04	0.01	0.01	0.00
Females	1.17	0.31	0.15	0.06	0.04	0.03	0.01	0.00	0.00	0.00

Table 5.1.1-23: Plasmakinetics (oral application, T-label)

Males	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
[mg/kg bw]	**[µg Eq/g plasma]**										
5	3.04	1.94	1.41	1.13	0.47	0.15	0.07	0.03	0.02	0.01	0.01
40	23.08	16.50	9.45	9.31	3.98	1.17	0.44	0.15	0.07	0.05	0.04
120	53.73	43.27	30.08	25.42	13.67	4.08	1.27	0.40	0.20	0.13	0.10
360	57.08	50.32	50.53	55.45	55.96	18.76	6.23	1.35	0.67	0.37	0.26
Females	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
[mg/kg bw]	**[µg Eq/g plasma]**										
5	2.07	0.79	0.53	0.29	0.25	0.12	0.02	0.01	0.01	0.01	0.00
40	13.77	7.88	4.50	4.41	1.12	0.25	0.13	0.05	0.04	0.04	0.02
120	34.37	23.21	13.32	20.41	5.48	1.06	0.28	0.12	0.10	0.08	0.05
360	20.32	12.25	13.71	29.19	28.62	5.75	1.33	0.53	0.26	0.24	0.13

For individual animals and high dose groups, two maxima in plasmakinetics were observed and might be explained by a potential enterohepatic recirculation. Radioactivity declined rapidly post dosing and a comparable time course of radioactivity was found for blood as for plasma in both sexes.
The AUC values (area under the curve, a parameter for internal dose) indicate a tentatively sex dependent internal exposure that is higher for male than for female animals (see Table 5.1.1-24). Increasing the dose from target values of 5 to 40 mg/kg bw (by a factor of 8) resulted in an increase of the AUC by a factor of about 8 in males and 7 in females. Further increase of the target dose from 40 to 120 mg/kg bw and 120 to 360 mg/kg bw resulted in an increase of the AUC by a total factor of 8.9 in males and 9.6 in females. In females, the AUC factor between doses of 120 to 360 mg/kg bw is 2.5.

Table 5.1.1-24: Plasmakinetic parameters (T-label)

Dose [mg/kg bw]	**Route, no. of doses**	**Cmax [µg Eq/g]**		**Tmax [h]**		**half life [h]**		**AUC [µg Eq x h/g]**	
		♂	♀	♂	♀	♂	♀	♂	♀
0.4	1x i.v.	1.35	1.17	Directly	Directly	12.46	10.03	3.6	1.3
5	1x oral	3.04	2.07	1	1	43.83	34.11	38	17
40	1x oral	23.08	13.77	1	1	20.43	41.94	296	119
120	1x oral	53.73	34.37 20.41	1	1 8	17.39	58.38	886	467
360	1x oral	57.08 55.96	20.32 29.19	1 24	1 8	30.05	38.72	2629	1148

Based on the plasmakinetic parameters, a high absorption can be assumed as shown by the AUC ratios of oral (5 mg/kg bw) versus intravenous (0.4 mg/kg bw) administration that yielded in dose corrected ratios of about 80% and 111% for males and females, respectively, according to the following formula:

$$(AUC / dose)_{5\ mg/kg\ bw\ oral} / (AUC / dose)_{0.4\ mg/kg\ bw\ i.v.}$$

Especially the value of female animals demonstrates a potential hepatic first pass effect.

Plasma kinetics of ^{14}C-BAS 750 F within the current study demonstrated high absorption, potential enterohepatic recirculation, fast excretion and a more or less linear correlation of the internal exposure to the oral dose.

2. Balance and excretion pattern of ^{14}C-BAS 750 F

After single oral administration of a target dose of 5.0 mg/kg bw of ^{14}C-BAS 750 F, mean total recoveries of radioactivity were about 100% for male and females rats. No relevant portions of the administered radioactivity were excreted as CO_2 in exhaled air. Within 168 hours after single oral administration of 5.0 mg/kg bw, 41% and 15% of the administered radioactivity were excreted in urine for males and females, and the total amount of radioactivity excreted via feces was found to be 59% and 87% for males and females, respectively. The time course of the amount of radioactivity found in urine and feces indicates excretion predominantly within the first four days after administration.

After a single oral administration of a target dose of 180 mg/kg bw of ^{14}C- BAS 750 F, mean total recoveries of radioactivity were about 100% for both sexes (see Table 5.1.1-25). After 168 hours total amounts of radioactivity excreted in urine were 19 and 11% of dose in males and females, and about 81% and 90% of the administered radioactivity were excreted via feces, respectively. The time course of the amount of radioactivity found in urine and feces indicates an excretion that occurred predominantly within the first three days after dosing. The pattern of excretion after repeated oral administration (14 oral administrations with unlabeled BAS 750 F at target doses of 180 mg/kg bw and one oral administration with labeled ^{14}C- BAS 750 F at nominally 180 mg/kg bw) showed slightly higher amounts of radioactivity excreted in urine (23% versus 19% of dose and 17% versus 11% of dose for males and females) than in the single dose experiment, giving an indication that changes in kinetics / metabolism occur after multiple dosing.

Table 5.1.1-25: Excretion balance (% of administered radioactivity, T-label)

Balance/Excretion			5 mg/kg bw male		5 mg/kg bw female		180 mg/kg bw male		180 mg/kg bw female		(14+1) 180 mg/kg bw male		(14+1) 180 mg/kg bw female	
				(cum.)		*(cum.)*		*(cum.)*		*(cum.)*		*(cum.)*		*(cum.)*
Urine	0	- 6 h	4.44	*4.4*	3.49	*3.5*	0.71	*0.7*	1.07	*1.1*	1.32	*1.3*	0.96	*1.0*
	6	- 12 h	8.40	*12.8*	3.58	*7.1*	1.65	*2.4*	1.71	*2.8*	2.08	*3.4*	1.56	*2.5*
	12	- 24 h	11.10	*23.9*	4.27	*11.3*	5.61	*8.0*	3.27	*6.1*	7.12	*10.5*	4.55	*7.1*
	24	- 48 h	10.93	*34.9*	2.74	*14.1*	6.61	*14.6*	3.13	*9.2*	7.51	*18.0*	5.77	*12.8*
	48	- 72 h	3.64	*38.5*	0.72	*14.8*	2.62	*17.2*	0.96	*10.1*	2.88	*20.9*	2.52	*15.4*
	72	- 96 h	1.19	*39.7*	0.29	*15.1*	0.95	*18.2*	0.22	*10.4*	0.95	*21.9*	0.79	*16.2*
	96	- 120 h	0.74	*40.4*	0.12	*15.2*	0.38	*18.5*	0.12	*10.5*	0.37	*22.2*	0.20	*16.4*
	120	- 144 h	0.37	*40.8*	0.08	*15.3*	0.15	*18.7*	0.07	*10.6*	0.20	*22.4*	0.16	*16.5*
	144	- 168 h	0.19	*41.0*	0.05	*15.3*	0.06	*18.7*	0.07	*10.6*	0.10	*22.5*	0.09	*16.6*
Subtotal Urine			**41**		**15**		**19**		**11**		**23**		**17**	
Feces	0	- 24 h	18.94	*18.9*	35.18	*35.2*	33.79	*33.8*	35.2	*35.2*	36.70	*36.7*	23.03	*23.0*
	24	- 48 h	30.10	*49.0*	37.46	*72.6*	34.25	*68.0*	39.5	*74.7*	28.99	*65.7*	40.34	*63.4*
	48	- 72 h	6.71	*55.8*	10.23	*82.9*	10.12	*78.2*	12.6	*87.3*	6.02	*71.7*	12.42	*75.8*
	72	- 96 h	2.14	*57.9*	3.42	*86.3*	2.40	*80.6*	1.92	*89.2*	1.18	*72.9*	4.79	*80.6*
	96	- 120 h	0.63	*58.5*	0.45	*86.7*	0.63	*81.2*	0.72	*90.0*	0.82	*73.7*	0.88	*81.5*
	120	- 144 h	0.20	*58.7*	0.22	*87.0*	0.12	*81.3*	0.09	*90.0*	0.10	*73.8*	0.30	*81.8*
	144	- 168 h	0.04	*58.8*	0.07	*87.0*	0.03	*81.3*	0.04	*90.1*	0.02	*73.8*	0.36	*82.1*
Subtotal Feces			**59**		**87**		**81**		**90**		**74**		**82**	
Cage wash			**0.60**		**0.18**		**0.11**		**0.55**		**0.47**		**0.30**	
Blood cells			0.01		0.01		0.00		0.00		0.00		0.00	
Plasma			0.00		0.00		0.00		0.00		0.00		0.00	
Lung			0.00		0.00		0.00		0.00		0.00		0.00	
Heart			0.00		0.00		0.00		0.00		0.00		0.00	
Spleen			0.00		0.00		0.00		0.00		0.00		0.00	
Kidney			0.00		0.00		0.00		0.00		0.00		0.00	
Adrenals			0.00		0.00		0.00		0.00		0.00		0.00	
Testes/Ovaries			0.00		0.00		0.00		0.00		0.00		0.00	
Uterus			---		0.00		---		0.00		---		0.00	
Muscle			0.00		0.00		0.00		0.00		0.00		0.00	
Brain			0.00		0.00		0.00		0.00		0.00		0.00	
Adipose Tissue			0.00		0.00		0.00		0.00		0.00		0.00	
Bone			0.00		0.00		0.00		0.00		0.00		0.00	
Bone marrow			0.00		0.00		0.00		0.00		0.00		0.00	
Thyroid			0.00		0.00		0.00		0.00		0.00		0.00	
Pancreas			0.00		0.00		0.00		0.00		0.00		0.00	
Stomach cont.			0.00		0.00		0.00		0.00		0.00		0.00	
Stomach			0.00		0.00		0.00		0.00		0.00		0.00	
Gut cont.			0.03		0.03		0.01		0.01		0.01		0.03	
Gut			0.01		0.01		0.01		0.01		0.01		0.01	
Liver			0.07		0.09		0.01		0.02		0.01		0.02	
Skin			0.30		0.06		0.11		0.04		0.18		0.13	
Carcass			0.76		0.15		0.09		0.08		0.09		0.08	
Subtotal Tissues			**1.18**		**0.35**		**0.23**		**0.16**		**0.30**		**0.27**	
Total recovery			**101.54**		**102.90**		**100.42**		**101.41**		**97.16**		**99.28**	

3. Biliary excretion

The bile excretion study was performed after bile catheterization as a balance experiment. Bile, urine and feces were collected up to 72 hours from rats that were dosed by oral gavage with ^{14}C-BAS 750 F at 5 mg/kg bw and 180 mg/kg bw. Based on the data of excreted radioactivity via bile and urine as well as on the remaining radioactive residue in carcass, the oral absorption of ^{14}C- BAS 750 F within 72 hours after administration of ^{14}C-BAS 750 F was calculated. Results are presented in Table 5.1.1-26.

At the low dose level (5 mg/kg bw), excretion via bile was found to be 71% and 74% of the administered radioactivity in males and females, respectively. Total excretion of radioactivity via urine after 72 hours was 11% for males and 10% for females.

At a dose level of 180 mg/kg bw, mean excretion via bile was found to be 42% and 46% of the administered radioactivity in males and females, respectively. Mean total excretion of radioactivity via urine after 72 hours was 7% for males and 11% for females.

Based on the amounts of radioactivity excreted via bile and urine, as well as the radioactive residues found in cage wash and carcass, the oral absorption of ^{14}C- BAS 750 F in rats was calculated to be 84% and 85% of the administered low dose (5.0 mg/kg bw), and 50% and 58% of the administered high dose (180 mg/kg bw) for males and females, respectively.

Table 5.1.1-26: Bile excretion balance (% of administered radioactivity, T-label)

				5 mg/kg bw				180 mg/kg bw			
				male		female		male		female	
Balance/Excretion					***(cum.)***		***(cum.)***		***(cum.)***		***(cum.)***
Urine	0	-	24 h	7.84	*7.8*	9.13	*9.1*	4.41	*4.4*	8.10	*8.1*
	24	-	48 h	2.82	*10.7*	0.85	*10.0*	1.79	*6.2*	2.18	*10.3*
	48	-	72 h	0.67	*11.3*	0.48	*10.5*	0.35	*6.6*	0.75	*11.0*
Subtotal Urine				**11**		**10**		**7**		**11**	
Bile	0	-	24 h	69.47	*69.5*	72.05	*72.1*	39.86	*39.9*	44.57	*44.6*
	24	-	48 h	1.10	*70.6*	1.39	*73.4*	1.68	*41.5*	1.44	*46.0*
	48	-	72 h	0.40	*71.0*	0.12	*73.5*	0.09	*41.6*	0.11	*46.1*
Subtotal Bile				**71**		**74**		**42**		**46**	
Feces	0	-	24 h	12.35	*12.4*	5.19	*5.2*	33.13	*33.1*	17.50	*17.5*
	24	-	48 h	0.93	*13.3*	2.19	*7.4*	4.63	*37.7*	6.39	*23.9*
	48	-	72 h	0.35	*13.6*	0.67	*8.1*	0.15	*37.9*	0.52	*24.4*
Subtotal Feces				**14**		**8**		**38**		**24**	
Cage wash				**0.51**		**0.26**		**0.30**		**0.72**	
Stomach cont.				0.01		2.01		2.62		4.29	
Stomach				0.01		0.15		0.14		0.18	
Gut cont.				0.10		0.54		1.53		1.47	
Gut				0.01		0.08		0.10		0.11	
Carcass				0.88		0.38		1.06		0.95	
Total recovery				**97.45**		**95.33**		**91.83**		**88.73**	
Bioavailability				**84**		**85**		**50**		**58**	

4. Tissue distribution

1 hour after administration of 5.0 mg/kg bw ^{14}C-BAS 750 F to male and female rats highest tissue concentrations (means) were found in the GI-tract/GI-tract contents (see Table 5.1.1-27). With the exception of the GI-tract (including its content), highest residues (means) in male rats of this dose group were found in liver and adrenal glands. Lowest mean radioactive residues at this time point were measured in blood cells, spleen, brain, skin, carcass, adipose tissue, muscle, testes and bone. With the exception of the GI-tract (including its content), highest residues (means) in female rats of the high dose group 1 hour post dose were found in liver and adrenal glands. Lowest mean radioactive residues at this time point were measured in bone marrow, heart, uterus, brain, spleen, adipose tissue, carcass, skin, muscle, blood cells and bone. In male and female animals, radioactive residue concentrations declined generally in organs and tissues from the 1 h time point on and during the following 28 and 24 hours parallel to the radioactive residues in plasma. Exceptions on this general trend are radioactive residues in testes that increased to 4 h and then decreased to later observation time points. In females, radioactive residues in ovaries and uterus increased from 1 hour to 2 hours and then decreased to later time points. Also radioactive residues in adipose tissues of female animals increased from 1 to 4 hours to reach a maximum of 0.96 µg Eq/g at 4 hours and decreased to 0.17 µg Eq/g at the 24 hours observation time point.

Table 5.1.1-27: Mean tissue concentration of radioactivity after single oral administration of ^{14}C-BAS 750 F at a dose level of 5 mg/kg bw (T-label)

Dose: 5 mg/kg bw	Mean tissue concentration [µg Eq/g] Males				Mean tissue concentration [µg Eq/g] Females			
Time after administration	C_{max} 1 h	$C_{max}/2$ 4 h	$C_{max}/4$ 18 h	$C_{max}/8$ 28 h	C_{max} 1 h	$C_{max}/2$ 2 h	$C_{max}/4$ 4 h	$C_{max}/8$ 24 h
Blood cells	0.88	0.55	0.33	0.20	0.30	0.12	0.14	0.04
Plasma	2.99	1.20	0.42	0.29	1.53	0.48	0.42	0.09
Lung	1.47	0.85	0.41	0.29	1.01	0.44	0.37	0.11
Heart	1.29	0.67	0.36	0.25	0.87	0.34	0.27	0.07
Spleen	0.76	0.61	0.40	0.26	0.55	0.36	0.32	0.08
Kidney	1.98	1.78	0.54	0.49	1.66	0.89	0.84	0.26
Adrenal glands	4.68	1.63	0.64	0.50	3.72	1.47	0.86	0.18
Testes/Ovaries	0.57	0.70	0.40	0.26	1.06	2.01	0.82	0.11
Uterus	---	---	---	---	0.64	1.50	0.76	0.14
Muscle	0.58	0.61	0.38	0.26	0.41	0.22	0.20	0.07
Brain	0.72	0.53	0.33	0.22	0.59	0.26	0.19	0.06
Adipose tissue	0.62	0.36	0.11	0.05	0.54	0.70	0.96	0.17
Bone	0.32	0.29	0.16	0.12	0.16	0.09	0.08	0.02
Bone marrow	1.07	0.79	0.48	0.34	0.97	0.39	0.34	0.10
Thyroid	2.71	1.42	0.84	0.68	2.53	0.84	0.64	0.14
Pancreas	1.36	0.74	0.53	0.24	1.33	1.37	1.09	0.09
Stomach content	131.04	78.59	1.16	2.41	94.05	71.23	41.77	0.28
Stomach	14.23	10.87	0.96	0.67	19.73	15.47	6.23	0.30
Gut content	43.59	49.46	13.02	8.05	34.33	32.87	63.74	11.88
Gut	6.42	7.42	2.97	1.00	15.48	23.05	12.18	2.08
Liver	16.35	6.74	1.64	1.07	9.13	4.24	4.14	0.68
Skin	0.69	0.57	0.39	0.27	0.48	0.31	0.23	0.07
Carcass	0.67	0.55	0.36	0.24	0.52	0.55	0.37	0.10

Following a single oral dose of ^{14}C-BAS 750 F at a dose level of 180 mg/kg bw, tissue distribution was measured 1, 24, 36 and 48 hours post-dosing in males and 1, 8, 24 and 34 hours post-dosing in females. At the low dose level of 5.0 mg/kg bw, the corresponding radioactivity measurements were performed 1, 4, 18 and 28 hours as well as 1, 2, 4 and 24 hours after administration in males and females, respectively.

1 hour after administration of 180 mg/kg bw ^{14}C- BAS 750 F to male and female rats highest tissue concentrations (means) were found in the GI-tract/GI-tract contents (see Table 5.1.1-28). With the exception of the GI-tract (including its content), highest residues (means) in male rats were found in liver, adrenal glands, plasma, thyroid, kidney and pancreas and lowest mean radioactive residues at this time point were measured in bone. With the exception of the GI-tract (including its content), highest residues (means) of the high dose group in female rats 1 hours post dosing were found in liver, adrenal glands, pancreas, thyroid, lung, kidney, plasma and ovaries. Lowest mean radioactive residues at this time point were measured in bone. In male and female animals, radioactive residue concentrations generally declined in organs and tissues from the 1 h time point on and parallel to the radioactive residues in plasma.

Table 5.1.1-28: Mean tissue concentration of radioactivity after single oral administration of ^{14}C-BAS 750 F at a dose level of 180 mg/kg bw (T-label)

Dose: 180 mg/kg bw	**Mean tissue concentration [µg Eq/g] Males**				**Mean tissue concentration [µg Eq/g] Females**			
Time after administration	**C_{max} 1 h**	**C_{max}/2 24 h**	**C_{max}/4 36 h**	**C_{max}/8 48 h**	**C_{max} 1 h**	**C_{max}/2 8 h**	**C_{max}/4 24 h**	**C_{max}/8 34 h**
Blood cells	21.60	13.61	9.16	8.98	17.10	8.40	4.04	2.57
Plasma	70.96	18.03	11.20	10.43	52.86	17.32	6.93	2.85
Lung	47.87	15.63	10.10	11.21	70.49	20.16	6.24	3.15
Heart	45.94	14.37	9.17	18.55	48.22	18.09	5.13	2.30
Spleen	31.44	15.03	9.98	5.26	34.78	13.35	5.59	2.49
Kidney	65.36	22.06	13.37	13.54	69.81	26.98	8.90	4.13
Adrenal glands	123.94	26.44	12.71	13.15	144.30	52.10	11.04	4.70
Testes/Ovaries	22.00	14.45	9.46	9.61	52.19	25.67	7.89	2.34
Uterus	---	---	---	---	31.22	16.63	7.88	2.79
Muscle	22.69	13.67	8.88	8.85	23.06	9.62	4.35	2.15
Brain	37.38	12.61	8.27	8.70	47.77	15.09	3.59	1.86
Adipose tissue	22.82	3.44	1.46	3.88	40.50	44.88	2.83	1.18
Bone	6.99	5.46	2.33	3.23	8.09	2.82	1.92	0.63
Bone marrow	35.12	18.65	10.73	11.25	34.22	13.20	5.45	3.15
Thyroid	70.82	36.53	16.11	17.07	74.52	29.11	25.12	5.76
Pancreas	59.52	14.30	9.18	9.60	76.61	25.21	6.91	2.58
Stomach content	6558.92	52.53	54.38	38.23	4056.53	725.01	22.33	16.68
Stomach	671.23	33.77	19.33	22.86	1373.12	95.21	17.38	6.01
Gut content	1349.46	909.28	572.58	185.46	1273.02	2395.29	978.73	550.58
Gut	201.79	104.52	59.29	24.84	399.59	178.25	139.13	38.93
Liver	267.46	41.53	29.38	17.11	221.46	91.19	29.87	13.83
Skin	21.49	14.28	11.65	12.61	34.50	14.38	4.32	2.56
Carcass	24.87	15.86	12.08	18.24	28.59	14.99	6.45	3.64

In contrast to the AUC-dose correlation, the tissue distribution experiments showed a sublinear correlation between the radioactive residues in organs and tissues and the external dose and an increase of the dose by a factor of 36 resulted in general in a lower ratio of organ and tissue concentrations.

c. Metabolism study (C-, TFMP- and T-label)

1. Balance and excretion pattern of ^{14}C-BAS 750 F

Excretion balances are additionally provided for dose groups DX (C- and T-label), supporting those of the dose groups that have been performed within the two biokinetics studies.

Table 5.1.1-29: Excretion balance (in % of administered radioactivity) at up to 170 hours post dosing

Matrix	Time Interval	Dose Group DX Single high dose (180 mg/kg bw) C-label		Dose Group DX Single high dose (180 mg/kg bw) T-label	
		Males	Females	Males	Females
Urine	0 - 6 h	0.62	1.55	0.71	1.47
	6 - 12 h	1.21	1.32	2.00	2.26
	12 - 24 h	1.24	2.10	4.41	2.24
	24 - 48 h	0.90	0.96	4.68	2.39
	48 - 72 h	0.34	0.35	1.96	0.70
	72 - 96 h	0.13	0.19	1.01	0.30
	96 - 120 h	0.07	0.09	1.05	0.32
	120 - 144 / 146 h	0.04	0.06	0.14	0.13
	146 - 168 / 170 h	0.04	0.08	0.07	0.06
Subtotal Urine [1)]	***0 - 72 h***	***4.31***	***6.29***	***13.76***	***9.08***
	72 - 168 / 170 h	***0.07***	***0.10***	***0.33***	***0.01***
Faeces	0 - 12 h	0.01	0.01	8.94	5.45
	12 - 24 h	38.90	36.58	43.53	50.11
	24 - 48 h	29.96	27.39	22.03	21.69
	48 - 72 h	6.71	4.74	6.46	4.24
	72 - 96 h	1.76	0.94	1.38	0.78
	96 - 120 h	0.43	0.22	0.20	0.17
	120 - 144 / 146 h	0.14	0.08	0.14	0.07
	146 - 168 h	0.08	0.06	0.04	0.02
Subtotal Faeces [1)]	***0 - 72 h***	***75.58***	***68.71***	***80.96***	***83.31***
Cage Wash	total	0.38	0.79	1.36	1.28
Total	**0 - 168 / 170 h**	**82.96 [2)]**	**77.51 [2)]**	**100.11**	**93.70**

1) % of the dose measured

2) For the C-label, the obtained values were accepted since the pattern of the chromatographies was comparable to dose groups DM and DF of the same label where >90% of the dose were recovered (compare biokinetics study). Consequently, no deviations in sampling are expected and the corresponding samples of dose groups DXM and DXF are considered valid.

2. Distribution of radioactivity in selected tissues

Table 5.1.1-30: Distribution of radioactivity in plasma and selected tissues at tmax of plasma level (1 hour, C-label)

Matrix	Dose group (nominal dose)							
	Dose group V 5.0 mg/kg bw				Dose group W 180.0 mg/kg bw			
	Males		Females		Males		Females	
	% Dose	*µg/g equiv*	% Dose	µg/g equiv	% Dose	µg/g equiv	% Dose	µg/g equiv
Liver	8.50	*11.15*	6.46	*8.62*	5.57	*262.40*	4.43	*210.90*
Kidney	0.23	*1.70*	0.31	*2.14*	0.31	*75.80*	0.30	*71.36*
Plasma	0.33	*2.70*	0.24	*1.96*	0.38	*97.37*	0.34	*94.48*
Fat	0.11	*0.41*	0.25	*0.95*	0.21	*30.70*	0.29	*36.84*

Table 5.1.1-31: Distribution of radioactivity in plasma and selected tissues at tmax of plasma level (1 hour, T-label)

Matrix	Dose group (nominal dose)							
	Dose group V 5.0 mg/kg bw				Dose group W 180.0 mg/kg bw			
	Males		Females		Males		Females	
	% Dose	µg/g equiv	% Dose	µg/g equiv	% Dose	µg/g equiv	% Dose	µg/g equiv
Liver	9.49	*12.22*	6.71	*9.48*	3.29	*162.56*	2.78	*131.69*
Kidney	0.26	*1.73*	0.24	*1.83*	0.16	*41.87*	0.16	*39.47*
Plasma	0.25	*1.94*	0.15	*1.36*	0.15	*47.60*	0.13	*36.12*
Fat	0.13	*0.36*	0.12	*0.49*	0.09	*10.50*	0.15	*17.95*

3. Extractability

4. Metabolite patterns

In total, BAS 750 F as well as 68 metabolites have been identified.

Table 5.1.1-32: Proportions of metabolites identified in rat urine (biokinetics study, C-label)

Dose	Percent of administered dose					
	Group B Single low dose (5 mg/kg bw)		Group D Single high dose (180 mg/kg bw)		Group C Repeated high dose (14 + 1 x 180 mg/kg bw)	
Compound M750-	Males (0-48 h)	Females (0-48 h)	Males (0-48 h)	Females (0-48 h)	Males (0-48 h)	Females (0-48 h)
F052	n.d.	n.d.	0.139	n.d.	0.476	n.d.
F049 [a]	2.603	2.156	0.951	n.d.	0.450	n.d.
F052 / F049 (19.0)	n.d.	n.d.	n.d.	n.d.	n.d.	0.906
F050	n.d.	n.d.	0.725	0.468	0.675	0.661
F079 (30.0)	n.d.	n.d.	0.099	0.338	0.231	n.d.
F063 (22.3) / F063 (23.1)	n.d.	2.363	n.d.	n.d.	n.d.	n.d.
F063 (22.3) / F079 (30.0) / F087 (21.4)	n.d.	n.d.	n.d.	n.d.	n.d.	2.221
F015 / F057 (26.9) - isomer 1	n.d.	0.161	n.d.	0.619	n.d.	0.243
F015 / F058 / F067 (31.2)	n.d.	n.d.	n.d.	n.d.	0.369	n.d.
F058 / F081 (34.4)	n.d.	n.d.	n.d.	n.d.	n.d.	0.025
F016	n.d.	n.d.	n.d.	n.d.	0.715	n.d.
F017	n.d.	n.d.	0.596	n.d.	n.d.	n.d.
F016 / F017 / F057 (26.9) - isomer 2	n.d.	0.769	n.d.	n.d.	n.d.	n.d.
F016 / F017 / F059 (28.9)	n.d.	n.d.	n.d.	1.054	n.d.	n.d.
F016 / F017 / F096 (32.6)	n.d.	n.d.	n.d.	n.d.	n.d.	0.656
F098 (31.1)	n.d.	0.568	n.d.	n.d.	n.d.	n.d.
F067 (31.2)	n.d.	n.d.	0.292	n.d.	n.d.	n.d.
F058	n.d.	n.d.	n.d.	0.634	n.d.	n.d.
F058 / F081 (34.4)	0.902	n.d.	0.500	n.d.	n.d.	n.d.
F082 (39.6)	n.d.	n.d.	0.128	n.d.	n.d.	n.d.
F082 (43.5)	n.d.	n.d.	0.256	n.d.	n.d.	n.d.
F083	n.d.	0.303	0.858	0.988	n.d.	n.d.
F082 (39.6) / F083	n.d.	n.d.	n.d.	n.d.	1.104	n.d.
F098 (34.5)	n.d.	0.744	n.d.	n.d.	n.d.	n.d.
F038 / F066 (36.9) / F083 / F098 (31.1)	n.d.	n.d.	n.d.	n.d.	n.d.	0.763
F066 (41.4)	n.d.	0.079	n.d.	n.d.	n.d.	n.d.
F059 (35.5) / F066 (36.9)/ F066 (39.9) / F098 (34.5) / F099 (44.0) - isomer 1	n.d.	n.d.	n.d.	0.092	n.d.	n.d.
F059 (35.5) / F059 (41.4) / F066 (39.9) / F066 (41.4) / F082 (39.6) / F098 (34.5) / F099 (44.0) - isomer 1 / F099 (44.0) - isomer 2	n.d.	n.d.	n.d.	n.d.	n.d.	2.585
F099 (44.0) - isomer 2	n.d.	n.d.	n.d.	0.239	n.d.	n.d.
F059 (41.4)	n.d.	1.521	n.d.	n.d.	n.d.	n.d.
F043	n.d.	n.d.	n.d.	n.d.	n.d.	0.298
Total identified	**3.505**	**8.662**	**4.544**	**4.433**	**4.020**	**0.298**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers
[a] includes isomer of metabolite M750F049 at 19.0 min

Table 5.1.1-33: Proportions of metabolites identified in rat urine (metabolism study, C-label)

Dose	**Percent of administered dose Group DX single high dose (180 mg/kg bw), C-label**	
Compound M750-	Males (0-168 h)	Females (0-168 h)
F047	0.297	n.d.
F047 / F048 (18.0)	n.d.	0.206
F046 / F048 (18.4)	0.269	0.147
F048 (17.3) / F052	0.169	0.089
F049 (19.0)	0.640	0.530
F048 (19.7)	0.102	0.093
F050	n.d.	0.228
F050 / F076	0.501	n.d.
F044 / F045 / F087 (21.4) / F087 (21.9)	n.d.	1.008
F053 (25.1) / F062 (24.8)	n.d.	0.044
F077 / F078 (25.9)	0.065	n.d.
F078	n.d.	n.d.
F015 / F055 (26.1) / F078 (26.8)	0.203	0.330
F016 / F017 / F078 (27.2)	0.312	n.d.
F016 / F017 / F061 (27.2) / F078 (27.2) / F089 (27.2)	n.d.	0.920
F067 (31.2) / F079 (31.5) / F090 / F095 / F097 (32.1)	n.d.	0.176
F058	0.760	n.d.
F058 / F092 (33.0) / F093 / F094 / F096 (33.6)	n.d.	0.383
F081 (34.4)	0.067	n.d.
F057 (36.9) / F066 (36.9) / F073	n.d.	0.849
F055 (40.5) / F082 (39.6) / F083	0.244	n.d.
F060 (39.7) / F066 (39.9) / F083 / F097 (39.7)	n.d.	0.220
F055 (40.5) / F057 (40.5) / F060 (40.5)	n.d.	0.365
F057 (41.4) / F059 (41.4) / F066 (41.4) / F100	n.d.	0.529
F082 (44.2) / F099	n.d.	0.064
Total identified	**3.628**	**6.180**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-34: Proportions of metabolites identified in rat urine (biokinetics study, TFMP-label)

Dose	Percent of administered dose			
	Group D Single high dose (180 mg/kg bw)		Group S Single high dose (180 mg/kg bw)	
Compound M750-	Males (0-168 h)	Females (0-168 h)	Males (0-72 h)	Females (0-72 h)
F054	1.650	2.074	0.410	0.479
F084	n.d.	n.d.	n.d.	0.133
F091	n.d.	n.d.	0.841	0.779
F101 (17.3) / F101 (18.1)	0.567	n.d.	n.d.	n.d.
F003 / F049 (19.0)	2.836	2.074	n.d.	n.d.
F003 / F049 (19.0) / F071 (20.4)	n.d.	n.d.	0.465	n.d.
F003 / F049 (19.0) / F108 (19.1)	n.d.	n.d.	n.d.	0.609
F063 (22.3)/ F063 (23.1)/ F087 (21.4)	n.d.	0.632	n.d.	n.d.
F063 (22.3) / F087 (21.4)	n.d.	n.d.	2.572	n.d.
F063 (23.1)	n.d.	n.d.	2.656	n.d.
F035 / F044 / F045 / F065 / F087 (21.4) / F087 (21.9)	n.d.	n.d.	n.d.	4.253
F071 (19.4) / F071 (20.4)	3.052	n.d.	n.d.	n.d.
F053 (25.1) / F062 (24.8)	n.d.	n.d.	n.d.	0.328
F053 (25.1) / F067 (26.2)	n.d.	n.d.	n.d.	0.054
F015	0.680	0.680	1.163	n.d.
F015 / F089 (26.3)	n.d.	n.d.	n.d.	0.240
F015 / F067 (26.2)	n.d.	n.d.	n.d.	0.974
F016 / F017	0.400	1.446	1.911	n.d.
F016 / F017 / F089 (27.2)	n.d.	n.d.	n.d.	1.892
F098 (31.1)	n.d.	0.845	n.d.	n.d.
F059	n.d.	1.949	n.d.	n.d.
Total identified	**9.185**	**9.700**	**10.018**	**9.739**

n.d. = not detected / identified

() numbers in brackets denote retention times of isomers

[a] includes isomer of metabolite M750F049 at 19.0 min

Table 5.1.1-35: Proportions of metabolites identified in rat urine (biokinetics study, T-label)

Dose	Percent of administered dose					
	Group B Single low dose (5 mg/kg bw)		Group D Single high dose (180 mg/kg bw)		Group C Repeated high dose (14 + 1 x 180 mg/kg bw)	
Compound M750-	Males (0-48 h)	Females (0-48 h)	Males (0-48 h)	Females (0-48 h)	Males (0-48 h)	Females (0-48 h)
F001	19.964	3.193	9.583	3.097	13.834	7.700
F054	4.342	2.705	1.134	1.455	0.748	1.575
F049 [a]	n.d.	3.744	n.d.	n.d.	n.d.	n.d.
F003	n.d.	n.d.	1.526	n.d.	1.863	n.d.
F003 / F049 (19.0)	3.118	n.d.	n.d.	1.096	n.d.	1.374
F063 (22.3) / F063 (23.1)	n.d.	1.756	n.d.	n.d.	n.d.	n.d.
F063 (22.3) / F087 (21.9)	n.d.	n.d.	n.d.	0.733	n.d.	n.d.
F071	6.698	n.d.	2.061	n.d.	1.585	n.d.
F015	n.d.	0.412	n.d.	0.402	n.d.	0.380
F016 / F017	n.d.	1.315	n.d.	n.d.	n.d.	1.042
F016 / F017 / F059 (28.9)	n.d.	n.d.	n.d.	1.060	n.d.	n.d.
F098 (31.1)	n.d.	0.089	n.d.	n.d.	n.d.	0.178
F098 (31.1) / F098 (33.8)	n.d.	n.d.	n.d.	0.505	n.d.	n.d.
F098 (33.8) / F098 (34.5)	n.d.	0.270	n.d.	n.d.	n.d.	n.d.
F098 (34.5)	n.d.	n.d.	n.d.	n.d.	n.d.	0.171
F057 (26.9)	n.d.	n.d.	n.d.	0.010	n.d.	n.d.
F059 (41.4)	n.d.	0.596	n.d.	0.601	n.d.	n.d.
Total identified	**34.123**	**14.080**	**14.304**	**8.958**	**18.030**	**12.420**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers
[a] includes isomer of metabolite M750F049 at 19.0 min

Table 5.1.1-36: Proportions of metabolites identified in rat urine (metabolism study, T-label)

Dose	Percent of administered dose Group DX single high dose, (180 mg/kg bw), T-label	
Compound M750-	Males (0-170 h)	Females (0-168 h)
F001	10.491	3.320
F054	2.282	1.837
F049 [a]	0.962	0.777
F044 / F087 (21.4) / F045 / F087 (21.9)	n.d.	1.217
F071	2.021	n.d.
F015	n.d.	0.309
F016 / F017	n.d.	0.649
F073	n.d.	1.013
F057 (41.4) / F059 (41.4)	n.d.	0.229
F057 (42.1)	n.d.	0.341
Total identified	**15.756**	**9.692**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers
[a] includes isomer of metabolite M750F049 at 19.0 min

Table 5.1.1-37: Proportions of metabolites identified in rat faeces (biokinetics study, C-label)

Dose	Percent of administered dose					
	Group B Single low dose (5 mg/kg bw)		Group D Single high dose (180 mg/kg bw)		Group C Repeated high dose (14 + 1 x 180 mg/kg bw)	
Compound M750-	Males (0-72 h)	Females (0-72 h)	Males (0-72 h)	Females (0-72 h)	Males (0-48 h)	Females (0-48 h)
F062 (24.8)	3.972	3.663	n.d.	n.d.	n.d.	n.d.
F015	25.252	26.787	12.776	23.391	21.019	30.239
F016 / F017	32.121	23.621	23.754	17.896	31.686	29.139
F000	4.546	5.435	27.865	21.102	19.580	15.544
Total identified	**65.891**	**59.506**	**64.395**	**62.390**	**72.285**	**74.922**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-38: Proportions of metabolites identified in rat faeces (biokinetics study, TFMP-label)

Dose	Percent of administered dose Group D Single high dose (180 mg/kg bw)	
Compound M750-	Males (0-48 h)	Females (0-48 h)
F003	2.451	2.576
F015	14.722	30.181
F016 / F017	28.955	21.781
F000	17.220	21.142
Total identified	**63.348**	**75.680**

n.d. = not detected / identified

Table 5.1.1-39: Proportions of metabolites identified in rat faeces (biokinetics study, T-label)

Dose	Percent of administered dose					
	Group B Single low dose (5 mg/kg bw)		Group D Single high dose (180 mg/kg bw)		Group C Repeated high dose (14 + 1 x 180 mg/kg bw)	
Compound M750-	Males (0-72 h)	Females (0-72 h)	Males (0-72 h)	Females (0-72 h)	Males (0-48 h)	Females (0-48 h)
F003	4.319	4.314	1.796	3.454	1.290	1.087
F062 (24.8)	6.016	4.578	n.d.	n.d.	n.d.	n.d.
F062 (25.1)	0.833	1.615	n.d.	n.d.	n.d.	n.d.
F015	10.198	41.037	13.931	25.488	10.947	19.898
F016 / F017	21.702	26.499	23.763	18.148	20.390	19.957
F000	3.079	1.381	25.842	29.889	28.914	23.898
Total identified	**46.147**	**79.424**	**65.332**	**76.979**	**61.541**	**64.840**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-40: Proportions of metabolites identified in rat faeces (metabolism study, C- and T-label)

Dose	Percent of administered dose			
	Group DX single high dose (180 mg/kg bw) C-label		Group DX single high dose (180 mg/kg bw) T-label	
Compound M750-	Males (0-72 h)	Females (0-72 h)	Males (0-72 h)	Females (0-72 h)
F003	n.d.	n.d.	1.371	1.164
F062 (24.8)	3.032	3.772	n.d.	n.d.
F015	16.663	26.510	11.349	20.372
F016 / F017	28.491	20.652	19.980	15.500
F000	12.726	7.560	35.233	30.069
Total identified	**60.912**	**58.494**	**67.933**	**67.105**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers
[a] includes isomer of metabolite M750F049 at 19.0 min

Table 5.1.1-41: Proportions of metabolites identified in rat bile (biokinetics study, C-label)

Dose	Percent of administered dose				
	Group R Single low dose (5 mg/kg bw)		Group S Single high dose (180 mg/kg bw)		
Compound M750-	Males (0-24 h)	Females (0-24 h)	Males (0-24 h)	Females, 1 (0-24 h)	Females, 2 (0-24 h)
F084	2.477	4.012	2.281	1.164	0.929
F091	1.904	n.d.	n.d.	n.d.	n.d.
F069 (18.1)	n.d.	n.d.	n.d.	0.392	5.475
F069 (18.1) / F091	n.d.	n.d.	4.928	n.d.	n.d.
F049 (19.0) / F104	2.389	3.067	n.d.	n.d.	0.532
F104	n.d.	n.d.	n.d.	n.d.	n.d.
F075 (19.7) / F075 (20.1)	2.110	n.d.	n.d.	n.d.	0.563
F049 (19.0) / F104 / F075 (19.7) / F075 (20.1)	n.d	n.d.	3.147	n.d.	n.d.
F044 / F049 (21.5) / F087 (21.4) / F035 / F045 / F049 (21.9) / F049 (22.2)	53.172	49.069	22.040	37.494	45.401
F105	0.445	n.d.	0.673	n.d.	n.d.
F015	n.d.	n.d.	n.d.	0.139	n.d.
F060 (31.4)	0.578	n.d.	0.652	n.d.	n.d.
Total identified	**62.984**	**56.148**	**33.720**	**39.189**	**52.900**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-42: Proportions of metabolites identified in rat bile (biokinetics study, TFMP-label)

Dose	**Percent of administered dose Group S Single high dose (180 mg/kg bw)**	
Compound M750-	Males (0-24 h)	Females (0-24 h)
F054	0.551	n.d.
F084	2.069	n.d.
F069 (18.1) / F085	6.933	n.d.
F069 (18.3)	0.606	n.d.
F069 (18.1) / F085 / F069 (18.3)	n.d.	3.616
F049 (19.0)	1.811	3.263
F075 (19.7)	n.d.	0.484
F075 (19.7) / F075 (20.1)	1.302	n.d.
F044 / F087 (21.4)	21.523	n.d.
F035 / F045 / F049 (21.9)	18.815	n.d.
F044 / F049 (21.5) / F087 (21.4) / F035 / F045 / F049 (21.9) / F049 (22.2)	n.d.	47.556
F015	1.451	n.d.
F110 (22.5)	n.d.	1.638
Total identified	**55.061**	**56.557**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-43: Proportions of metabolites identified in rat bile (biokinetics study, T-label)

Dose	**Percent of administered dose Group R Single low dose (5 mg/kg bw)**		**Group S Single high dose (180 mg/kg bw)**	
Compound M750-	Males (0-15 h)	Females (0-21 h)	Males (0-24 h)	Females (0-24 h)
F001	0.203	0.040	0.232	0.024
F054	1.489	0.709	0.609	n.d.
F084	n.d.	1.572	n.d.	n.d.
F091	3.315	3.314	3.959	0.146
F069 (17.2)	n.d.	0.305	n.d.	n.d.
F003 / F049 (19.0)	7.035	5.497	1.819	0.537
F075 (19.7) / F075 (20.1)	10.503	5.192	5.452	5.437
F044 / F049 (21.5)	10.395	n.d.	n.d.	n.d.
F044 / F049 (21.5)/ F087 (21.4)	n.d.	23.980	13.010	n.d.
F044 / F049 (21.5)/ F087 (21.4) / F035 / F045 / F049 (21.9) / F049 (22.2) / F087 (21.9)	n.d.	n.d.	n.d.	30.691
F035 / F045 / F049 (21.9)	12.230	n.d.	n.d.	n.d.
F035 / F045 / F049 (21.9) / F049 (22.2)	n.d.	17.464	12.134	n.d.
F015	9.485	9.966	3.020	3.812
F016 / F017	12.323	6.507	3.131	2.306
Total identified	**66.977**	**74.547**	**43.367**	**42.954**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-44: Proportions of metabolites identified in rat liver analyzed at tmax of plasma level (1 hour, metabolism study, C-label)

Dose	Percent of administered dose			
	Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound M750-	VM	VF	WM	WF
F049 (19.0)	0.463	n.d.	0.281	0.131
F015 / F055 (26.1) / F067 (26.2) / F078 (26.8)	3.597	2.359	1.727	1.825
F016 / F017 / F061 (27.2) / F078 (27.2) / F089 (27.2)	1.610	2.068	0.608	0.884
BAS 750 F	1.664	1.011	1.881	2.025
Total Identified	**7.334**	**5.438**	**4.496**	**4.866**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-45: Proportions of metabolites identified in rat liver analyzed at tmax of plasma level (1 hour, metabolism study, T-label)

Dose	Percent of administered dose			
	Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound M750-	VM	VF	WM	WF
F049 (19.0)	0.513	n.d.	n.d.	n.d.
F015	3.252	2.664	0.948	0.907
F016 / F017	1.663	2.202	0.438	0.509
BAS 750 F	1.412	1.243	1.075	1.231
Total Identified	**6.840**	**6.109**	**2.460**	**2.647**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-46: Proportions of metabolites identified in rat kidney analyzed at tmax of plasma level (1 hour, metabolism study, C-label)

Dose	Percent of administered dose			
	Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound M750-	VM	VF	WM	WF
F015 / F055 (26.1) / F067 (26.2) / F078 (26.8)	0.055	0.063	0.046	0.042
F016 / F017 / F061 (27.2) / F078 (27.2) / F089 (27.2)	n.d.	0.030	n.d.	0.015
BAS 750 F	0.112	0.106	0.188	0.237
Total Identified	**0.167**	**0.199**	**0.234**	**0.293**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-47: Proportions of metabolites identified in rat kidney analyzed at tmax of plasma level (1 hour, metabolism study, T-label)

Dose	Percent of administered dose Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound M750-	**VM**	**VF**	**WM**	**WF**
F001	0.049	n.d.	0.007	n.d.
F054	n.d.	0.008	n.d.	n.d.
F049 (19.0)	0.016	n.d.	0.005	n.d.
F015	0.037	0.040	0.017	0.024
F016 / F017	0.012	0.032	n.d.	n.d.
BAS 750 F	0.082	0.092	0.130	0.144
Total Identified	**0.196**	**0.171**	**0.159**	**0.168**

n.d. = not detected / identified
() numbers in brackets denote retention times of isomers

Table 5.1.1-48: Proportions of metabolites identified in rat fat analyzed at tmax of plasma level (1 hour, metabolism study, C-label)

Dose	Percent of administered dose Group W Single high dose (180 mg/kg bw)	
Compound	**WM**	**WF**
BAS 750 F	0.135	0.224
Total Identified	**0.135**	**0.224**

Table 5.1.1-49: Proportions of metabolites identified in rat plasma (1 hour, metabolism study, C-label)

Dose	Percent of administered dose Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound	**VM**	**VF**	**WM**	**WF**
BAS 750 F	0.030	0.007	0.052	0.067
Total Identified	**0.030**	**0.007**	**0.052**	**0.067**

Table 5.1.1-50: Proportions of metabolites identified in rat plasma (1 hour, metabolism study, T-label)

Dose	Percent of administered dose			
	Group V Single low dose (5 mg/kg bw)		Group W Single high dose (180 mg/kg bw)	
Compound M750-	VM	VF	WM	WF
F001	0.037	0.002	0.014	0.006
F015	0.009	0.003	0.003	0.006
F000	0.016	0.003	0.025	0.026
Total Identified	**0.061**	**0.009**	**0.042**	**0.038**

5. Metabolic Pathway

The proposed metabolic pathway of BAS 750 F is shown from Figure 5.1/6 to Figure 5.1/13. An overview on quantified metabolites in numerical order is provided in Table 5.1.1-51. The identified metabolites comprise the following phase I and phase II conversions of the parent compound:

Phase I-Metabolism

1) Hydroxylation: mono, di- and trihydroxylation including CL-shift
(Figure 5.1/6, Figure 5.1/7, Figure 5.1/8, Figure 5.1/9 and Figure 5.1/13)

2) Methylation
(Figure 5.1/7 and Figure 5.1/12)

3) Cleavage of the ether group
(Figure 5.1/11 and Figure 5.1/13)

4) Cleavage of the triazole ring from the parent compound
(Figure 5.1/11 and Figure 5.1/13)

Phase II-Metabolism

Conjugation of Phase I metabolites by

a) Sulfation
(Figure 5.1/6, Figure 5.1/7, Figure 5.1/9, Figure 5.1/11, Figure 5.1/12 and Figure 5.1/13)

b) Glucuronidation
(Figure 5.1/6, Figure 5.1/7, Figure 5.1/9, Figure 5.1/11, Figure 5.1/12 and Figure 5.1/13)

c) GSH adduction and its decomposition products
(Figure 5.1/9, Figure 5.1/10, Figure 5.1/12 and Figure 5.1/13)

In detail, the following phase I metabolites were detected in quantitative amounts:

1) Hydroxylation: Mono, Di and Trihydroxylation including CL-shift:
M750F015, M750F016, M750F017, M750F078, M750F062
Hydroxylations may accompanied by a chlorine shift from para position to meta position at the chlorophenyl ring.

2) Methylation
M750F089

3) Cleavage of the ether group
M750F003

4) Cleavage of the triazole ring from the parent compound
M750F001

The following phase II metabolites were detected in quantitative amounts:

a) Sulfation
M750F043, M750F048, M750F055, M750F057, M750F058, M750F059, M750F060, M750F066, M750F067, M750F071, M750F079, M750F082, M750F096, M750F097, M750F101, M750F098 (includes M750F060 with potential Cl-shift)

b) Glucuronidation
M750F035, M750F044, M750F045, M750F046, M750F047, M750F049, M750F052, M750F054, M750F063, M750F108

c) GSH adduction and its decomposition products
M750F048, M750F050, M750F052, M750F053, M750F055, M750F061, M750F065, M750F069, M750F075, M750F079, M750F084, M750F085, M750F087, M750F091

Generally, the cysteine conjugates most likely result from glutathione conjugation with following degradation of this formation by glutamyltranspeptidase. In the next step, the resulting cysteinylglycine conjugates are then cleaved by a dipeptidase into glycine and the cysteine conjugates. It is consequently assumed that metabolites as M750F055 or M750F061 are further degradation products of this glutathione pathway, taking also into account the identified precursors (e.g. M750F079 and M750F083 as precursors for M750F055).

Few metabolites were only detected in trace amounts by HPLC-MS/MS, which can be either attributed to phase I (M750F005, M750F038) or phase II (M750F088, M750F100, M750F102, M750F107) as indicated above.

Figure 5.1/6 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 1)

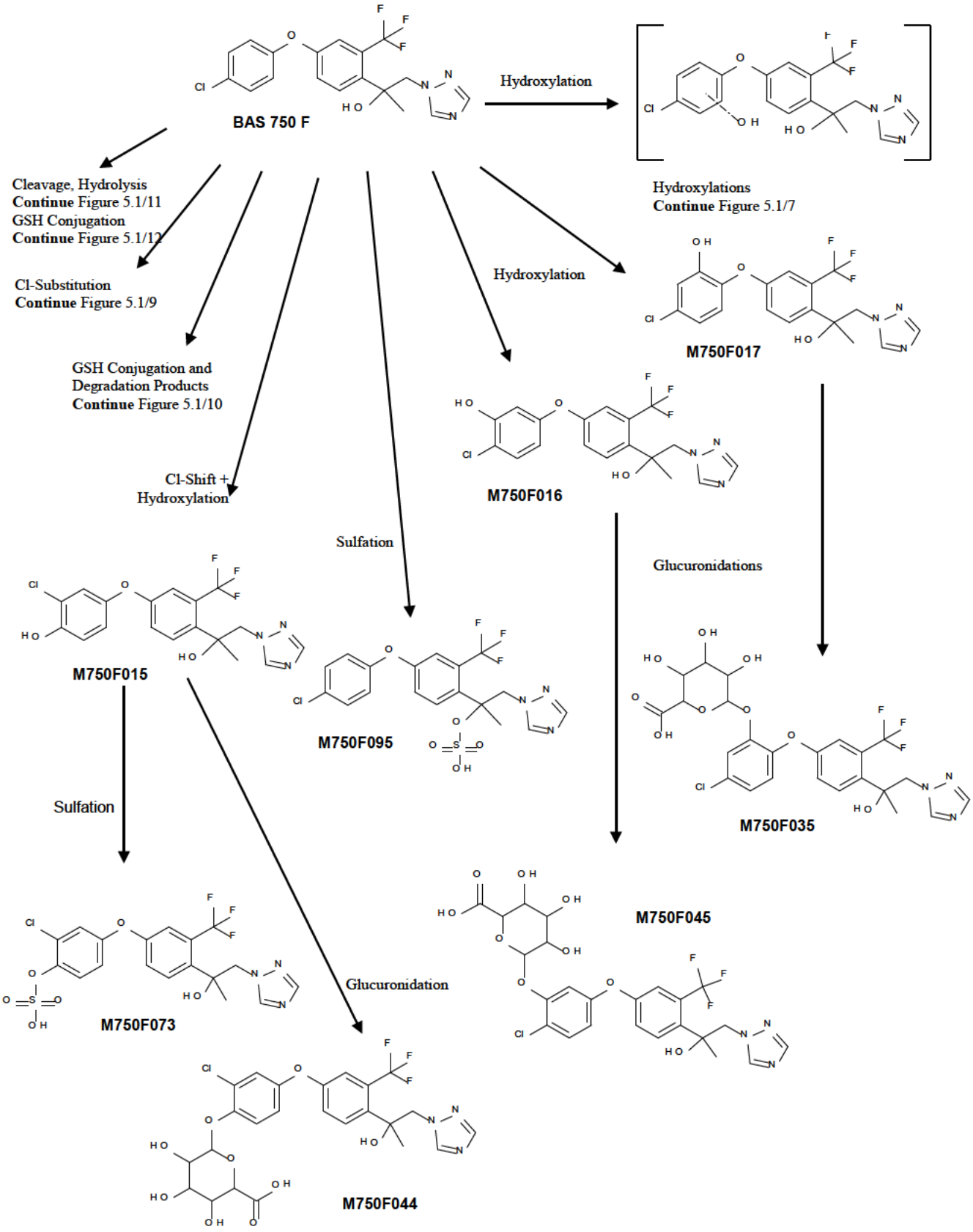


Figure 5.1/7 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 2, First And Second Hydroxylation, Multiple Phase I and Phase II)

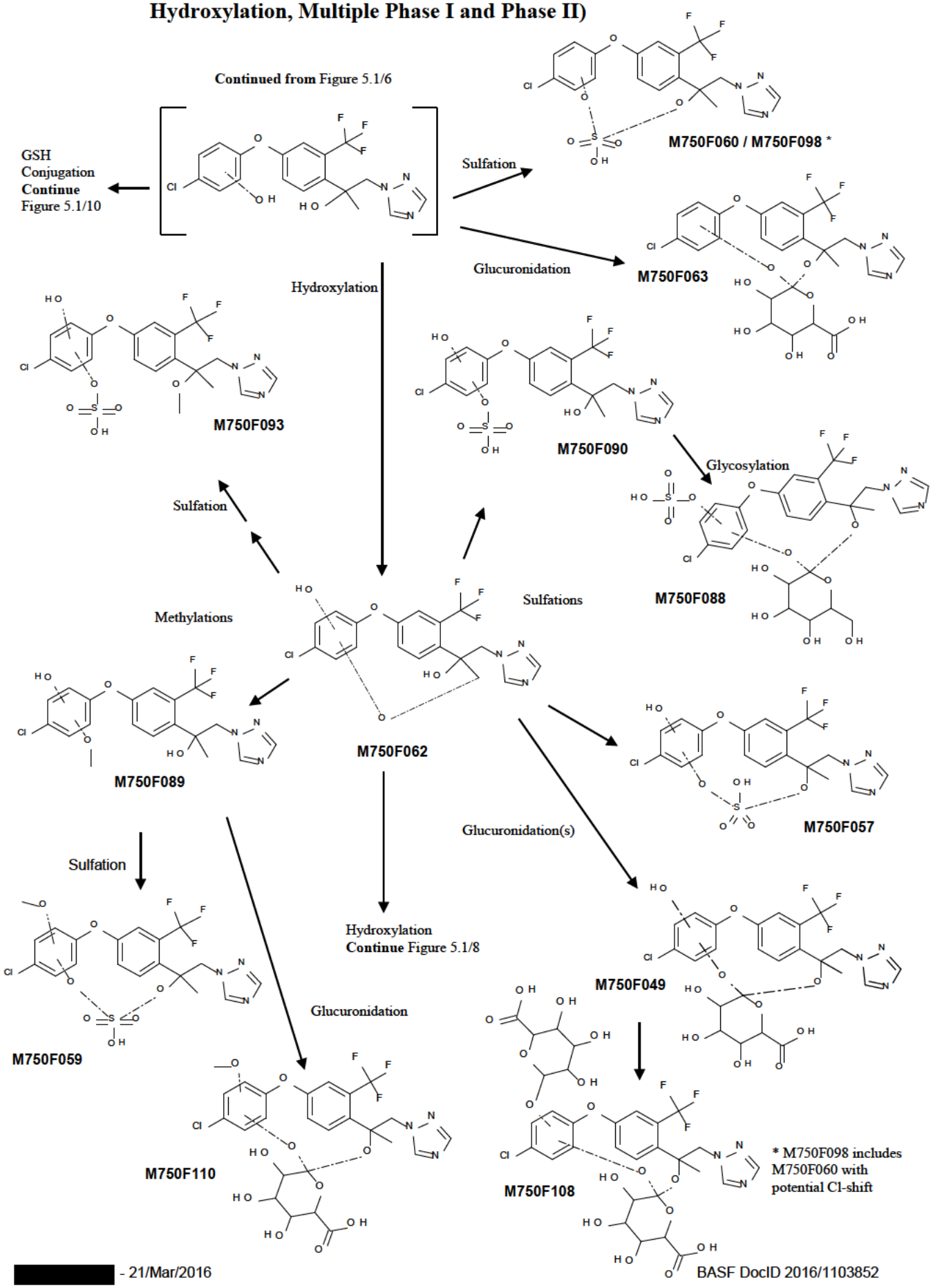


Figure 5.1/8 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 3, Third Hydroxylation, Multiple Phase I and Phase II)

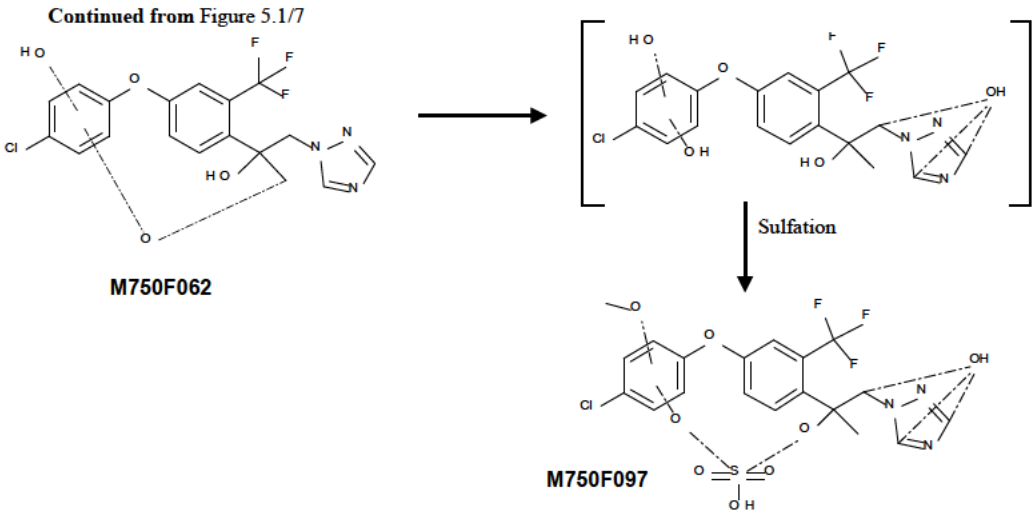


Figure 5.1/9 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 4, Cl-Substitution, Multiple Phase I and Phase II)

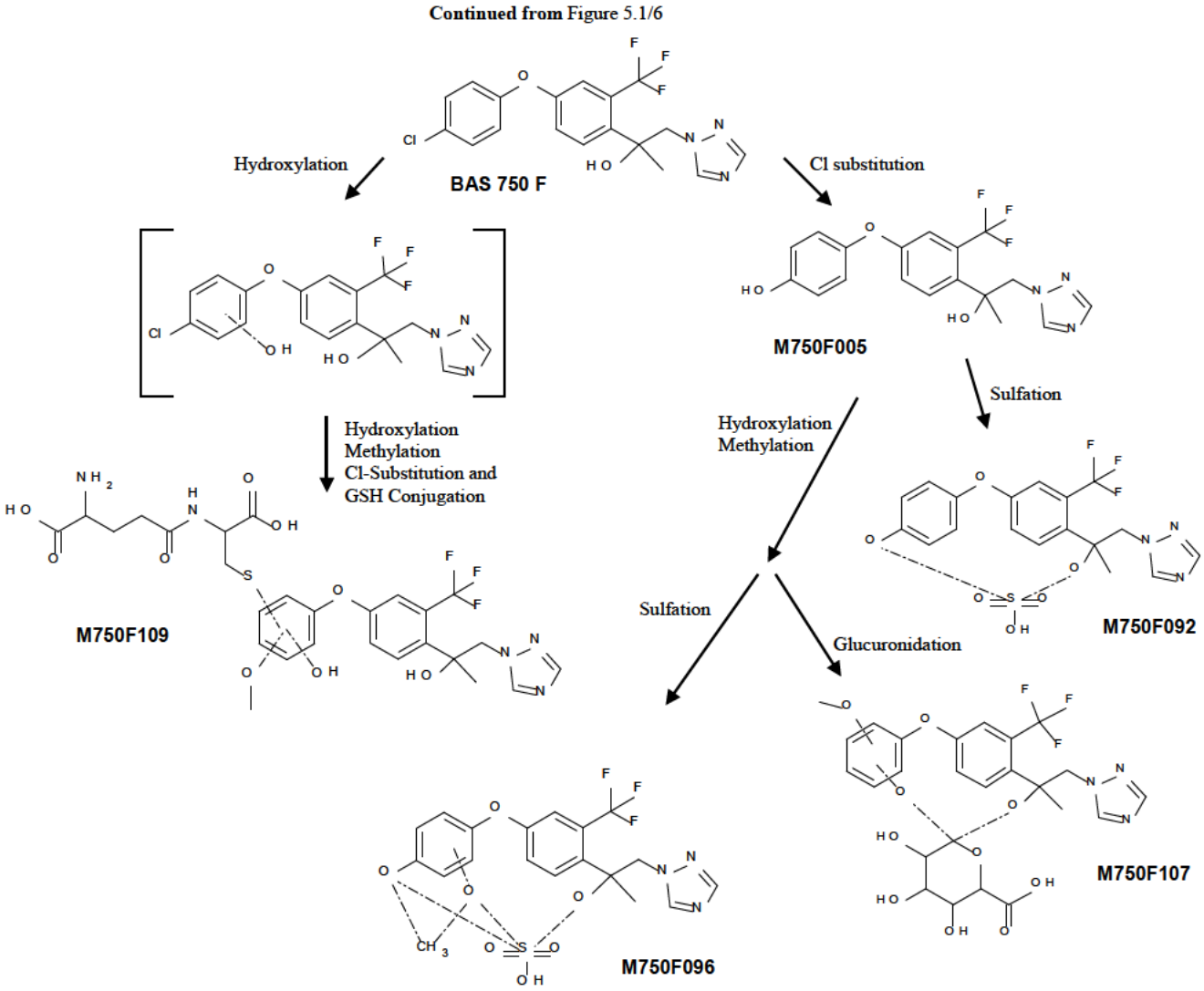


Figure 5.1/10 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 5, GSH Conjugation)

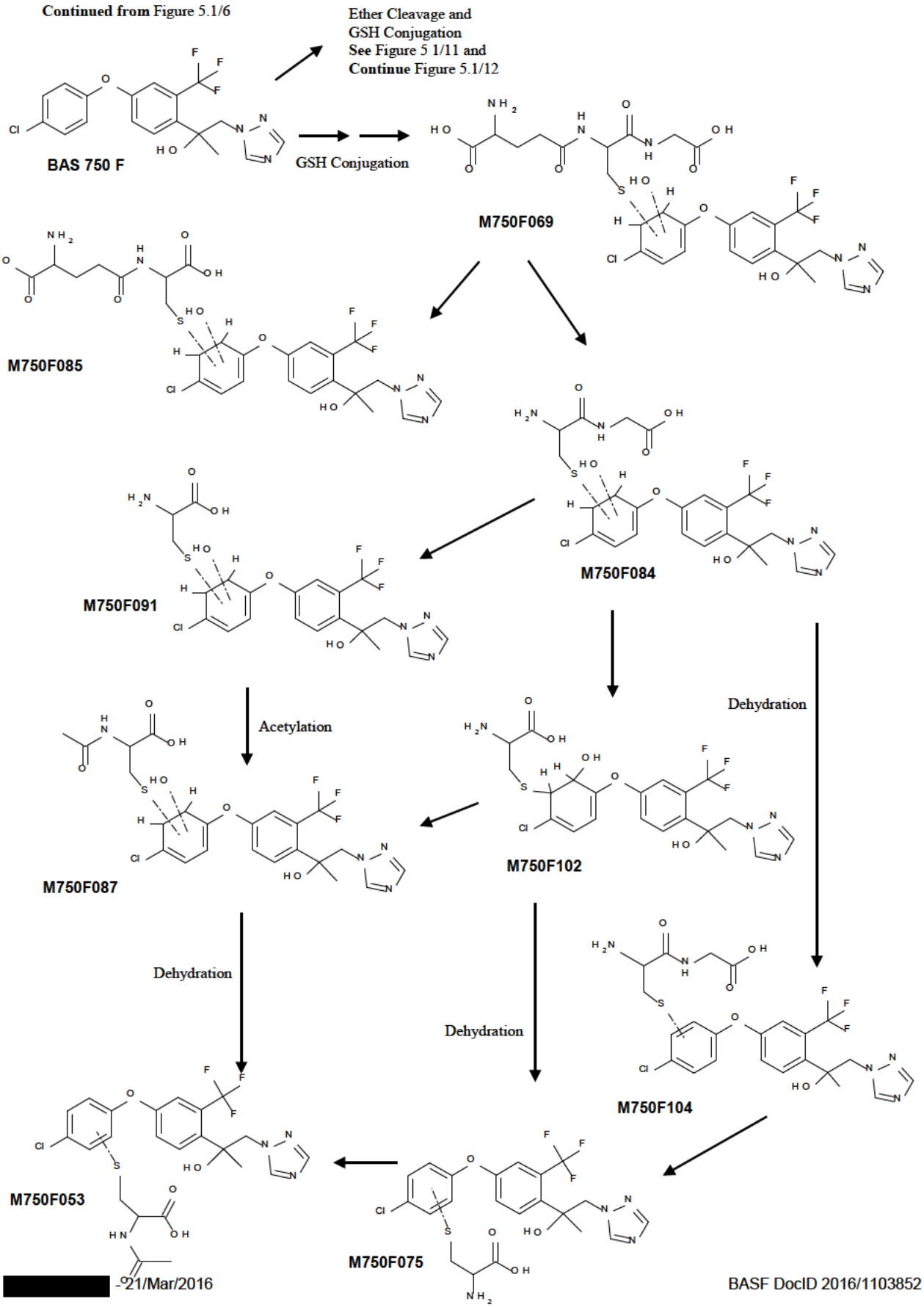


Figure 5.1/11 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 6, Ether Cleavage and Hydrolysis)

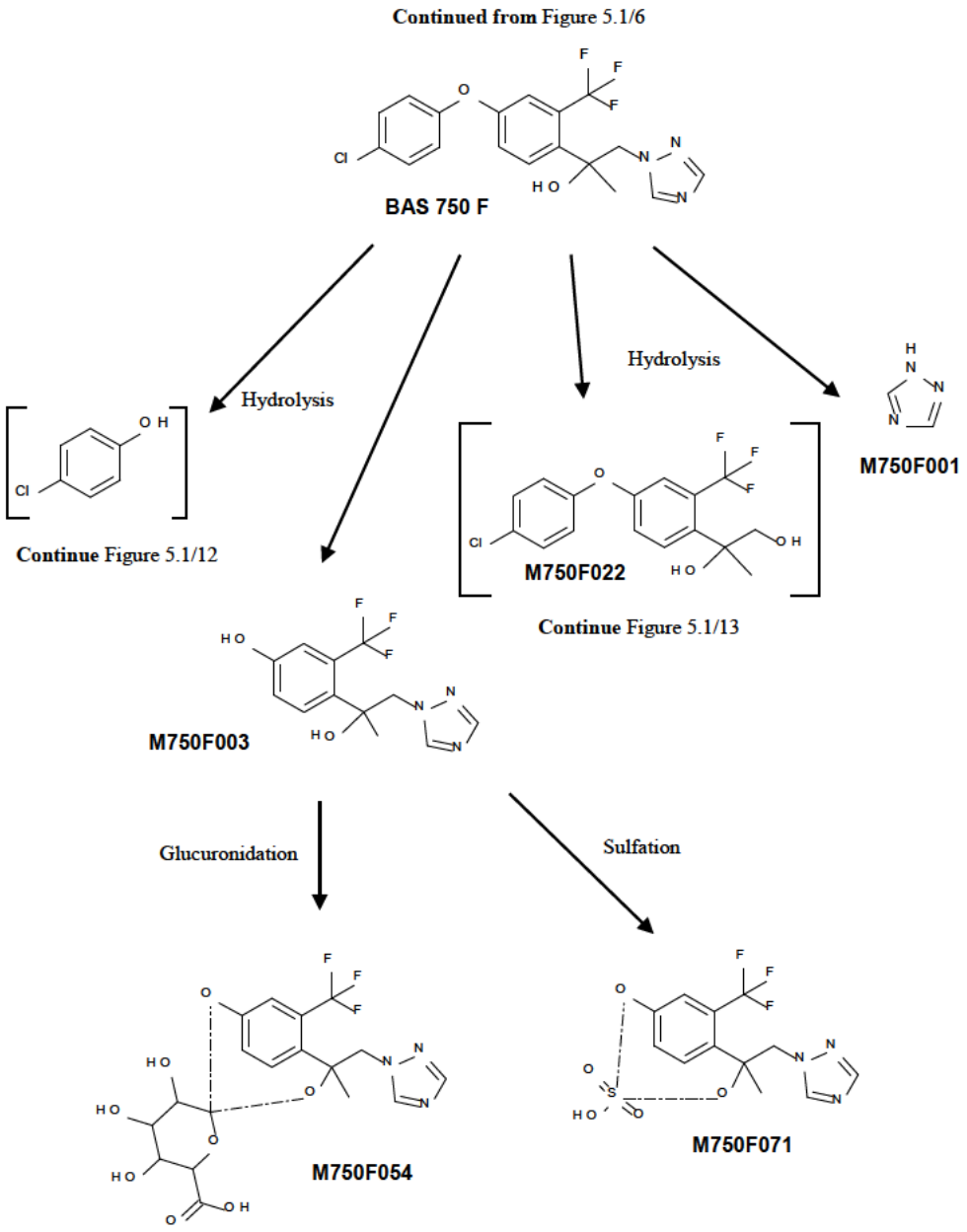


Figure 5.1/12 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 7, Ether Cleavage, GSH Conjugation)

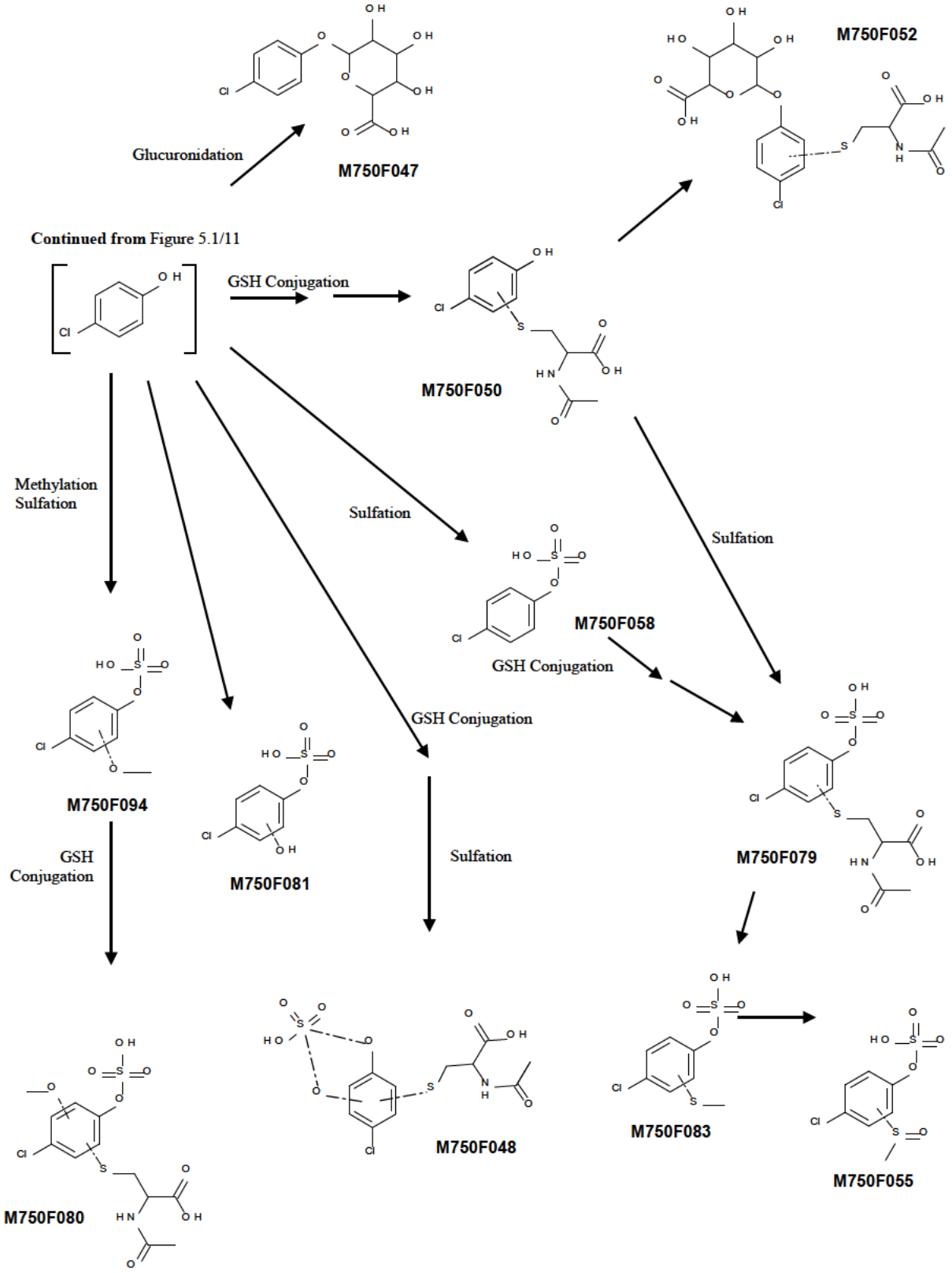


Figure 5.1/13 Proposed Metabolic Pathway of BAS 750 F in Rats (Part 8, Multiple Phase I and Phase II after Hydrolysis)

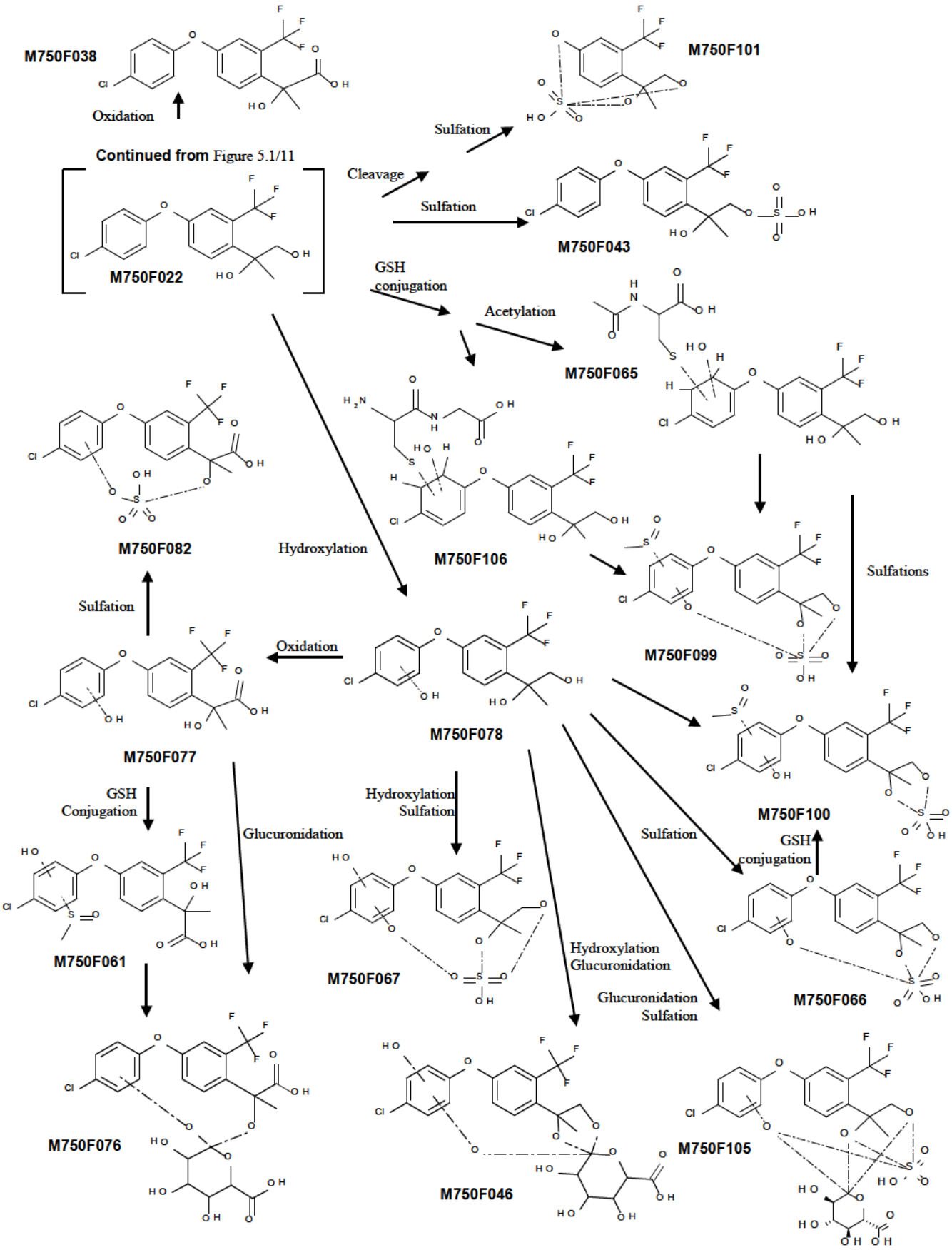
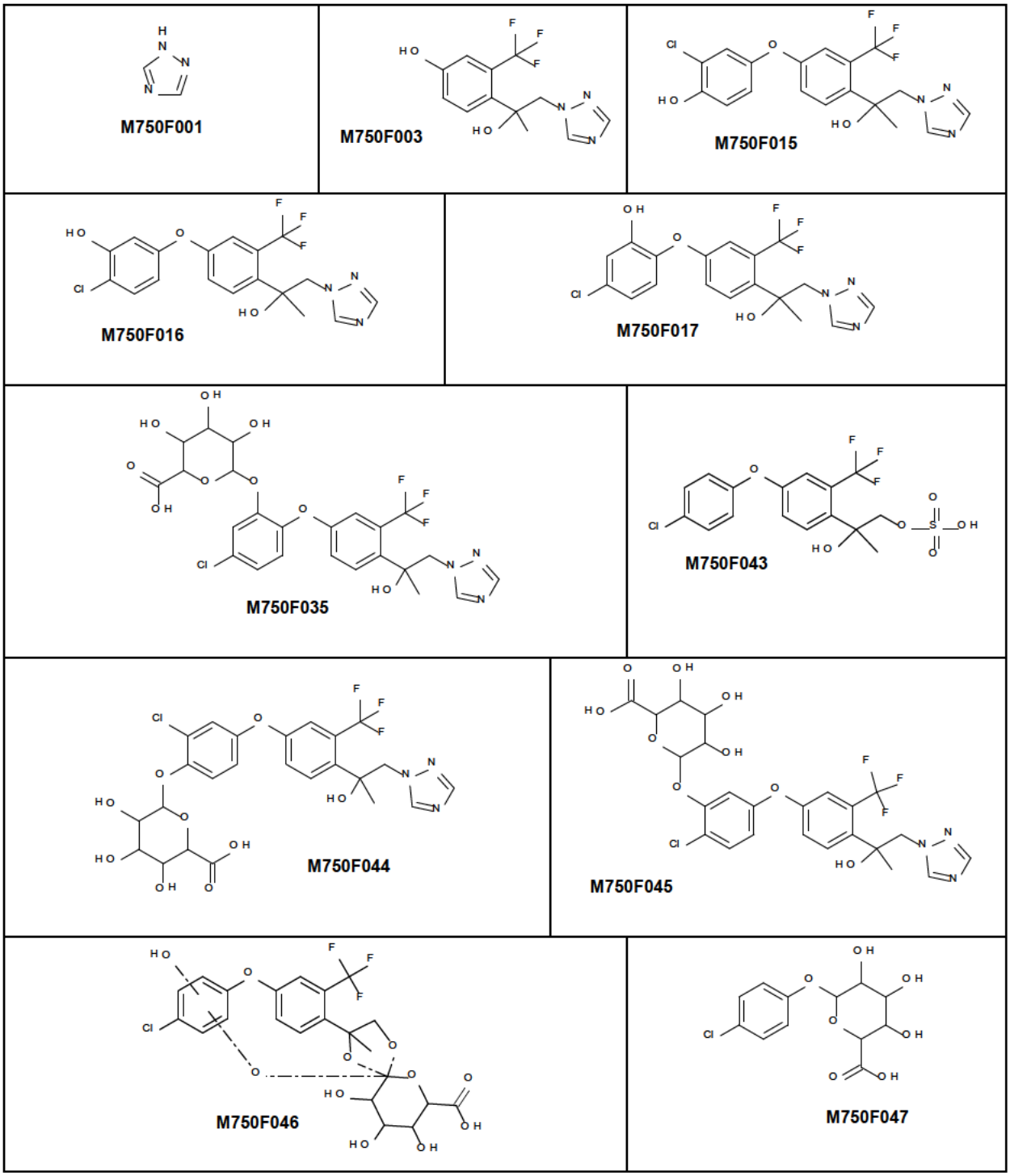
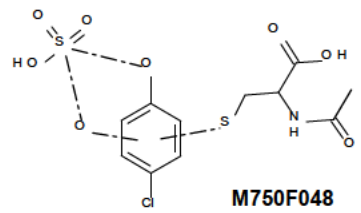
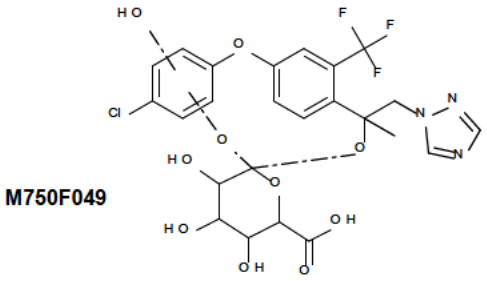


Table 5.1.1-51: Summary of identified and quantified metabolites

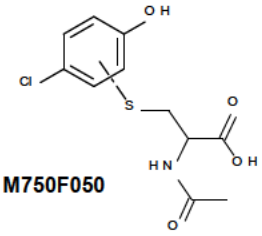




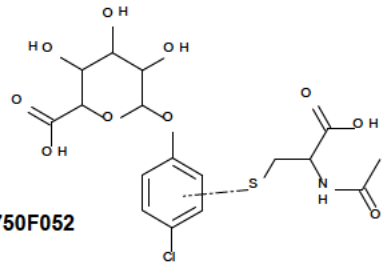
M750F048



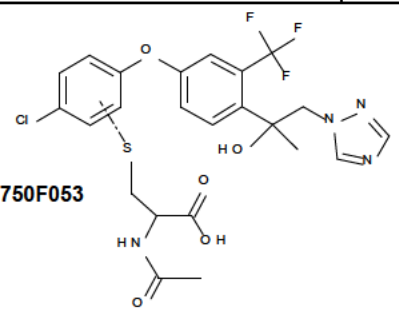
M750F049



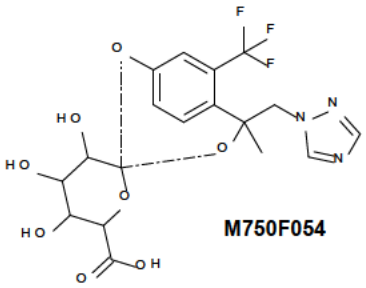
M750F050



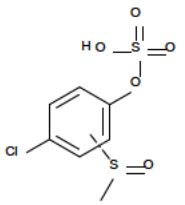
M750F052



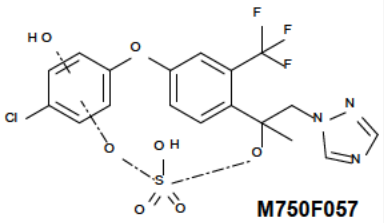
M750F053



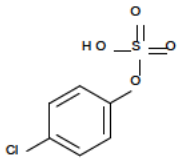
M750F054



M750F055



M750F057



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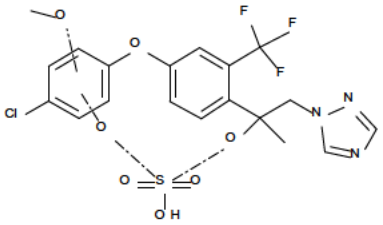

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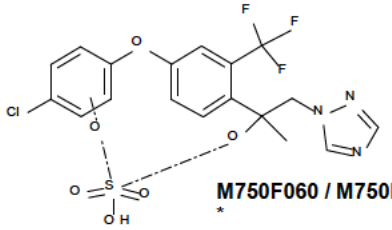
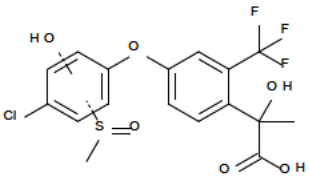


M750F060 / M750F098
*

* M750F098 includes M750F060 with potential Cl-shift



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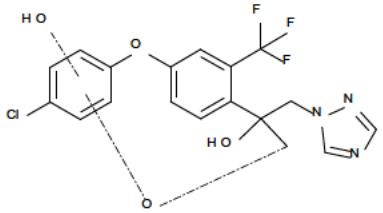

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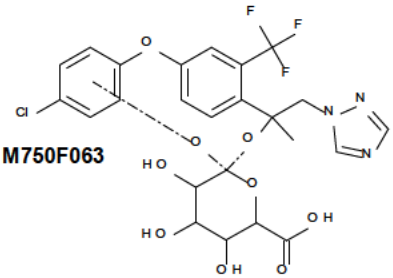

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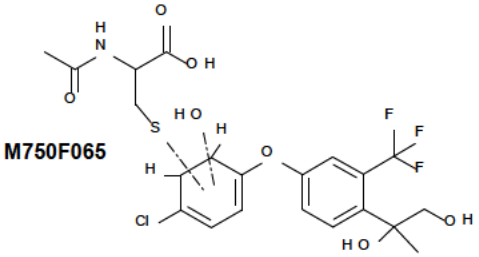

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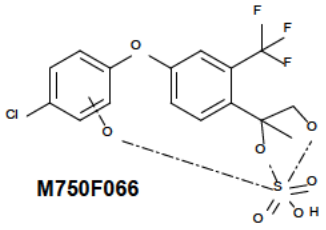

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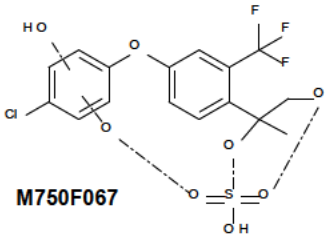

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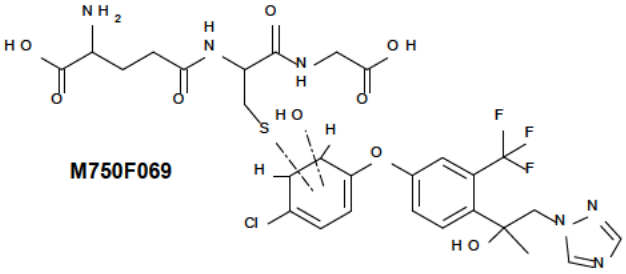

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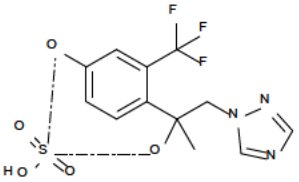

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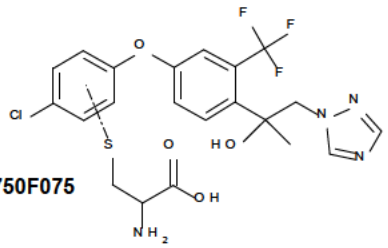

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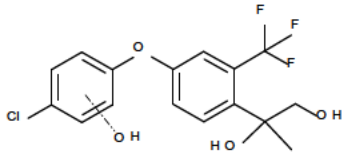

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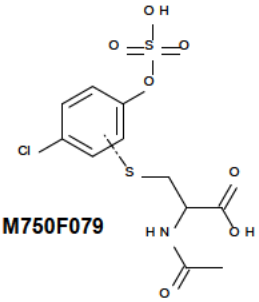
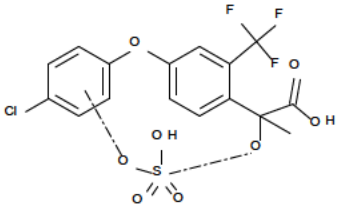
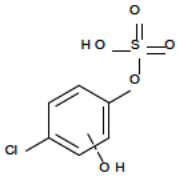


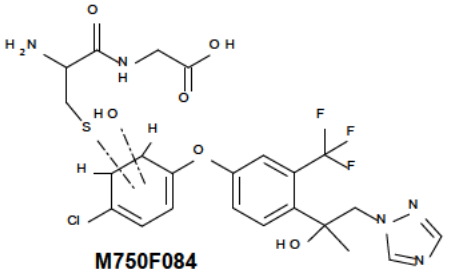
M750F079



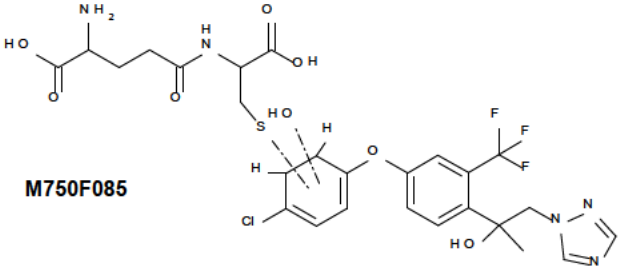
M750F082



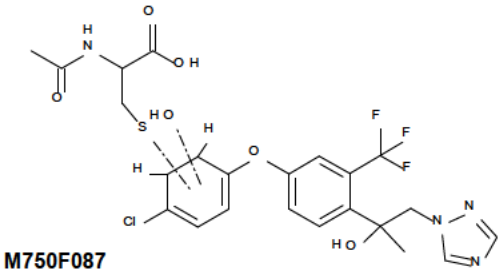
M750F081



M750F084



M750F085



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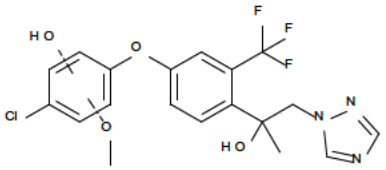
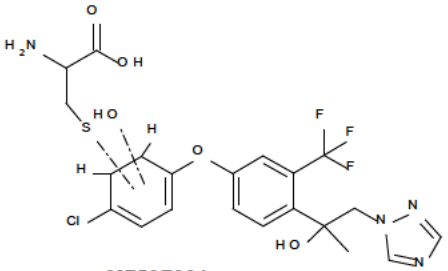
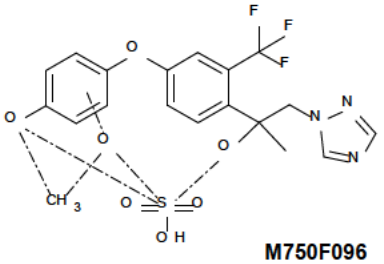


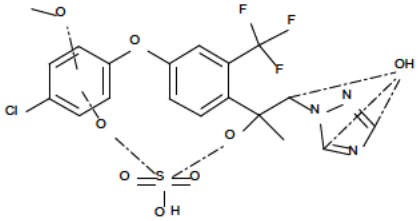
M750F089



M750F091

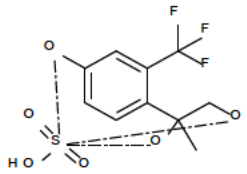


M750F096

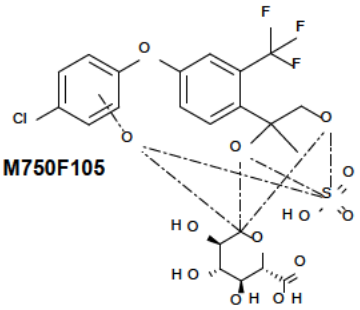


M750F097

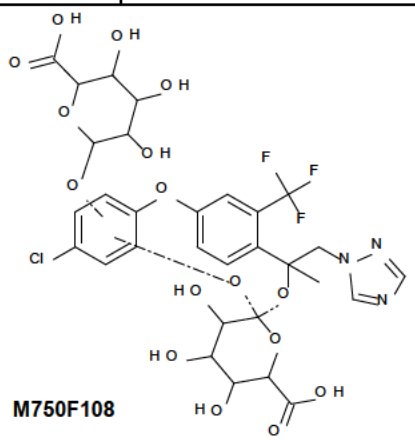
M750F098: see M750F060



M750F101



M750F105



M750F108

Table 5.1.1-52: Summary of metabolites in urine, faeces and bile (C-label)

Metabolite (M750F-)	Urine BM	Urine BF	Urine DM	Urine DF	Urine DXM	Urine DXF	Urine CM	Urine CF	Faeces BM	Faeces BF	Faeces DM	Faeces DF	Faeces DXM	Faeces DXF	Faeces CM	Faeces CF	Bile RM	Bile RF	Bile SM	Bile SF1	Bile SF2
	Composition of Radioactive Residues in % of the Dose																				
001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
054	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
084	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	4.0	2.3	1.2	0.9
106	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.9	-	4.9	-	-
069	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		0.4	5.5
085	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
047	-	-	-	-	0.3	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
046	-	-	-	-	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
048	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
052	-	-	0.1	-			0.5	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-
049 [1]	2.6	2.2	1.0	-	0.6	0.5	0.5		-	-	-	-	-	-	-	-	2.4	3.1	1.7	-	0.5
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				-	
003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
075	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	-	1.4	-	0.6
050	-	-	0.7	0.5	0.5	0.2	0.7	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-
076	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
065	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
079	-	-	0.1	0.3	-	-	0.2		-	-	-	-	-	-	-	-	-	-	-	-	-
063	-	2.4	-	-	-	-	-	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-
087	-	-	-	-	-	1.0	-		-	-	-	-	-	-	-	-	53	49	22	37	45
044	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-					
045	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-					
035	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
049 [2]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
071	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
062	-	-	-	-	-	+	-	-	4.0	3.7	-	-	3	4	-	-	-	-	-	-	-
053	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
105	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	-	0.7	-	-
077	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
078	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
015	-	0.2 [3]	-	0.6 [3]	0.2 [6]	0.3	0.4 [15]	0.2 [3]	25	27	13	23	17	27	21	30	-	-	-	0.1	-
055	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
088	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
061	-	-	-	-	-	0.9 [6]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
016	-	0.8 [3]	-	1.1 [4]	0.3 [6]		0.7	0.7 [16]	32	24	24	18	28	21	32	29	-	-	-	-	-
017	-		0.6														-	-	-	-	-
089	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
080	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
098	-	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Metabolite (M750F-)	Urine BM	Urine BF	Urine DM	Urine DF	Urine DXM	Urine DXF	Urine CM	Urine CF	Faeces BM	Faeces BF	Faeces DM	Faeces DF	Faeces DXM	Faeces DXF	Faeces CM	Faeces CF	Bile RM	Bile RF	Bile SM	Bile SF1	Bile SF2
	Composition of Radioactive Residues in % of the Dose																				
038	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
000	-	-	-	-	-	-	-	-	4.5	5.4	28	21	13	8	20	16	-	-	-	-	-
067	-	-	0.3	-	-	0.2[8]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
090	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
095	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
097	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
096	-	-	-	-	-	0.4[9]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
092	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
094	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
093	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
058	0.9	-	0.5	0.6	0.8	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
081		-		-	0.1	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-
060	-	-	-	-	-	0.4[10]	-	-	-	-	-	-	-	-	-	-	0.6	-	0.7	-	-
057	-	-	-	-	-	0.8[11]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
073	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
082	-	-	0.4	-	0.2[7]	-	1.1	3.3[17]	-	-	-	-	-	-	-	-	-	-	-	-	-
083	-	0.3	0.9	1.0		0 2[12]			-	-	-	-	-	-	-	-	-	-	-	-	-
066	-	0.1	-	0.3[5]	-		-		-	-	-	-	-	-	-	-	-	-	-	-	-
099	-	-	-		-	0 1[13]	-		-	-	-	-	-	-	-	-	-	-	-	-	-
059	-	1.5	-		-	0 5[14]	-		-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
043	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-

Metabolites were sorted by retention time.

\+ metabolite was assigned, but the % of dose value was equal or below 0.1%

\- metabolite was not quantitatively assigned

1) includes isomer of metabolite M750F049 at 19.0 min
2) includes isomers of metabolite M750F049 at 21.5 min, 21.9 min and 22.2 min
3) includes also metabolite M750F057
4) includes also metabolite M750F059
5) includes also metabolite M750F098
6) includes also metabolite M750F078
7) includes also metabolite M750F055
8) includes also metabolite M750F079
9) includes also metabolite M750F058
10) includes also metabolites M750F055 and M750F057
11) includes also metabolite M750F066
12) includes also metabolites M750F060 and M750F097
13) includes also metabolite M750F082
14) includes also metabolites M750F057 and M750F066
15) includes also metabolites M750F058 and M750F067
16) includes also metabolite M750F096
17) includes also metabolites M750F038 and M750F098

Table 5.1.1-53: Summary of metabolites in urine, faeces and bile (TFMP-label)

Metabolite (M750F-)	Urine DM		Urine DF	Urine SM	Urine SF	Faeces DM	Faeces DF	Bile SM	Bile SF
	Composition of Radioactive Residues in % of the Dose								
001	-		-	-	-	-	-	-	-
054	1.6		2.1	0.4	0.5	-	-	0.6	-
084	-		-	-	0.1	-	-	2.1	-
106	-		-	-	-	-	-	-	-
102	-		-	-	-	-	-	-	-
091	-		-	0.8	0.8	-	-	-	-
085	-		-	-	-	-	-	7.5	3.6
069	-		-	-	-	-	-		
101	0.6		-	-	-	-	-	-	-
047	-		-	-	-	-	-	-	-
046	-		-	-	-	-	-	-	-
048	-		-	-	-	-	-	-	-
052	-		-	-	-	-	-	-	-
049 [1)]	2.8		2.1	0.5 [4)]	0.6	-	-	1.8	3.3
003						2 5	2.6	-	-
108	-		-	-		-	-	-	-
109	-		-	-	-	-	-	-	-
104	-		-	-	-	-	-	-	-
075	-		-	-	-	-	-	1.3	0.5
050	-		-	-	-	-	-	-	-
076	-		-	-	-	-	-	-	-
110	-		-	-	-	-	-	-	1.6
107	-		-	-	-	-	-	-	-
065	-		-	-	-	-	-	-	-
079	-		-	-	-	-	-	-	-
063	-		0.6	5.2	-	-	-	-	-
087	-				4.3 [5)]	-	-	22	48
044	-		-	-		-	-		
045	-		-	-		-	-	19	
035	-		-	-		-	-		
049 [2)]	-		-	-	-	-	-		
071	3.1		-	-	-	-	-	-	-
062	-		-	-	0.4 [6)]	-	-	-	-
053	-		-	-		-	-	-	-
105	-		-	-	-	-	-	-	-
077	-		-	-	-	-	-	-	-
078	-		-	-	-	-	-	-	-
015	0.7		0.7	1.2	1.2 [7)]	15	30	1.5	-
055	-		-	-	-	-	-	-	-
088	-		-	-	-	-	-	-	-
061	-		-	-	-	-	-	-	-
016	0.4		1.4	1.9	1.9	29	22	-	-
017								-	-
089	-		-	-		-	-	-	-
080	-		-	-	-	-	-	-	-
098	-		0.8	-	-	-	-	-	-
038	-		-	-	-	-	-	-	-
000	-		-	-	-	17	21	-	-
067	-		-	-	-	-	-	-	-
090	-		-	-	-	-	-	-	-

Metabolite (M750F-)	Urine DM		Urine DF	Urine SM	Urine SF	Faeces DM	Faeces DF	Bile SM	Bile SF
		Composition of Radioactive Residues in % of the Dose							
095	-		-	-	-	-	-	-	-
097	-		-	-	-	-	-	-	-
096	-		-	-	-	-	-	-	-
092	-		-	-	-	-	-	-	-
094	-		-	-	-	-	-	-	-
093	-		-	-	-	-	-	-	-
058	-		-	-	-	-	-	-	-
081	-		-	-	-	-	-	-	-
060	-		-	-	-	-	-	-	-
057	-		-	-	-	-	-	-	-
073	-		-	-	-	-	-	-	-
082	-		-	-	-	-	-	-	-
083	-		-	-	-	-	-	-	-
066	-		-	-	-	-	-	-	-
059	-		1.9 [3)]	-	-	-	-	-	-
099	-		-	-	-	-	-	-	-
100	-		-	-	-	-	-	-	-
005	-		-	-	-	-	-	-	-
043	-		-	-	-	-	-	-	-

Metabolites were sorted by retention time.
- metabolite was not quantitatively assigned
1) includes isomer of metabolite M750F049 at 19.0 min
2) includes isomers of metabolite M750F049 at 21.5 min, 21.9 min and 22.2 min
3) includes also metabolite M750F098
4) includes also metabolite M750F071
5) includes also metabolite M750F065
6) includes also metabolite M750F067
7) includes also metabolite M750F067 and M750F089

Table 5.1.1-54: Summary of metabolites in urine, faeces and bile (T-label)

Metabolite (M750F-)	Urine BM	Urine BF	Urine DM	Urine DF	Urine DXM	Urine DXF	Urine CM	Urine CF	Faeces BM	Faeces BF		Faeces DM	Faeces DF	Faeces DXM	Faeces DXF	Faeces CM	Faeces CF	Bile RM	Bile RF	Bile SM	Bile SF
	Composition of Radioactive Residues in % of the Dose																				
001	20	3 2	9.6	3.1	10	3.3	14	7.7	-	-		-	-	-	-	-	-	0.2	+	0 2	+
054	4.3	2.7	1.1	1.5	2 3	1.8	0.7	1.6	-	-		-	-	-	-	-	-	1.5	0.7	0.6	-
084	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	1.6	-	-
106	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
091	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	3.3	3.3	4.0	0.1
069	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	0.3	-	-
085	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
047	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
046	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
048	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
052	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
049 [1)]	3.1	3.7	-	1.1	1.0	0.8	-	1.4	-	-		-	-	-	-	-	-	7.0	5.5	1.8	0.5
003		-	1.5		-	-	1.9		4.3	4.3		1.8	3.5	1.4	1.2	1.3	1.1				
104	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
075	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	11	5.2	5 5	5.4
050	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
076	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
065	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
079	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
063	-	1.8	-	0.7	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
087	-	-	-		-		-	-	-	-		-	-	-	-	-	-	-			
044	-	-	-	-	-	1.2	-	-	-	-		-	-	-	-	-	-				
045	-	-	-	-	-		-	-	-	-		-	-	-	-	-	-	23	41	25	31
035	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-				
049 [2)]	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-				
071	6.7	-	2.1	-	2.0	-	1.6	-	-	-		-	-	-	-	-	-	-	-	-	-
062	-	-	-	-	-	-	-	-	6.8	6.2		-	-	-	-	-	-	-	-	-	-
053	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
105	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
077	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
078	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
015	-	0.4	-	0.4	-	0.3	-	0.4	10	41		14	25	11	20	11	20	9.5	10	3.0	3.8
055	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
088	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
061	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
016	-	1 3	-	1.1 [3)]	-	0.6	-	1.0	22	26		24	18	20	15	20	20	12	6.5	3 1	2.3
017	-		-		-		-														
089	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
080	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
098	-	0.4	-	0.5	-	-	-	0.3	-	-		-	-	-	-	-	-	-	-	-	-

Metabolite (M750F-)	Urine BM	Urine BF	Urine DM	Urine DF	Urine DXM	Urine DXF	Urine CM	Urine CF	Faeces BM	Faeces BF		Faeces DM	Faeces DF	Faeces DXM	Faeces DXF	Faeces CM	Faeces CF	Bile RM	Bile RF	Bile SM	Bile SF
	Composition of Radioactive Residues in % of the Dose																				
038	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
000	-	-	-	-	-	-	-	-	3.1	1.4		26	30	35	30	29	24	-	-	-	-
067	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
090	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
095	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
097	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
096	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
092	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
094	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
093	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
058	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
081	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
060	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
057	-	-	-	+	-	0.3	-	-	-	-		-	-	-	-	-	-	-	-	-	-
073	-	-	-	-	-	1.0	-	-	-	-		-	-	-	-	-	-	-	-	-	-
082	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
083	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
066	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
099	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
059	-	0.6	-	0.6	-	0.2 [4)]	-	-	-	-		-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
005	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
043	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-

Metabolites were sorted by retention time.

\+ metabolite was assigned, but the % of dose value was equal or below 0.1%

\- metabolite was not quantitatively assigned

1) includes isomer of metabolite M750F049 at 19.0 min

2) includes isomers of metabolite M750F049 at 21.5 min, 21.9 min and 22.2 min

3) includes also metabolite M750F059

4) includes also metabolite M750F057

Table 5.1.1-55: Summary of metabolites in tissues and plasma (C-label)

Metabolite (M750F-)	Liver				Kidney				Fat		Plasma			
	VM	VF	WM	WF	VM	VF	WM	WF	WM	WF	VM	VF	WM	WF
	Composition of Radioactive Residues in % of the Dose													
001	-	-	-	-	-	-	-	-	-	-	-	-	-	-
054	-	-	-	-	-	-	-	-	-	-	-	-	-	-
084	-	-	-	-	-	-	-	-	-	-	-	-	-	-
106	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-
091	-	-	-	-	-	-	-	-	-	-	-	-	-	-
069	-	-	-	-	-	-	-	-	-	-	-	-	-	-
085	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-	-	-	-	-
047	-	-	-	-	-	-	-	-	-	-	-	-	-	-
046	-	-	-	-	-	-	-	-	-	-	-	-	-	-
048	-	-	-	-	-	-	-	-	-	-	-	-	-	-
052	-	-	-	-	-	-	-	-	-	-	-	-	-	-
049 [1)]	0.46	-	0.28	0.13	-	-	-	-	-	-	-	-	-	-
003	-	-	-	-	-	-	-	-	-	-	-	-	-	-
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-	-	-	-	-
075	-	-	-	-	-	-	-	-	-	-	-	-	-	-
050	-	-	-	-	-	-	-	-	-	-	-	-	-	-
076	-	-	-	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-	-	-	-	-
065	-	-	-	-	-	-	-	-	-	-	-	-	-	-
079	-	-	-	-	-	-	-	-	-	-	-	-	-	-
063	-	-	-	-	-	-	-	-	-	-	-	-	-	-
087	-	-	-	-	-	-	-	-	-	-	-	-	-	-
044	-	-	-	-	-	-	-	-	-	-	-	-	-	-
045	-	-	-	-	-	-	-	-	-	-	-	-	-	-
035	-	-	-	-	-	-	-	-	-	-	-	-	-	-
049 [2)]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
071	-	-	-	-	-	-	-	-	-	-	-	-	-	-
062	-	-	-	-	-	-	-	-	-	-	-	-	-	-
053	-	-	-	-	-	-	-	-	-	-	-	-	-	-
105	-	-	-	-	-	-	-	-	-	-	-	-	-	-
077	-	-	-	-	-	-	-	-	-	-	-	-	-	-
078	3.60 [3)]	2.36 [3)]	1.73 [3)]	1.83 [3)]	0.06 [3)]	0.06 [3)]	0.05 [3)]	0.04 [3)]	-	-	-	-	-	-
015									-	-	-	-	-	-
055									-	-	-	-	-	-
088	-	-	-	-	-	-	-	-	-	-	-	-	-	-
061	1.61 [4)]	2.07 [4)]	0.61 [4)]	0.88 [4)]	-	0.03 [4)]	-	0.01 [4)]	-	-	-	-	-	-
016					-		-		-	-	-	-	-	-
017					-		-		-	-	-	-	-	-
089					-		-		-	-	-	-	-	-
080	-	-	-	-	-	-	-	-	-	-	-	-	-	-
098	-	-	-	-	-	-	-	-	-	-	-	-	-	-
038	-	-	-	-	-	-	-	-	-	-	-	-	-	-
000	1.66	1.01	1.88	2.03	0.11	0.11	0.19	0.24	0 13	0 22	0.03	0.01	0.05	0.07

Metabolite (M750F-)	Liver				Kidney				Fat		Plasma			
	VM	VF	WM	WF	VM	VF	WM	WF	WM	WF	VM	VF	WM	WF
	Composition of Radioactive Residues in % of the Dose													
067	-	-	-	-	-	-	-	-	-	-	-	-	-	-
090	-	-	-	-	-	-	-	-	-	-	-	-	-	-
095	-	-	-	-	-	-	-	-	-	-	-	-	-	-
097	-	-	-	-	-	-	-	-	-	-	-	-	-	-
096	-	-	-	-	-	-	-	-	-	-	-	-	-	-
092	-	-	-	-	-	-	-	-	-	-	-	-	-	-
094	-	-	-	-	-	-	-	-	-	-	-	-	-	-
093	-	-	-	-	-	-	-	-	-	-	-	-	-	-
058	-	-	-	-	-	-	-	-	-	-	-	-	-	-
081	-	-	-	-	-	-	-	-	-	-	-	-	-	-
060	-	-	-	-	-	-	-	-	-	-	-	-	-	-
057	-	-	-	-	-	-	-	-	-	-	-	-	-	-
073	-	-	-	-	-	-	-	-	-	-	-	-	-	-
082	-	-	-	-	-	-	-	-	-	-	-	-	-	-
083	-	-	-	-	-	-	-	-	-	-	-	-	-	-
066	-	-	-	-	-	-	-	-	-	-	-	-	-	-
099	-	-	-	-	-	-	-	-	-	-	-	-	-	-
059	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
005	-	-	-	-	-	-	-	-	-	-	-	-	-	-
043	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Metabolites were sorted by retention time.
+ metabolite was assigned, but the % of dose value was equal or below 0.005%
- metabolite was not quantitatively assigned
1) includes isomer of metabolite M750F049 at 19.0 min
2) includes isomers of metabolite M750F049 at 21.5 min, 21.9 min and 22.2 min
3) includes also metabolite M750F067
4) includes also metabolite M750F078

Table 5.1.1-56: Summary of metabolites in tissues and plasma (T-label)

Metabolite (M750F-)	Liver				Kidney				Plasma			
	VM	VF	WM	WF	VM	VF	WM	WF	VM	VF	WM	WF
	Composition of Radioactive Residues in % of the Dose											
001	-	-	-	-	0.05	-	0.01	-	0.04	+	0.01	0.01
054	-	-	-	-	-	0.01	-	-	-	-	-	-
084	-	-	-	-	-	-	-	-	-	-	-	-
106	-	-	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-	-	-
091	-	-	-	-	-	-	-	-	-	-	-	-
069	-	-	-	-	-	-	-	-	-	-	-	-
085	-	-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-	-	-
047	-	-	-	-	-	-	-	-	-	-	-	-
046	-	-	-	-	-	-	-	-	-	-	-	-
048	-	-	-	-	-	-	-	-	-	-	-	-
052	-	-	-	-	-	-	-	-	-	-	-	-
049 [1)]	0.51	-	-	-	0.02	-	+	-	-	-	-	-
003	-	-	-	-	-	-	-	-	-	-	-	-
104	-	-	-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-	-	-
075	-	-	-	-	-	-	-	-	-	-	-	-
050	-	-	-	-	-	-	-	-	-	-	-	-
076	-	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-	-	-
065	-	-	-	-	-	-	-	-	-	-	-	-
079	-	-	-	-	-	-	-	-	-	-	-	-
063	-	-	-	-	-	-	-	-	-	-	-	-
087	-	-	-	-	-	-	-	-	-	-	-	-
044	-	-	-	-	-	-	-	-	-	-	-	-
045	-	-	-	-	-	-	-	-	-	-	-	-
035	-	-	-	-	-	-	-	-	-	-	-	-
049 [2)]	-	-	-	-	-	-	-	-	-	-	-	-
071	-	-	-	-	-	-	-	-	-	-	-	-
062	-	-	-	-	-	-	-	-	-	-	-	-
053	-	-	-	-	-	-	-	-	-	-	-	-
105	-	-	-	-	-	-	-	-	-	-	-	-
077	-	-	-	-	-	-	-	-	-	-	-	-
078	-	-	-	-	-	-	-	-	-	-	-	-
015	3.25	2.66	0.95	0.91	0.04	0.04	0.02	0.02	0.01	+	+	0.01
055	-	-	-	-	-	-	-	-	-	-	-	-
088	-	-	-	-	-	-	-	-	-	-	-	-
061	-	-	-	-	-	-	-	-	-	-	-	-
016	1.66	2.20	0.44	0.51	0.01	0.03	-	-	-	-	-	-
017							-	-	-	-	-	-
089	-	-	-	-	-	-	-	-	-	-	-	-
080	-	-	-	-	-	-	-	-	-	-	-	-
098	-	-	-	-	-	-	-	-	-	-	-	-
038	-	-	-	-	-	-	-	-	-	-	-	-
000	1.41	1.24	1.07	1.23	0.08	0.09	0.13	0.14	0.02	+	0.02	0.03

Metabolite (M750F-)	Liver				Kidney				Plasma			
	VM	VF	WM	WF	VM	VF	WM	WF	VM	VF	WM	WF
	Composition of Radioactive Residues in % of the Dose											
067	-	-	-	-	-	-	-	-	-	-	-	-
090	-	-	-	-	-	-	-	-	-	-	-	-
095	-	-	-	-	-	-	-	-	-	-	-	-
097	-	-	-	-	-	-	-	-	-	-	-	-
096	-	-	-	-	-	-	-	-	-	-	-	-
092	-	-	-	-	-	-	-	-	-	-	-	-
094	-	-	-	-	-	-	-	-	-	-	-	-
093	-	-	-	-	-	-	-	-	-	-	-	-
058	-	-	-	-	-	-	-	-	-	-	-	-
081	-	-	-	-	-	-	-	-	-	-	-	-
060	-	-	-	-	-	-	-	-	-	-	-	-
057	-	-	-	-	-	-	-	-	-	-	-	-
073	-	-	-	-	-	-	-	-	-	-	-	-
082	-	-	-	-	-	-	-	-	-	-	-	-
083	-	-	-	-	-	-	-	-	-	-	-	-
066	-	-	-	-	-	-	-	-	-	-	-	-
099	-	-	-	-	-	-	-	-	-	-	-	-
059	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-
005	-	-	-	-	-	-	-	-	-	-	-	-
043	-	-	-	-	-	-	-	-	-	-	-	-

Metabolites were sorted by retention time.
+ metabolite was assigned, but the % of dose value was equal or below 0.005%
- metabolite was not quantitatively assigned
1) includes isomer of metabolite M750F049 at 19.0 min
2) includes isomers of metabolite M750F049 at 21.5 min, 21.9 min and 22.2 min

6. Enantiomer ratio of BAS 750 F

In order to analyse whether one isomer of BAS 750 F was preferably metabolized in rats, enantiomer-specific HPLC analyses were representatively performed for different dose groups, time periods and matrices with a sufficient amount of the parent compound. The results for the different dose groups, time periods and matrices are compiled in Table 5.1.1-57.

Table 5.1.1-57: Isomer ratio of BAS 750 F in matrices of rat metabolism studies (T-label)

Matrix [1]		Males		Females	
		S-enantiomer [%]	R-enantiomer [%]	S-enantiomer [%]	R-enantiomer [%]
1x 5 mg/kg bw[Dose group codes: feces – BM; liver/kidney/plasma – VM / VF]					
faeces	[0-24 h]	45.65	54.35	–	–
liver	[1 h]	34.59	65.41	18.85	81.15
kidney	[1 h]	24.21	75.79	13.99	86.01
1x 180 mg/kg bw [Dose group code: feces – DXM / DXF; liver/kidney/plasma – WM / WF]					
application formulation		–	–	51.55	48.45
faeces	[0-72 h]	49.42	50.58	51.01	48.99
plasma	[1 h]	30.46	69.54	23.72	76.28
liver	[1 h]	31.28	68.72	20.50	79.50
kidney	[1 h]	26.92	73.08	18.88	81.12
(14+1) x 180 mg/kg bw [Dose group code: feces – CM / CF]					
faeces	[0-24 h]	50.97	49.03	49.67	50.33
	[24-48 h]	48.94	51.06	50.50	49.50

1) Enantiomer-specific analyses were representatively performed for different dose groups, time periods and matrices with a sufficient amount of the parent compound.

The relative amounts of the isomers were approximately 1:1 in the application formulation and in the methanol extracts of faeces. In detail, the relative amounts were 51.6 : 48.5 % for the S-enantiomer and the R-enantiomer in the application formulation. In faeces for dose groups BM (0 - 24 h), CM (0 - 24 h), CM (24 - 48 h), CF (0 - 24 h), CF (24 - 48 h), DXM (0 - 72 h) and DXF (0 - 72 h), the relative amounts of both enantiomers remained similar and ranged from 45.7 : 54.4 % to 51.0 : 49.0 % for the S-enantiomer and the R-enantiomer.

The ratio between S- and R-enantiomer shifted in the methanol extracts of liver and kidney as well as in plasma towards a higher relative amount of the R-enantiomer. The corresponding samples however only contained minor amounts of radioactive residues (< 1 % dose).

In detail, the relative amounts in extracts of liver ranged from 18.9 : 81.2 % to 34.6 : 65.4 % (S-enantiomer : R-enantiomer) and in extracts of kidney from 14.0 : 86.0 % to 26.9 : 73.1 % for the S-enantiomer and the R-enantiomer (both matrices dose groups VM, VF, WM and WF, 1 h). The radioactivity accounted only for up to 0.9 % dose in the corresponding liver samples and for up to 0.1 % dose in the corresponding kidney samples.

In plasma (dose groups WM and WF), the relative amounts ranged from 23.7 : 76.3 % to 30.5 : 69.5 % (S-enantiomer : R-enantiomer). The radioactivity in the corresponding plasma samples however only accounted for 0.02 % of the administered dose.

III. CONCLUSION

The study was conducted according to OECD 417 and was divided into three separate studies. The biokinetics parts were performed separately for the C- / TFMP-label and the T-label, while the metabolism part was performed for all three labels (C- / TFMP- and T-label).

Biokinetics (C- / TFMP-label)
^{14}C- BAS 750 F, either dosed labeled at the chlorophenyl or the trifluoromethyl moiety, was rapidly absorbed from the gastrointestinal tract after oral administration to male and female rats and was fast excreted via urine and feces. Excretion was more pronounced via feces. After multiple administration of the test substance (14 oral administrations with unlabeled BAS 750 F at 180 mg/kg bw and one oral administration with labeled [^{14}C] chlorophenyl - BAS 750 F at 180 mg/kg bw), urinary excretion was comparable to single dosing.

Investigations on plasma kinetics demonstrate an internal exposure that is clearly correlated to the dosing regimen of ^{14}C- BAS 750 F. In addition, male rats showed roughly a twice higher internal dose compared to female rats. Based on bile excretion experiments, absorption of ^{14}C-BAS 750 F in male and female rats after single oral dosing was comparable for both labels, but slightly lower for the high dose and was calculated to be about 78 and 85% of dose for males and females at a dose level of 5 mg/kg bw (C-label), about 67 and 64% of dose for males and females at a dose level of 180 mg/kg bw (C-label) and 71% of dose for both sexes at a dose level of 180 mg/kg bw (T-label).

Biokinetics (T-label)
After single oral administration, ^{14}C- BAS 750 F was rapidly absorbed from the gastro-intestinal tract. Based on the bile excretion experiments, the bioavailability of ^{14}C-BAS 750 F in male and female rats was calculated to be about 50% and 58% of the applied dose at a dose level of 180 mg/kg bw and about 84% and 85% of the applied dose at a dose level of 5.0 mg/kg bw. The excretion of radioactivity occurred mainly within three days after dosing with a lower excretion in urine than in feces at the higher dose groups. After repeated oral administration (14 oral administrations with non-radiolabeled BAS 750 F at 180 mg/kg bw and one oral administration with labeled ^{14}C- BAS 750 F at 180 mg/kg bw) urinary excretion was slightly higher for male animals and significant higher for female animals than after single oral administration. At the low dose of 5 mg/kg bw the excretion occurred mainly within three days after dosing with a slightly lower urinary excretion than in feces for male animals but a significant lower urinary excretion than in feces for female animals. This difference is based on the high bioavailability and biliary excretion rates for the low dose group and reabsorption of the radioactivity which is excreted mainly via urine for male animals of the low dose group indicating different metabolism between the genders.

The AUC values indicate a more or less sex-dependent internal exposure. Increasing the dose by a factor of 8 (from 5 to 40 mg/kg bw) resulted in an increase of the AUC-values by a factor of about 7.8 in males and about 7 in females. Further increase of the dose by a factor of 3 (from 40 to 120 mg/kg bw and 120 to 360 mg/kg bw) resulted in an increase of the AUC-values by a factor of about 3 in males and about 3.9 and 2.5 in females which indicates a potential saturation of kinetics for female animals present at a dose level of 360 mg/kg bw.

Taken together, the data indicate a clear correlation of the internal exposure with increasing the external dose. In contrast to the AUC-dose correlation, the tissue distribution experiments showed a sublinear correlation between the radioactive residues in organs and tissues and the external dose and an increase of the dose by a factor of 36 resulted in general in a lower ratio of organ and tissue concentrations.

Metabolism

After oral dosing to rats, BAS 750 F was metabolised to a high extent. The excretion of radioactive residues *via* urine and faeces within this study (dose groups DXM and DXF, C- and T-label, respectively) was rapid and comparable for both sexes. The major excretion of radioactive residues was observed within 48 hours, supporting the results in the reference studies. The majority of the radioactive residues (69% - 83% of the dose) were excreted *via* faeces, smaller portions were excreted *via* urine (4% - 14% of the dose) for dose groups DXM and DXF.

For both male and female rats, the unchanged parent compound BAS 750 F was exclusively detected in excretions *via* faeces (up to 35% of the dose, all labels). To a lower extent, the parent compound was detected in concentrations of up to 2.0% of the dose in the investigated tissues and plasma, respectively (highest amount in liver extracts of female rats of dose group WF, C-label).

Transformation of BAS 750 F includes phase I conversions of the parent compound as mono, di- and trihydroxylation (including CL-shift), methylation, cleavage of the ether group or of the triazole ring from the parent compound. For phase II metabolism, phase I metabolites are either subjected to sulfation, glucuronidation and / or GSH adduction, which also includes corresponding decomposition products.
The experiments indicate that predominately glucuronide and sulfate conjugates of mono-, di- or trihydroxylated BAS 750 F are excreted *via* urine. *Via* bile, predominately glucuronide conjugates of mono- or dihydroxylated BAS 750 F are excreted, whereas most of the fecal metabolites were hydroxylated.

For urine of the C-label, comparable metabolite pattern for the dose groups D and DX were observed for male and female rats, respectively. For the dose groups DM and DXM, the main common metabolites were detected in comparable abundances of up to 1% of the dose, respectively: M750F049 - 19.0 min, M750F050 and M750F058 / M750F081 - 34.4 min. For female rats a broader spectrum of assigned metabolites was detected. Despite the variety of metabolites detected in female rats, the overall metabolic pathway of the two genders is comparable for urine of the C-label.

For urine of the TFMP-label and the T-label, the metabolite pattern were largely comparable. For the TFMP-label, the main components in dose groups DM and DF were M750F054 and M750F049 - 19.0 min / M750F003 (up to 2.8% of the dose).

For urine of the T-label and comparable dose groups DM and DXM, the main common metabolites were detected in highly similar concentrations: M750F001 (9.6% and 10.5% of the dose, respectively), M750F071 and M750F054 (<5% of the dose, respectively).
This was also the case for dose groups DF and DXF and the metabolites M750F001 and M750F054 (<5% of the dose, respectively). Additional metabolites were detected in both groups, DF and DXF, albeit in varying metabolite combinations at smaller general concentrations. For the investigated dose groups of the T-label, the main compound detected was M750F001 (up to 20% of the dose), with the exception of dose group BF, where the abundance of M750F001 ranged slightly below M750F049 - 19.0 min. Usually, the abundance of M750F001 was higher in male rats than in female rats. Further abundant components were M750F054 and M750F049 - 19.0 min / M750F003 (up to 4.3% of the dose).
In general, the metabolite pattern in urine of all dose groups for the T-label was in good accordance to each other.

For urine of all labels, all metabolites were excreted either as hydroxylated and / or cleaved parent compounds or as glucuronide or sulfate conjugates of the mono- or dihydroxylated parent compound or as glutathione conjugates of parent compound. Overall, the quality and quantity of the detected metabolites in urine of all three labels show high concordance.

For faeces, only metabolites that either have been once subjected to hydroxylation or which result from cleavage of the parent compound were excreted besides the parent compound. Overall the metabolic pathway of males and females of all labels is highly comparable in faeces. All dose groups of all labels showed a comparable metabolite pattern with M750F015 (ranging from 10 - 41% of the dose), M750F016 / M750F017 (ranging from 15 - 32% of the dose) and the parent compound BAS 750 F (ranging from 3 - 35% of the dose) as the major components. Metabolite M750F003 was detected at lower amounts (less than 4.3% of the dose) in all dose groups of the T- and the TFMP-label. Except for dose group BM and BF, the parent compound was the most abundant component for the T-label. For the C- and TFMP-label, M750F015 and / or M750F016 / M750F017 were usually more abundant than the parent compound.

The metabolite patterns in bile were comparable for samples of all labels for both male and female rats. Metabolites M750F035, M750F044, M750F045, M750F049 (including isomers) and M750F087 constituted the main portions of radioactive residues in bile samples of all labels. These metabolites are either once or twice hydroxylated parent compounds, which have been subsequently subjected to glucuronidation. For all labels, these compounds were usually detected in large amounts within 0 - 6 hours. For dose groups RM, SM and SF1 (C-label) and SM (TFMP-label), hydroxylated parent compound or sulfate conjugates were detected in minor traces (up to 0.7% of the dose). Consequently, the quality and quantity of the detected metabolites in bile of all three labels show high concordance.

The metabolite patterns in tissues and plasma were also comparable for the C- and the T-label. The major portion of metabolites were detected as hydroxylated parent compounds or as unchanged parent compound. Minor amounts were detected as glucuronide or sulfate conjugates of dihydroxylated parent compound. No gender specific differences were observed in the residue composition of tissues and plasma. The portions of radioactive residues were highest for liver (up to 7.9% of the dose) and lowest for plasma (0.1% of the dose), whereby higher portions of radioactive residues were usually detected in samples for the low dose groups VM and VF compared to the high dose groups WM and WF (except for kidney and plasma of the C-label).

The relative amounts of the isomers were approximately 1:1 in the application formulation and remained highly similar in the methanol extracts of faeces. In the methanol extracts of liver and kidney as well as in plasma, the ratio between S- and R-enantiomer shifted towards a higher relative amount of the R-enantiomer.

Report:	CA 5.1.1/4 [redacted] 2014 a 14C-BAS 750 F - Study on plasma kinetics in C57BL/6 J Rj mice 2014/1018105
Guidelines:	(EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, OECD 417 (July 2010), EPA 870.7485, JMAFF Guidelines on the Compiling of Test Results on Toxicity - Tests on In Vivo Fate in Animals (2001)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The plasma kinetics of 14C-BAS 750 F were investigated in male and female C57BL/6JRj mice. Groups of 4 male and female mice were dosed once orally by gavage at target dose levels of 10, 50 and 75 mg/kg bw. Blood samples were taken before and at 0.5, 1, 3 (or 2, 4), 8, 24, 48, 72, 96, 120, 144 and 168 h after test substance administration. Blood was separated in plasma and blood cells and radioactivity was determined to calculate the concentrations of ^{14}C-BAS 750 F in each sample. The maximum plasma concentrations showed a clear dose dependency with a sublinear c_{max}-dose relationship. At the high dose in male animals, c_{max} values of comparable magnitude were obtained at sampling time points of 0.5, 3 and 8 h post dosing; for female animals c_{max} values were 21.48 and 26.62 µg Eq/g at sampling time points of 0.5 and 8 h post dosing. For the mid dose, c_{max} values were 19.78 and 19.18 µg Eq/g as well as 17.24 µg Eq/g for males and females and occurred 1 h (males) and 8 h (males and females) post dosing. In the low dose in males, c_{max} values were 5.66 µg Eq/g and occurred 8 h post dosing whereas in females c_{max} values of 3.98 and 5.31 µg Eq/g were reached 1 h and 4 h post dosing. The observation that more than one c_{max} value was present in the high dose groups of males and females, in the mid dose group of males as well as in the low dose group of females indicates a potential enterohepatic (re)circulation of the test substance and/or potential metabolites thereof.

The calculated $AUC_{inf\ obs}$ values in the experiments for male as well as for female mice were 151, 694 and 958 as well as 127, 478 and 1012 µg Eq x hour/g for target doses of 10, 50 and 75 mg/kg bw, respectively. As can be seen from AUC/dose ratios and from the AUC versus dose ratio relationships (AUC ratios of the higher dose levels compared to the AUC of the low dose experiments), the internal dose (indicated by the AUC) is clearly correlated to the oral dose administered. The ratio between the AUC-values and the chosen dose levels is slightly underproportional to the oral nominal actual doses for males. This effect is assessed to be based on reduced absorption of the test substance with increased dose. For females, the AUC/dose ratio is about 4% lower for the low than for the high dose but is about 30% lower for the mid dose when compared to the low dose. Correspondingly, the AUC ratio of the high dose versus the low dose is 8.0 and is therewith slightly higher than the corresponding ratio of the actual nominal doses of 7.6.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material:

- Radiolabelled:	^{14}C- BAS 750 F (batch: 1062-2001; purity: >95%)
	Label: triazole-3(5)-C14; specific activity: 5.46 MBq/mg
- Non-radiolabelled:	BAS 750 F (batch: COD-001740; purity: 98.8%)
Stability of test compounds:	the stability of the test substance in the test substance preparations over the test period was verified by Radio-HPLC analyses. Concentrations of the test substance in the test substance preparations were in close agreement with the target and nominal concentrations according to LC-UV and radiochemical analyses.

2. Vehicle: 0.5% Carboxymethylcellulose (CMC)

3. Test animals:

Species:	Mouse
Strain:	C57BL/6 J Rj
Sex:	male and female
Age (Day 0):	49 – 54 days at dosing
Weight on day 0:	about 17 – 23 g
Source:	Janvier Labs SAS, Le Genest Saint Isle, France
Acclimatization period:	at least 7 days
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Acclimatization and prior to dosing: Females were housed in groups in Macrolon cages (type M III), while male animals were housed alone in Macrolon cages (type M III), in both cases using dust-free wooden bedding. Nest-building material (Nestlets NES 3600) and red transparent mouse tunnel were provided as enrichment (Plexx b.v., Elst, Netherlands)
	During the experiment animals were housed individually in steel wire mesh cages (type DK III).
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

4. Preparation of dosing solutions

In order to achieve the required specific radioactivity appropriate amounts of the prepared radio-labeled test-substance solution (specific activity: 277.1 MBq/g; concentration: 50.8 mg/g test-substance solution in acetonitrile) was taken and the organic solvent was evaporated to dryness. Respective amounts of the non-labeled material and the aqueous vehicle [0.5% carboxymethylcellulose (CMC) in tap water] were added. The blending ratio of labeled and unlabeled compound was 1+4 (75 mg/kg bw) and 2+5 (50 mg/kg bw) for the high- and mid-dose, respectively. Due to the low concentration of the low dose (10 mg/kg bw) this test-substance preparation was made with the pure radiolabeled material and respective amounts of CMC suspension. The preparations were homogenized by ultrasonification for about 75 minutes and were further stirred with a magnetic stirrer overnight. Before administration, the homogenization by ultrasonification was repeated for about 40 min; the test-substance preparations were stirred until and during administration.

B. STUDY DESIGN

1. Dates of work: 05-Nov-2013 to 12-Dec-2013

2. General study design

The experimental design is summarized in Table 5.1.1-58

Table 5.1.1-58: Design of blood / plasma level study

Experiment	Route, no. of doses	MBq / mouse	Dose (mg/kg bw)	Number of rats / gender	Remarks
1	oral, 1x	1.26 – 1.72	75	4 ♂+ 4 ♀	Analysis of total radioactivity in blood/plasma
1A	oral, 1x	1.66 – 1.77	75	4 ♂	
2	oral, 1x	1.28 – 1.71	50	4 ♂+ 4 ♀	
3	oral, 1x	0.90 – 1.25	10	4 ♂+ 4 ♀	

Dose volume oral: 10 mL/kg bw

The body weight of all animals was determined on the day of administration (prior to dosing). Groups of 4 mice/sex were treated with ^{14}C-BAS 750 F and then placed in steel wire mesh cages and blood samples (about 20-100 µL) were taken from the *vena facialis* at defined time points. Since animals died as a result of incidental high blood losses especially during the first day of the experiment, incidental blood losses were strictly avoided and sampling volumes were set to about 20 µL with restriction to an upper maximum of 50 µL during the ongoing experiment. To stabilize the health condition, mice received 500 µL drinking water (by gavage) after the 24-hour blood sampling time point and 300 µL sterile saline s.c. between 48-hour and 72-hourh sampling. In additional high-dose group males (experiment A1), the mice were dosed with sterile saline s.c. additionally before the 3-h blood sample was taken.

Blood was collected from all mice at the following time points: before administration and 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours after dosing.

For additional male animals of experiment A1 blood sampling time points changed on the first day (one time point less): before administration and 0.5, 1, 3, 8, 24, 48, 72, 96, 120, 144 and 168 hours after dosing.

Animals were sacrificed in isoflurane anesthesia after 168 h without further examination.

Whole blood samples were separated into plasma and blood cells by centrifugation. Appropriate aliquots of samples were mixed with scintillator (Hionic Fluor cocktail, Perkin Elmer) and the concentrations of the radioactive residues in plasma and blood cells were measured by liquid scintillation counting (LSC).

II. RESULTS AND DISCUSSION

1. Clinical observations and mortality

Incidental bleeding and blood loss occurred in individual animals after blood sampling, i.e. one male and female mouse each of the low-dose group (nos. 19 and 23) and mid-dose group (Nos. 11 and 16), and three high-dose males of experiment 1 (no.1, 3 and 4). Male no. 1 died 24 h and animals no. 2 and 4 died 48 hours after test substance administration. Therefore the high dose group was repeated for male mice with additional 4 animals. Animal No. 19 died 24 h and animal no. 23 died 72 h after test substance administration. It is assumed that incidental bleeding and blood loss after blood sampling paralleled by secondary effects such as dehydration and/or anemia are responsible for mortalities. After these symptoms became aware, water substitution was introduced consequently as described in Materials and Methods. In comparison with other repeated dose toxicity studies in the same species and with the repeated experiment for male mice (high dose group), mortalities are assessed not to be test substance related.

2. Plasma and blood levels of ^{14}C-BAS 750 F

Plasma kinetics were investigated after single oral (p.o.) administration. Target dose levels were 10, 50 and 75 mg/kg bw. Plasma concentration data are summarized in Table 5.1.1-59.

Table 5.1.1-59: Mean plasma concentration of radioactivity (in µg Eq/g plasma)

Dose	10 mg/kg bw		50 mg/kg bw		75 mg/kg bw	
Sex	male	female	male	female	male	female
Experiment	3	3	2	2	1+1A	1
Sampling time point [h]						
0.5	2.84	2.96	18.21	11.49	24.80	21.48
1	3.40	3.98	19.78	11.92	23.18	18.52
2	3.51	3.11	18.39	14.68	20.06	20.99
3	–	–	–	–	26.02	–
4	3.81	5.31	18.01	15.33	20.16	23.61
8	5.66	3.22	19.18	17.24	26.85	24.62
24	3.07	2.09	17.24	9.83	21.04	21.89
48	0.19	0.96	1.27	0.58	4.81	6.70
72	0.06	0.06	0.18	0.14	0.26	0.86
96	0.05	0.02	0.19	0.09	0.45	0.20
120	0.03	0.04	0.10	0.07	0.19	0.17
144	0.03	0.02	0.09	0.06	0.07	0.26
168	0.03	0.01	0.07	0.06	0.05	0.07

The parameters derived from the plasma kinetics are presented in the following table:

Table 5.1.1-60: Plasmakinetic parameters in mice after single oral gavage dosing

Sex	Dose [mg/kg bw]	c_{max} [µg Eq/g]	T_{max} [h]	Terminal half-life [h]	$AUC_{0\rightarrow 168\ h}$ [µg Eq*h/g]	$AUC_{0\rightarrow\infty}$ [µg Eq*h/g]
Male	**10**	5.66	8	80.4	147	151
	50	19.78; 19.18	1; 8	65.2	687	694
	75	24.80; 26.02; 26.85	0.5; 3 8	31.8	955	958
Female	**10**	3.98; 5.31	1; 4	54.2	126	127
	50	17.24	8	40.1	475	478
	75	21.48; 24.62	0.5; 8	34.6	1008	1012

The maximum plasma concentrations showed a clear dose dependency with a sublinear c_{max}-dose relationship. At the high dose in male animals, c_{max} values of comparable magnitude were obtained at sampling time points of 0.5, 3 and 8 h post dosing; for female animals c_{max} values were 21.48 and 26.62 µg Eq/g at sampling time points of 0.5 and 8 h post dosing. For the mid dose, c_{max} values were 19.78 and 19.18 µg Eq/g as well as 17.24 µg Eq/g for males and females and occurred 1 h (males) and 8 h (males and females) post dosing. In the low dose in males, c_{max} values were 5.66 µg Eq/g and occurred 8 h post dosing whereas in females c_{max} values of 3.98 and 5.31 µg Eq/g were reached 1 h and 4 h post dosing. The observation that more than one c_{max} value was present in the high dose groups of males and females, in the mid dose group of males as well as in the low dose group of females indicates a potential enterohepatic (re)circulation of the test substance and/or potential metabolites thereof.

The calculated $AUC_{inf\ obs}$ values in the experiments for male as well as for female mice were 151, 694 and 958 as well as 127, 478 and 1012 µg Eq x hour/g for target doses of 10, 50 and 75 mg/kg bw, respectively. As can be seen from AUC/dose ratios and from the AUC versus dose ratio relationships (AUC ratios of the higher dose levels compared to the AUC of the low dose experiments), the internal dose (indicated by the AUC) is clearly correlated to the oral dose administered. The ratio between the AUC-values and the chosen dose levels is slightly under-proportional to the oral nominal actual doses for males. This effect is assessed to be based on reduced absorption of the test substance with increased dose. For females, the AUC/dose ratio is about 4% lower for the low than for the high dose but is about 30% lower for the mid dose when compared to the low dose. Correspondingly, the AUC ratio of the high dose versus the low dose is 8.0 and is therewith slightly higher than the corresponding ratio of the actual nominal doses of 7.6.

Generally, a comparable time course of radioactivity was found for blood as for plasma in both sexes with the tendency to slightly higher blood/plasma ratios at later sampling time points, indicating that parts of the test substance and/or its metabolites may be bound to blood constituents (see Table 5.1.1-61).

Table 5.1.1-61: Ratio of blood / plasma concentration of radioactivity

Dose	**10 mg/kg bw**		**50 mg/kg bw**		**75 mg/kg bw**	
Sex	male	female	male	female	male	female
Experiment	3	3	2	2	1+1A	1
Sampling time point [h]						
0.5	0.25	0.28	0.40	0.40	0.24	0.28
1	0.60	0.54	0.43	0.41	0.37	0.36
2	0.43	1.09	0.32	0.47	0.54	0.39
3	–	–	–	–	0.46	–
4	0.68	0.85	0.52	0.65	0.37	0.35
8	0.62	0.72	0.57	0.45	0.57	0.54
24	1.19	1.82	0.83	0.86	0.74	0.67
48	1.10	2.40	1.00	0.96	0.90	0.46
72	1.34	5.31	1.06	0.80	0.71	1.08
96	0.70	4.99	0.64	1.33	0.84	0.60
120	0.69	1.46	0.81	0.86	0.86	0.86
144	1.72	2.73	0.76	0.90	1.00	0.70
168	2.52	0.92	0.94	1.27	1.00	0.71

III. CONCLUSION

The plasmakinetics of ^{14}C- BAS 750 F in male and female mice were assessed after single oral gavage administration. Maximum plasma concentrations that were reached 0.5 to 8 hours post dosing showed a clear dose dependency with a sublinear c_{max}-dose relationship. Observations for more than one c_{max} value being present in selected dose groups of males and females indicate a potential enterohepatic (re)circulation of the test substance and/or potential metabolites thereof. AUC values were slightly under-proportional to the single oral dose for male animals, whereas the ratio was – taken experimental variability into account – assessed to be more or less proportional to dose for female mice.

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

Report: CA 5.1.2/1
Funk D. et al., 2016 a
Comparative in-vitro-metabolism with 14C-BAS 750 F
2015/1020123
Guidelines: <none>
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

A comparative in-vitro metabolism study was performed with BAS 750 F. The objective of this study was the qualitative comparison of the metabolite patterns of ^{14}C-BAS 750 F formed after incubation with human, rat and mouse hepatocytes. For the comparison with human hepatocytes, the species rat and mouse were chosen, because they were used in toxicological studies with BAS 750 F. The radiolabelled test item (Triazole, Chlorophenyl or Trifluoromethylphenyl Label) was incubated with hepatocytes from humans, rat or mouse (all mixed genders) at a final concentration of 1 µM. The viability of the hepatocytes was determined after a 180 min incubation using a luminescent cell viability assay. Negative and positive controls were run in parallel to prove the absence of non-metabolic degradation and the metabolic activity of the hepatocytes (phase I and phase II metabolic reactions), respectively. The control experiments yielded the expected results. After incubation for 10, 30, 60 or 180 min, the reaction was terminated by addition of ice-cold ethanol and the resulting supernatant after concentration was analysed by LSC and HPLC. Selected samples were additionally investigated by HPLC-MS. If the supernatant contained less than 90 % of the applied radioactivity (% AR), the corresponding pellet was extracted with acetonitrile. The radioactive residues in the resulting extract and in the corresponding pellet were determined by LSC. The pellet extract was also analysed by HPLC.

The radio-HPLC analyses of human, rat and mouse cell samples were compared in order to determine whether a unique human peak occurred or not. Selected human, rat and mouse supernatant samples were also analysed by HPLC-MS to assign m/z values to prominent peaks representing more than 5 % AR in human samples.

After the incubation of BAS 750 F with human hepatocytes, up to two peaks were detected in the radio-chromatograms. Both peaks represented more than 5 % AR. One of these signals represented the unchanged active substance BAS 750 F. The second peak (peak at 7.6 min; m/z 590) corresponded to a metabolite of BAS 750 F.

Up to two peaks were detected in human hepatocyte samples, which were both also detected in rat hepatocyte samples. In conclusion, no unique human metabolite was observed. After incubation of BAS 750 F with mouse hepatocytes, no significant biotransformation of the test item was observed.

All relevant metabolites detected after incubation with human hepatocytes were also found after incubation with rat hepatocytes.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material: BAS 750 F

- Batch # / purity: see Table below

Test item	Batch No	Radiochemical purity [%]	Chemical purity [%]	Specific radioactivity a.s. [1] (MBq/mg)	Species
chlorophenyl-U-C14	1075-1001	99.3	88.0	8.11	human and rat
chlorophenyl-U-C14	1075-1101	99.5	98.1	8.11	mouse
trifluoromethylring-U-C14	CFQ42039	98.3	96.3	8.288 (solid)	human and rat
trifluoromethylring-U-C14	1125-2101	99.9	82.8	6.83	mouse
triazole-3(5)-C14	1062-2101	99.2	95.7	5.57	human and rat
triazole-3(5)-C14	1062-2201	99.4	98.2	5.47	mouse
chlorophenyl-1-C13	RS4-2012-173A2	-	97.7	-	human, rat and mouse
propyl-2-C13	1126-1006	-	99.5	-	human, rat and mouse
triazole-3(5)-C13	1077-1001	-	97.1	-	human, rat and mouse
unlabeled BAS 750 F	COD-001740	-	98.8	-	human, rat and mouse

1 active substance

- Stability of test compound: The test item was stable over the test period. Stability controls without cells showed nearly identical HPLC profiles that contained only peaks corresponding to the unchanged active substance BAS 750 F.

2. Positive controls: Hepatocytes were incubated with 7-ethoxycoumarin and testosterone instead of the active substance to validate the metabolic activity of the hepatocytes.

3. Test animals:

Species / Strain: Mouse (C57BL/6 J Rj),
Rat (Wistar)
Human (Xenotech, Germany)

B. STUDY DESIGN AND METHODS

The study was carried out at the Agricultural Research Centre of BASF SE in Limburgerhof, Germany.

1. Dates of work: August 05, 2014 – December 18, 2015

2. Test system preparation

Test items
The radiolabeled and non-radiolabeled test compound BAS 750 F was solved in acetonitrile. The concentration of the ^{14}C-labeled stock solutions accounted for approximately 2 mg/mL for experiments performed on human and rat hepatocytes and the concentration was about 0.5 mg/mL for experiments on mouse hepatocytes. Concentrations of the stock solutions containing ^{13}C-labeled BAS 750 F ranged from 0.6-0.7 mg/mL. For unlabeled BAS 750 F the concentration was 1.6 mg/mL in the stock solution.
The stock solution for testosterone contained about 0.5 mg/mL ^{14}C-labeled test material dissolved in ethanol. For the ^{14}C-ethoxycoumarin stock solution, 0.4 mg/mL of the radio-labeled substance were dissolved in dimethylsulphoxide (DMSO).

For the preparation of the application solution for experiments with 1 µM BAS 750 F or 5 µM BAS 750 F (T-label for cell viability experiments), a specific amount of the respective ^{14}C-labeled test item was concentrated to dryness in a stream of nitrogen and re-dissolved in DMSO. For the preparation of the application solutions for experiments with 10 µM BAS 750 F (T-label for cell viability experiments), calculated amounts of ^{14}C-labeled, ^{13}C-labeled and unlabeled test item were mixed, concentrated to dryness in a stream of nitrogen and re-dissolved in DMSO. The isotope ratio (^{14}C:^{13}C:^{12}C) was 80:10:10 for the T-label. The actual concentrations of radiolabeled BAS 750 F in the application solutions were determined by liquid scintillation counting (LSC). The purity of each application solution was confirmed by HPLC analysis. In some cases, the isotope ratio and the identity of the test item was confirmed by MS analysis.

Hepatocytes
Cryopreserved hepatocytes from human, rat and mouse were stored in liquid nitrogen. On each incubation day, the cells were thawed according to a protocol provided by the cell supplier using appropriate kits. Slight modifications were conducted for mouse hepatocytes. Aliquots of the resulting cell suspensions in hepatocyte incubation medium were diluted with phosphate-buffered saline (PBS) and the number of viable cells was determined using an automated cell counter. The cell suspensions were then adjusted with incubation medium to the desired cell density of 2 x 10^6 viable cells per mL. In the case of rat and mouse hepatocytes, male and female cells were combined in this final step in a ratio of 1:1. The human hepatocytes were purchased as a mixture of male and female cells.

Viability tests
The viability of human hepatocytes after incubation with 1 µM, 5 µM and 10 µM BAS 750 F (Triazole Label) was tested in order to select the appropriate concentration of the test item. Therefore, 250 µL of the respective application medium were incubated in a 24-well plate with 250 µL hepatocyte cell suspension at 37 °C and 5% CO_2 for 180 min. The cell viability (in relative luminescence units, RLU) was determined using a luminescent cell viability assay. The viability of the human hepatocytes was highest at a final concentration of 1 µM amounting to 87 %. Therefore, the final concentration of 1 µM BAS 750 F for each label was chosen.
For the experiments with 1 µM BAS 750 F (each label), the viability of the hepatocytes was determined for each incubation date by a luminescent cell viability assay.

3. In-vitro assays

Human, rat or mouse hepatocytes were incubated with BAS 750 F at a final concentration of 1 µM (each label). For testosterone and 7-ethoxycoumarin, the incubations were performed at a concentration of 10 µM.

On each incubation day, the application solutions in DMSO were diluted with the hepatocytes incubation medium by a factor of 100 to prepare the respective application media for hepatocyte incubations. Each sample comprised of equal amounts of the application medium and of the hepatocyte cell suspension in one well of a 24-well cell culture plate (maximum concentration of DMSO: 0.5%).

The reactions were performed for 10 min, 30 min, 60 min and 180 min at approximately 37°C, except for rat hepatocytes of the C- or TFMP-label, where samples were generated after 180 min. Incubations were terminated by pipetting an aliquot of the incubation mixture into a weighed tube containing ice-cold ethanol (final concentration of ethanol 70%) and cell lysis was assisted by ultra-sonication.

In addition, two negative controls (“stability control” and “zero incubation control”), two positive controls and a blank control (application medium with DMSO instead of test item) were performed for each species. No biotransformation should occur under these conditions. For the “stability control”, the application medium was mixed only with incubation medium instead of the cell suspension. For the “zero incubation control” (t = 0 min), the reaction was stopped immediately after addition of the cell suspension.

For the positive controls, 10 µM testosterone or 7-ethoxycoumarin instead of the active substance was incubated with hepatocytes from the different species to prove the metabolic activity of the different hepatocytes.

In each experimental setup, the incubation of the substrates as well as all control assays were performed in triplicates.

4. Work-up of samples

The terminated incubation mixtures were centrifuged and concentrated to volumes of approximately 1 mL by a centrifugal evaporator. The supernatant and the pellet were frozen separately. All samples were stored in a freezer at -18°C or below.

Analysis of the supernatants
All supernatants were analyzed by reversed phase HPLC. The metabolites detected in the supernatants of human hepatocyte samples at relative concentrations equal or above 5% of the applied radioactivity (5% AR) were further investigated by HPLC-MS. For all other samples, the radio-HPLC peaks were assigned to the identified metabolites by comparison of retention times and metabolite patterns, as well as by HPLC-MS analysis of at least one of three replicate supernatant samples. The metabolite patterns formed by incubation of the animal hepatocytes were compared to the metabolite pattern formed by incubation of human hepatocytes.

Work-up of the residual pellet
If the radioactive residues of the supernatants after concentration and centrifugation of the terminated incubation mixtures yielded less than 90% AR, the pellets after centrifugal evaporation were re-suspended in 50 µL water and mixed with 250 µL acetonitrile (assisted by ultra-sonication). After centrifugation, the radioactive residues in the supernatants of the extracts were determined by LSC measurements of aliquots. The remaining pellets were re-suspended in 320 µL water and the radioactive residues were measured by LSC.

II. RESULTS AND DISCUSSION

1. Control Experiments

The blank controls performed for each species without the test item showed no significant amounts of radioactivity (LSC measurements of the supernatants after addition of ethanol to the incubation mixtures and concentration). Furthermore, no radioactive peaks were detected by HPLC analysis.

The triplicates of each negative control showed nearly identical HPLC profiles that contained only one peak corresponding to the unchanged active substance BAS 750 F (some pellet extracts of rat and mouse hepatocyte samples showed two closely adjacent peaks both most likely representing BAS 750 F, and a minor peak at approximately 10 min was also observed in some application media). Hence, no metabolisation or degradation of BAS 750 F occurred without the influence of hepatocytes.

The positive controls with testosterone showed that the metabolic activity of the hepatocytes with respect to phase I metabolic reactions was sufficiently high. The mean portions of metabolised testosterone reached values above 90 % AR after incubation with human and rat hepatocytes and values above 84 % AR after incubation with mouse hepatocytes.

HPLC analyses of the positive controls with ethoxycoumarin revealed portions of approximately 8-9 % AR of unchanged ethoxycoumarin after incubation with human hepatocytes, no unchanged ethoxycoumarin (incubation date: 22.01.2015) or portions of approximately 0-12 % AR (incubation date: 11.11.2014) after incubation with rat hepatocytes and portions of approximately 0-8 % AR (incubation date: 08.10.2015) or no unchanged ethoxycoumarin (incubation date: 10.12.2015) after incubation with mouse hepatocytes. The phase I metabolite 7-hydroxycoumarin was found after the incubation with mouse hepatocytes (incubation date: 10.12.2015). The conjugated metabolites 7-hydroxycoumarin β-D-glucuronide and 7-hydroxycoumarin sulphate were found after the incubation with hepatocytes of all three species (except for mouse hepatocytes, incubation date 10.12.2015: no 7-hydroxycoumarin β-D-glucuronide). Compared to rat and mouse hepatocytes, higher portions of 7-hydroxycoumarin β-D-glucuronide were detected in human hepatocytes. Vice versa, higher portions of 7-hydroxycoumarin sulphate were detected after the incubation with rat hepatocytes or mouse hepatocytes compared to human hepatocytes. Thus, the positive controls with ethoxycoumarin indicate that the metabolic activity of the hepatocytes was high for the three species.

2. Viability of the Hepatocytes

In order to select an appropriate concentration of the test item with a sufficiently high viability of the cells, concentrations of 1 µM, 5 µM and 10 µM of BAS 750 F (T-label) were tested with human hepatocytes. The viability of the human hepatocytes was highest at a final concentration of 1 µM amounting to 87 % of the viability of the cells incubated without the test item. Therefore, the final concentration of 1 µM BAS 750 F for each label was chosen.

The hepatocyte suspensions were adjusted to a cell density of 2×10^6 viable cells per mL to achieve a final cell density of approximately 1×10^6 cells per mL in the incubation assays. After the incubation for 180 min, the viability of the cells was determined using the luminescent cell viability assay.

The viability of the human hepatocytes incubated with 1 µM BAS 750 F was 106%, 84% and 79% of the viability of the cells incubated without the test item for the T-label, C-label and TFMP-label. The viability of the rat hepatocytes incubated with 1 µM BAS 750 F was 86%, 90% and 123% of the viability of the cells incubated without the test item for the T-label, C-label and TFMP-label. The viability of the mouse hepatocytes incubated with 1 µM BAS 750 F was 136%, 122% and 101% of the viability of the cells incubated without the test item for the T-label, C-label and TFMP-label Workup Procedures.

In some samples, the radioactive residues recovered in the concentrated supernatants after termination of the incubation of BAS 750 F with the hepatocytes (or incubation medium) were below 90% AR (especially the zero incubation controls, For this reason, the respective residual pellets (pellet 1) were extracted with acetonitrile. The radioactive residues in the acetonitrile extracts were below or equal to 24.29% AR in all experiments with BAS 750 F, and the radioactive residues in the final pellets of all experiments with BAS 750 F were below or equal to 3.19% AR. Therefore, no further workup was performed.

3. Comparison of Metabolic Profiles

Each in-vitro assay was performed in triplicates, and selected samples were further investigated by HPLC-MS to assign m/z values to peaks representing more than 5% AR in the human hepatocytes assays (for the incubation with rat and mouse hepatocytes, the HPLC-MS data are exemplarily presented in the report for the incubation times of 0 and 180 min).

A minor peak eluting at approximately 10 minutes observed in some samples after incubation with mouse hepatocytes was also detected in stability controls and zero incubation controls as well as in application media (data not shown in the report). Two closely adjacent peaks probably both representing parent BAS 750 F, which occurred in some extracts of pellet 1 after incubation with rat or mouse hepatocytes, were also detected in stability controls and zero incubation controls

The peaks detected in human samples are compared with those detected in rat and mouse samples (% AR values are presented as mean % AR values, if applicable).

4. Kinetics of the Biodegradation of BAS 750 F

Based on the retention time and the m/z value, the ^{14}C peak at approximately 11.6 min was assigned to BAS 750 F. The respective peak representing the unchanged active substance is present in all human samples. The mean % AR amounted to 87.53 % (Triazole Label), 85.80 % (Chlorophenyl Label) and 93.51 % (Trifluoromethylphenyl Label) at 0 min, 95.21 % (Triazole Label), 93.01 % (Chlorophenyl Label) and 93.66 % (Trifluoromethylphenyl Label) after 10 min, 92.15 % (Triazole Label), 91.76 % (Chlorophenyl Label) and 90.70 % (Trifluoromethylphenyl Label) after 30 min, 92.64 % (Triazole Label), 91.63 % (Chlorophenyl Label) and 92.83 % (Trifluoromethylphenyl Label) after 60 min and 72.84 % (Triazole Label), 74.49 % (Chlorophenyl Label) and 70.31 % (Trifluoromethylphenyl Label) after 180 min of incubation with 1 µM BAS 750 F.

After incubation with rat hepatocytes, BAS 750 F was detected in all zero incubation control experiments (between 88.07 % and 96.23 % AR; In the experiments with Triazole-labelled BAS 750 F, the mean % AR decreased to 41.04 % after 10 min, 15.74 % after 30 min and 5.79 % after 60 min. No BAS 750 F was detected after incubation periods of 180 min (all labels). The transformation of BAS 750 F thus proceeded faster in rat hepatocytes.

After incubation with mouse hepatocytes, BAS 750 F was detected in all zero incubation control experiments (between 88.54 % and 92.57 % AR). In the experiments with all three labels, the portion of parent BAS 750 F showed no noticeable change after incubation for 10 to 180 min (range of 86.69 % to 96.33 % AR). BAS 750 F was thus not transformed in mouse hepatocytes.

5. Peaks Detected after Incubation of BAS 750 F with Hepatocytes

Radio-HPLC and HPLC-MS analyses of human hepatocyte samples lead to the detection of two peaks (peak at 7.6 min and the peak at approximately 11.6 min representing the unchanged parent compound. The peak at 7.6 min was detected after an incubation period of 180 min and amounted to 18.92 % AR (Triazole Label), 18.71 % AR (Chlorophenyl Label) and 21.47 % AR (Trifluoromethylphenyl Label).

In rat hepatocyte samples, the peak at 7.6 min was already present after an incubation period of 10 min and amounted to 2.89 % AR (Triazole Label). The mean % AR of the peak at 7.6 min increased to 12.14 % after 30 min, 22.34 % after 60 min and 43.36 % after 180 min (all Triazole Label). For incubations with the Chlorophenyl- and the Trifluoromethylphenyl-labelled BAS 750 F, the mean % AR of the peak at 7.6 min amounted to 31.41 % and 31.95 % after 180 min of incubation. The peak at 7.6 min (m/z 590) was represented by two closely adjacent peaks in the HPLC-MS analyses of rat hepatocyte samples.

In mouse hepatocyte samples, no peak representing a biotransformation product of BAS 750 F was detected above 5 % AR.

In conclusion, the only metabolite detected in human samples was also present in rat samples, and no significant biotransformation of BAS 750 F by mouse hepatocytes was observed.

Table 5.1.2-1: Comparison of peaks detected after incubation of human, rat and mouse hepatocytes with BAS 750 F

Incubation time [min]	Analyte / Peak	Human [Mean % AR]	Rat [Mean % AR]	Mouse [Mean % AR]
C-LABEL				
0	BAS 750 F	85.80	91.45	88.54
10	BAS 750 F	93.01	not applied	89.84
30	BAS 750 F	91.76		87.53
60	BAS 750 F	91.63		86.69 [1]
180	BAS 750 F	74.49	–	90.51
	Peak at 7.6 min	18.71	31.41	–
TFMP-LABEL				
0	BAS 750 F	93.51	88.07	92.57
10	BAS 750 F	93.66	not applied	96.33
30	BAS 750 F	90.70		94.59
60	BAS 750 F	92.83		91.67
180	BAS 750 F	70.31	–	89.75 [1]
	Peak at 7.6 min	21.47	31.95	–
T-LABEL				
0	BAS 750 F	87.53	96.23	90.53
10	BAS 750 F	95.21	41.04	93.00
	Peak at 7.6 min	–	2.89	–
30	BAS 750 F	92.15	15.74	93.32
	Peak at 7.6 min	–	12.14	–
60	BAS 750 F	92.64	5.79	93.21
	Peak at 7.6 min	–	22.34	–
180	BAS 750 F	72.84	–	95.48 [2]
	Peak at 7.6 min	18.92	43.36	–

1 Sum of mean % AR values of two evaluated replicates.
2 Only replicate 3 was evaluated.

III. CONCLUSION

In the present comparative in-vitro metabolism study, the only human metabolite (peak at 7.6 min; m/z 590) was present in samples after incubation with human and rat hepatocytes. Thus, this study did not result in the detection of any human metabolite that was not found in animals.

Generally, only minor differences concerning the metabolic degradation of BAS 750 F were noted between the test species human and rat, and no significant biotransformation was observed after incubation of BAS 750 F with mouse hepatocytes.

The metabolic degradation of BAS 750 F was faster in rat hepatocytes compared to human cells. In deviation to human samples, lower amounts of the parent compound were detected after incubation periods of 10, 30 and 60 min. BAS 750 F was not detected in rat hepatocyte samples after an incubation period of 180 min.

In summary, the metabolism of ^{14}C- BAS 750 F was similar in the tested species, especially in rat and human. The BAS 750 F metabolite found after the incubation with human hepatocytes was also detected after the incubation with rat hepatocytes.

OVERALL CONCLUSION

In the toxicokinetic studies, BAS 750 F was extensively and rapidly absorbed. After a single low oral dose (5 mg/kg bw) oral absorption was estimated to be greater than 84%, taking into account the dose of radioactivity excreted within 48 hours in male/female urine (10/11%) and bile (71/74%), and subsequent recoveries in cage wash (1.4/0.7%) and carcass (0.9 – 0.4%). There was no evidence of accumulation. BAS 750 F was widely distributed: beside gastrointestinal tract and its contents, highest residues around T_{max} of 1 hour were found in the liver, and to a lesser extent in the adrenal gland. BAS 750 F was extensively metabolised (unchanged parent was found only in faeces) and rapidly excreted (at the low dose 94-98% within 72 hours) mainly via feces and to a lesser extent in urine (about 39% in males and 15% in females).

The main metabolic pathway identified were transformation reactions resulting in hydroxylation of the chlorophenyl ring, and cleavage of the three-ring parent backbone at the ether bridge and/or cleavage of the triazole ring from the parent molecule. Parent and the transformation products were subject to extensive Phase II metabolism (glucuronidation, sulfatation, methylation, glutathione conjugation). In combination, these transformation reactions resulted in the formation of more than 100 rat metabolites.

Differences in the metabolic degradation of BAS 750 F enantiomers were observed in the rat: while in feces, the enantiomer ratio remained 1:1 and thus highly similar to the administered radiolabel, the ratios in the methanol extracts of liver, kidney and plasma shifted towards a higher relative amount of the R-enantiomer. An enantiomer shift in the same direction occurred also in goat, but not in poultry metabolism (see MCA 6.2 and 6.7).

Taken together, although the extensive BAS 750 F metabolism in the rat appears to produce a complex metabolic profile, the metabolic routes followed in the rat are rather basic, which allows to form several groups of structurally closely related metabolites. Importantly, the metabolic routes identified in plant and livestock metabolism are all represented by rat metabolism (see sections MCA 5.8.1, and MCA 6.7.1).

No unique human metabolite is expected based on the results of comparative in vitro metabolism investigations in human, rat and mouse primary hepatocyte cultures. Metabolic profiles between rat and human were more similar than between mouse and rat.

CA 5.2 Acute Toxicity

The acute health effects of BAS 750 F were assessed using the studies listed in Table 5.2-1.

Table 5.2-1: Summary of acute toxicity studies with BAS 750 F

Annex II point	Study / method	Species	Result	GHS Classification	Reference (DocID)
5.2.1	Acute oral toxicity (OECD 423)	Wistar rat ♀	LD_{50} > 2000 mg/kg bw	–	[redacted] 2013c (2013/1149656)
5.2.2	Acute dermal toxicity (OECD 402)	Wistar rat ♂ + ♀	LD_{50} > 5000 mg/kg bw	–	[redacted] 2013b (2013/1149657)
5.2.3	Acute inhalation toxicity (OECD 403) – nose only	Wistar rat ♂ + ♀	LC_{50} > 5.314 mg/l	–	[redacted], 2014a (2014/1127433)
5.2.4	Skin irritation (OECD 404)	NZW rabbit ♀	Not a skin irritant	–	[redacted] 2013c (2013/1150122)
5.2.5	Eye irritation (OECD 405)	NZW rabbit ♀	Not an eye irritant	–	[redacted] 2013a (2013/1150121)
5.2.6	Skin sensitization (OECD 406) – Maximization test	Guinea pig ♀	Skin sensitizer	Skin Sens. 1B H 317	[redacted] 2013a (2013/1150123) Stinchcombe 2016 a (2016/1028946)
5.2.7	Phototoxicity (OECD 432)	Balb/c 3T3 cells	Not a phototoxicant	–	Cetto and Landsiedel, 2015a (2015/1117503)

BAS 750 F has low acute toxicity by the oral, percutaneous, and inhalation routes of administration. The test substance is neither a skin nor an eye irritant.

In the *in vitro* 3T3 NRU phototoxicity test using Balb/c 3T3 cells (OECD 432), BAS 750 F did not show evidence of phototoxicity.

In a skin sensitization study with guinea pigs (Maximisation test), BAS 750 F was found to be a skin sensitizer, following intradermal/topical induction concentrations of 5% / 60%, and challenge with a 50% substance concentration. Results of Local lymph node assays with formulations containing BAS 750 F at concentrations of up to 400 g/L, and LLNA data available for structurally closely related BAS 750 F precursors [see KCA 5.2.6/3 2016/1028946] support the conclusion that BAS 750 F has a weak skin sensitizing potential. Therefore, the following classification of BAS 750 F is proposed according to CLP and UN-GHS classification criteria:

Skin Sens 1B; H317

"May cause an allergic skin reaction"

Note on skin and eye irritation, and skin sensitisation testing of BAS 750 F:

The acute toxicity of BAS 750 F was investigated in a suite of studies, including both *in vitro* and *in-vivo* toxicological investigations, to comply with global data requirements, including Regulation (EU) No 283/2013.

The European data requirements for registration of pesticide active ingredients and plant protection products strongly promote alternative non-animal testing approaches and reduction and refinement methods for *in vivo* testing. Strictly following the data requirements of Regulation (EU) No 283/2013, the following acute toxicity tests with BAS 750 F would not have been necessary and could have been waived:

- Acute dermal toxicity study: not required since the acute oral toxicity LD_{50} of BAS 750 F is greater than 2000 mg/kg bw.
- Skin/Eye irritation in rabbits: Waiving possibility since validated *in-vitro* test methods for assessment of skin/eye corrosion and irritation are available. Moreover, the lack of any signs of skin irritation in the (available) acute dermal toxicity study with BAS 750 F would have been sufficient reason to waive the need for any dermal irritation studies.**1)**
- Skin sensitisation testing in guinea pigs: the Local Lymph Node Assay (LLNA) in mice is the preferred test method for skin sensitisation testing according to Regulation (EU) 283/2013; a justification needs to be given for the alternative testing using the Guinea Pig Maximization test method.

However, as mentioned above, global data requirements had to be considered in addition to European data requirements for toxicological testing of BAS 750 F. Therefore, the acute dermal toxicity test in rats, and *in vivo* skin irritation and eye irritation tests in rabbits had to be performed with BAS 750 F, to comply with Pesticide Regulations of non-European countries (*e.g.* United States, Canada, various countries in South America and in Asia).

The Guinea Pig Maximization Test (GPMT) had to be performed, because at the time of testing in 2013 the LLNA was not accepted as a full replacement of the GPMT in major Asian countries**2)**. Therefore, it was decided to perform only the GPMT, which is accepted as skin sensitisation test method on a global scale, in order to avoid double animal testing for skin sensitisation in mice and guinea pig studies.

1) Uncertainty remains in Regulation (EU) 283/2013 regarding the general requirement of in-vivo testing for skin and eye irritation, since in case of "insufficient data" for deciding on the basis of a weight-of-evidence analysis, the EU data requirements specifically foresee a tiered testing approach comprising a sequence from validated in vitro studies to in-vivo tests in rabbits (see sections 5.2.4 and 5.2.5). This tiered testing ruling seems to be in obvious contradiction to the general statement of the EU Regulation that tests on vertebrate animals shall be undertaken only where no other validated methods are available. A clear statement that only in vitro testing for skin and eye irritation is acceptable would be preferred.

2) In Japan, the LLNA is not an accepted skin sensitisation test method for pesticide registration; the GPMT is required. In China, a **positive** test result in the LLNA will trigger a further skin sensitisation study in guinea pigs in order to allow for Chinese-specific severity categorization of the skin sensitiser. In India, additional testing in guinea pigs is required in case of a **negative** test result in the LLNA.

CA 5.2.1 Oral

Report:	CA 5.2.1/1 [redacted] 2013 c BAS 750 F - Acute oral toxicity study in rats 2013/1149656
Guidelines:	OECD 423 (2001), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, EPA 870.1100, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)
Report:	CA 5.2.1/2 Becker M.,Kamp H., 2013 a BAS 750 F - Homogeneity and concentration control analyses in corn oil 2013/1395622
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In an acute oral toxicity study two groups of 3 young adult female Wistar rats were sequentially administered a single oral dose via gavage of BAS 750 F (Batch: COD-001740; Purity: 98.8%) suspended in corn oil at a dose level of 2000 mg/kg bw. Animals were observed for 14 days.

After administration of 2000 mg/kg bw of BAS 750 F all animals survived until the termination of the study. Accordingly, the oral LD_{50} was found to be greater than 2000 mg/kg bw.

Rat acute oral LD_{50} > 2000 mg/kg bw

Clinical signs were first noted 2 hours after treatment in four of six animals and comprised, impaired general state and piloerection; two rats were additionally found in a cowering position. These symptoms were noted on the day of treatment only. There were no apparent effects on body weight development throughout the 14-day post-exposure period. No apparent abnormalities were observed in any animal at necropsy performed at the termination of the study.

According to classification criteria of UN-GHS and EU CLP Regulation 1272/2008, no classification is warranted as to acute oral toxicity for BAS 750 F.

(DocID 2013/1149656)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: Corn oil

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han) SPF
Sex:	female
Age (day 0):	ca. 10 weeks
Weight at dosing (mean):	174 ± 4 g (I); 186 ± 3 g (II)
Source:	Charles River Wiga GmbH, Sulzfeld, Germany
Acclimation period:	At least 5 days
Diet:	VRF1(P); SDS Special Diets Services, 67122 Altrip, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cage type III

Environmental conditions:

Temperature:	22 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 10-Jun-2013 to 10-Jul-2013 [in life phase: 11-Jun-2013 (treatment) to 02-Jul-2013 (day of last observation)]

2. Animal assignment and treatment:

Two groups of 3 female rats received a single starting dose of 2000 mg/kg bw of the test item administered as corn oil suspension (40 g / 100 mL) by oral gavage. The test item suspension was prepared shortly before administration; stability and homogeneity of the test item in the vehicle was verified indirectly by concentration control analysis. The animals were deprived of food at least 16 hours before dosing, but had free access to water. Food was offered again approximately 4 hours after administration. Test substance was given to the animals at a volume of 5 ml/kg bw. The dosing was performed sequentially, and as no deaths occurred after administration of the first group of three animals, the results were confirmed in three other females at the same dose level.

A check for any dead or moribund animals was made at least once each workday. Observations for clinical signs were performed several times on the day of administration. Thereafter, the animals were observed at least once a day for a total of 2 weeks.

Body weights were recorded at day 0 (prior to dosing), weekly thereafter and on the last day of observation.

The animals were sacrificed by carbon dioxide inhalation and subsequently subjected to macroscopic examination on the last day of the observation period.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred.

B. CLINICAL OBSERVATIONS

Clinical observations are summarized in Table 5.2.1-1. Clinical signs in the first 2000 mg/kg bw test group revealed in two out of three animals, cowering position at hour 2 after administration. Moreover all three animals showed impaired general state and piloerection, starting from hour 2 and lasting until hour 3, 4, or 5 in the three individual animals, respectively.

Clinical signs in the second 2000 mg/kg bw test group revealed in one out of three animals impaired general state and piloerection from hour 2 until hour 3 after administration. No clinical signs were observed in the other two animals.

Table 5.2.1-1: Acute oral toxicity in rats - clinical observations

Observation	Group 1: 2000 mg/kg bw (n=3)		Group 2: 2000 mg/kg bw (n=3)	
	Animals with signs	**Observed**	**Animals with signs**	**Observed**
Impaired general state	3	h2 – h3 h2 – h5 h2 – h4	1	h2 – h3
Piloerection	3	h2 – h3 h2 – h5 h2 – h4	1	h2 – h3
Cowering position	2	h2	0	–

C. BODY WEIGHT

The mean body weight of the test groups increased throughout the study period within the normal range.

D. NECROSCOPY

There were no macroscopic pathological findings in the animals sacrificed at the end of the observation period.

E. CONCENTRATION CONTROL ANALYSES

Three samples of the test substance preparation in corn oil (nominal concentration: 40 g/ 100 mL) were analyzed for BAS 750 F content with HPLC. The analyzed content was 98.2 ± 3.2% (mean ± RSD), demonstrating the homogeneity and correctness of the concentration of BAS 750 F in corn oil.

III. CONCLUSION

Under the conditions of this study the median lethal dose of BAS 750 F after oral administration was found to be greater than 2000 mg/kg bw in rats. Clinical signs of toxicity were seen only on the day of exposure. No effects on body weight development or necroscopy findings were observed.

CA 5.2.2 Dermal

Report:	CA 5.2.2/1 [redacted] 2013 b BAS 750 F - Acute dermal toxicity study in rats 2013/1149657
Guidelines:	OECD 402 (1987), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, EPA 870.1200, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)
Report:	CA 5.2.2/2 Becker M.,Kamp K., 2013 a Analytical report - BAS 750 F - Homogeneity and concentration control analyses in corn oil 2013/1395620
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In an acute dermal toxicity study one group of 5 male and 5 female Wistar rats were exposed to a single dermal dose of 5000 mg/kg bw BAS 750 F (Batch: COD-001740; Purity: 98.8%). BAS 750 F was administered as suspension in corn oil on to the clipped skin (dorsal and dorso-lateral parts of the trunk) and covered by semi-occlusive dressing for 24 hours. The application area comprised at least 10% of the total body surface area. The animals were observed for 14 days.

Based on the absence of mortality in this study the acute dermal LD_{50} was determined to be greater than 5000 mg/kg bw.

Rat acute dermal LD_{50} > 5000 mg/kg bw

No mortality occurred. There were no signs of systemic toxicity or skin effects observed in any animal. The mean body weights of the animals increased within the normal range throughout the study period. As generally known for females dermally applied, the weight gain stagnated during the first week or only slightly increased during the study period. No macroscopic pathologic abnormalities were noted in the animals examined at the end of the study.

According to classification criteria of UN-GHS and EU CLP Regulation 1272/2008, no classification is warranted as to acute dermal toxicity for BAS 750 F.

(DocID 2013/1149657)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: Corn oil

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han) SPF
Sex:	male and female
Age (day 0):	♂: ca.8 weeks; ♀: ca. 12 weeks
Weight at dosing (mean):	♂: 223 ± 5 g; ♀: 203 ± 6 g
Source:	Charles River Wiga GmbH, Sulzfeld, Germany
Acclimation period:	At least 5 days
Diet:	VRF1(P); SDS Special Diets Services, 67122 Altrip, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cage type III

Environmental conditions:

Temperature:	22 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 10-Jun-2013 to 09-Jul-2013 [in-life phase: 11-Jun-2013 (treatment) to 25-Jun-2013 (day of last observation)]

2. Animal assignment and treatment:

24 hours prior administration, the fur was removed by clipping from dorsal and dorsolateral parts of the skin of 5 male and 5 female Wistar rats. Shortly before treatment, a suspension of BAS 750 F in corn oil was prepared (60 g/100 mL) and kept homogeneous by stirring with a magnetic stirrer. A volume of 8.33 mL/kg bw of this suspension (equivalent to a dose of 5000 mg/kg bw BAS 750 F) was administered on to a clipped epidermis area corresponding to at least 10% of the estimated body surface (i.e. about 40 cm^2). The test item was covered with an air-permeable dressing (4 layers of absorbent gauze and stretch bandage). After the 24-hour exposure period under these semi-occlusive conditions, the dressing was removed and the application site was rinsed with warm water. Stability and homogeneity of BAS 750 F in corn oil was verified indirectly by concentration control analysis.

A check for dead or moribund animals was made at least once each workday. The animals were observed for clinical signs several times on the day of administration, and then at least once a day for a total of 14 days. Skin findings were scored 30-60 minutes according to J.H. Draize after removal of the dressing, weekly thereafter and on the last day of observation.

Individual body weights were determined shortly before administration (day 0), weekly thereafter and on the last day of administration.

The animals were sacrificed by carbon dioxide inhalation and subsequently subjected to macroscopic examination on the last day of the observation period.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred.

B. CLINICAL OBSERVATIONS

There were no signs of systemic toxicity or skin effects observed in any animal.

C. BODY WEIGHT

The mean body weights of the animals increased within the normal range throughout the study period. As generally known known to occur as a consequence of the dermal application procedure, the weight gain stagnated during the first week or only slightly increased during the study period.

D. NECROSCOPY

No macroscopic pathologic abnormalities were noted in the animals examined at the end of the study.

E. CONCENTRATION CONTROL ANALYSES

Three samples of the test substance preparation in corn oil (nominal concentration: 60 g/ 100 mL) were analyzed for BAS 750 F content with HPLC. The analyzed content was 94.2 ± 0.5% (mean ± RSD), demonstrating the homogeneity and correctness of the concentration of BAS 750 F in corn oil.

III. CONCLUSION

Under the conditions of this study the median lethal dose of BAS 750 F after dermal administration was found to be greater than 5000 mg/kg bw in rats.

CA 5.2.3 Inhalation

Report:	CA 5.2.3/1 [redacted] 2014 a BAS 750 F - Acute inhalation toxicity study in Wistar rats - 4-hour dust exposure (head-nose only) 2014/1127433
Guidelines:	OECD 403 (2009), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.2, EPA 870.1300
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In an acute inhalation toxicity study (limit test) groups of 5 male and 5 female Wistar rats were exposed (head and nose) to a dust aerosol of BAS 750 F (Batch: COD-001740; Purity: 98.8%) at a concentration of 5.314 mg/L (analytical concentration) for 4 hours. The animals were observed for 14 days.

Based on the absence of mortality in this study the acute inhalation LC_{50} was determined to be greater than 5.314 mg/L.

Rat acute inhalation LC_{50} > 5.314 mg/L

Clinical signs of toxicity consisted labored respiration, abdominal respiration, respiration sounds, eye encrusted, red and colorless discharge and/or red crusts of the noses, poor general state, hunched posture, hyper-excitability, no defecation, piloerection and substance contaminated fur. Findings were observed from hour 2 of exposure through to study day 11. No clinical signs and findings were observed from study day 12 onwards. The mean body weights of the animals decreased during the first post exposure observation day and increased from study day 3 onward. No gross pathological abnormalities were detected during the necropsy in the animals at the termination of the study.

Cascade impactor measurements resulted in particle size distributions with mass median aerodynamic diameters (MMADs) of 3.8 µm.

According to classification criteria of UN-GHS and EU CLP Regulation 1272/2008, no classification is warranted as to acute inhalation toxicity for BAS 750 F.

(DocID 2014/1127433)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001740 (milled)
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: 3% Aerosil® 200 and 1% Aerosil® R 972 was added to milled test substance in order to improve flow ability and to decrease hygroscopic potential

3. Test animals:

Species:	Rat
Strain:	Wistar / RccHan:WIST
Sex:	male and female (nulliparous and non-pregnant)
Age (day 0):	♂: approx.9 weeks, ♀: approx. 11 weeks
Weight at dosing (mean):	♂: 282 ± 7 g; ♀: 213 ± 9 g
Source:	Harlan Lab B.V., Horst, Netherlands
Acclimation period:	At least 5 days
Diet:	Kliba-Labordiät (Maus / Ratte Haltung "GLP), 10 mm pellets; Provimi Kliba GmbH, Kaiseraugst, Basel, Switzerland; ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cage type III (floor area about 800 cm²); housing in groups of 5 rats in Polysulfone cages (H-Temp [PSU], floor area ca. 2065 cm²) if animals were free from clinical signs and findings. Enriched with wooden gnawing blocks (Type NGM E-022); Abedd ® Lab. and Vet. Service GmbH Vienna, Austria; Play tunnel, large (Art. 14153, PLEXX b.v. Elst, Netherlands. Dust-free bedding

Environmental conditions:

Temperature:	20–24°C (continuous control and recording)
Humidity:	30–70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 04-Mar-2014 to 16-May-2014 [in-life phase: 05-Mar-2014 (treatment) to 19-Mar-2014 (day of last observation)]

2. Animal assignment and treatment:

For determination of the acute inhalation toxicity (single head-nose inhalation, 4-hour-exposure) groups of five male and five female rats were exposed to 5.314 mg/L of the test substance BAS 750 F, which was dosed as a dust aerosol. The animals were randomly selected from a pool of animals. After exposure, animals were observed for at least 14 days.

Individual body weights were recorded shortly before exposure (day 0), and at least once on days 1, 3, and 7, and at the end of the 14-day post exposure period.

Clinical observations were recorded for each animal separately at least once on the day of pre-exposure day, several times during exposure, and at least once daily during the observation period.

A check for any dead or moribund animal was made twice each workday and once on Saturdays, Sundays, and on public holidays. At the end of the observation period the surviving animals were sacrificed with CO_2-inhalation in a chamber with increasing concentration over time, and were subjected to gross-pathological examination.

3. Statistics / calculations:

For results of the type "LC_{50} greater than", "LC_{50} approx.", or "LC_{50} smaller than", the binomial test was used for statistical evaluation. [Steel R.G.D., Torrie J.H. (1984): Principles and procedures of statistics a biometrical approach. McGraw - Hill]

The calculation of the particle size distribution was carried out in the inhalation laboratory on the basis of mathematical methods for evaluating particle measurements. [DIN 66141: Darstellung von Korngrößenverteilungen, DIN 66161: Partikelgrößenanalyse (Beuth-Vertrieb GmbH, Berlin 30, FRG und Köln 1, Germany)].

4. Generation of the test atmosphere and exposure:

The test substance was desagglomerated in a mixer (mixing for 3x 10 sec) under addition of 3% Aerosil® 200 and 1% Aerosil® R 972, in order to improve dust formation. The dust aerosol was generated inside the inhalation system with a dosing-wheel dust generator (Gericke/BASF) and compressed air. The concentration was adjusted by varying the aperture width rotation of the dosing wheel.

A head nose inhalation system INA (glass steel construction, BASF SE, volume V ≈ 34 L) was used. The animals were restrained in glass tubes and their snouts projected into the inhalation system.

The exposure system was located inside an exhaust cabin in an air conditioned laboratory. Supply airflow (compressed air) of 1.2 m^3 / h was used for the exposure. The exhaust airflow was set at 0.6 m^3 / h. The lower amount of exhaust air, which was adjusted by means of a separate exhaust air system, achieved a positive pressure inside the exposure system. This ensured that the mixture of test substance and air was not diluted with laboratory air in the breathing zones of the animals. An air change of about 35 times per hour can be calculated by dividing the supply air flow by the volume of the inhalation system. The animals were exposed to the inhalation atmosphere for 4 hours plus an equilibration time for the inhalation system of about 8 minutes.

5. Analytical investigation:

The nominal concentration was calculated from the amount of substance dosed and the supply air flow. The concentration of the test substance in the inhalation atmosphere was determined via gravimetrical measurement. Pre-weighed filters were placed into the filtration equipment. By means of a vacuum pump (Millipore) metered volumes of the dust were drawn through the filter.

• Sampling devices:	Filtration equipment with probe, internal diameter: 7 mm, (Millipore)
• Filter:	MN 85/90 BF (d = 47 mm)
• Equipment:	Balance Mettler AT 205
• Sampling position:	immediately adjacent to the animals' noses
• Sampling flow:	3 L/min
• Sampling velocity:	1.25 m/s
• Sampling frequency:	4 samples at about hourly intervals
• Sampling volume:	3 L

For each sample the dust concentration in mg/L was calculated from the difference between the pre-weight of the filter and the weight of the filter after sampling with reference to the sample volume of the inhalation atmospheres. Mean and standard deviation for the concentration were calculated based on the results from individual measurements. The concentration was corrected for the amount of additive used.

6. Particle size analysis:

The particle size analysis was done using a Stack Sampler Mark III (Andersen) and a vacuum pump (Thomas). The sampling probe had an internal diameter of 6.9 mm.

Before sampling, the impactor stages were assembled with pre-weighed glass-fiber collecting discs and equipped with a backup particle filter. The impactor was connected to the vacuum pump and two samples were taken from the breathing zone of the animals starting approx. 30 minutes after the beginning of the exposure. The sample volume for each sample was 6 L.

After sampling the impactor was taken apart. The collecting discs and the backup particle filter were re-weighed. The amount of material adsorbed to the walls of the impactor and in the sampling probe (wall losses) was also determined quantitatively.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred at the tested concentration of 5.314 mg/l during the study period of 14 days. Therefore, the study satisfies the criteria of a limit test.

Based on the absence of mortality the following LC_{50} value was determined:

Acute 4-h inhalation LC_{50} (both sexes combined):	> 5.314 mg/l ($p < 0.01$)
Acute 4-h inhalation LC_{50} (male rats)	> 5.313 mg/l ($p < 0.05$)
Acute 4-h inhalation LC_{50} (female rats)	> 5.314 mg/l ($p < 0.05$)

B. CLINICAL OBSERVATIONS

Clinical observations are summarized in Table 5.2.3-1. No abnormalities were detected in the animals during the post exposure observation period from study day 12 onwards.

Table 5.2.3-1: Acute inhalation toxicity in rats – clinical observations

Observation	Male (n=5)		Female (n=5)	
	Animals with signs	Observed	Animals with signs	Observed
Respiration, labored	5	h2–h4	5	h2–h4
Respiration, sounds	4	d0–d2	5	d0–d5
Respiration, abdominal	5	d0–d11	5	d0–d10
Fur, substance contaminated	5	d0–d4	5	d0–d4
Piloerection	5	d0–d7	5	d0–d5
Poor general state	1	d1	–	–
Hunched exposure	–	–	1	d1
No defecation	1	d1	–	–
Nose, red discharge	3	d0–d8	–	–
Nose, red encrusted	1	d1	2	d1–d4
Nose, colorless discharge	2	d1–d8	–	–
Eye, encrusted	1	d1	–	–
Hyper-excitability	5	d7–d10	5	d7–d10

C. BODY WEIGHT

The mean body weights of the animals decreased during the first post exposure observation day and increased from study day 3 onward.

D. NECROSCOPY

No gross pathological abnormalities were detected during the necropsy in the animals at the termination of the study.

E. ANALYTICAL MEASUREMENTS

The exposure conditions are summarized in Table 5.2.3-2.

Table 5.2.3-2: Acute inhalation toxicity in rats – exposure conditions

Supply air (compressed) [m^3 / h]	Exhaust air (m^3/h)	Substance flow (g/h)	Temperature (°C)	Relative humidity (%)
1.2 ± 0.0	0.6 ± 0.0	40.3	21.0 ± 0.3	18.6 ± 1.3

The low relative humidity resulted from the need to use compressed air for dust generation. This deviation from the guideline recommendations (30% - 70% relative humidity; especially low relative humidity in dusts) is considered not of influence for the test results, because of the relatively short exposure time.

The result of the analytical concentration measurements are presented in Table 5.2.3-3.

Table 5.2.3-3: Acute inhalation toxicity in rats – analytical concentration measurements

Mean concentration [mg/l]	Standard deviation	Nominal concentration [mg/l]
5.314	0.436	33.6

The measurements of particle-size distribution revealed mass median aerodynamic diameters (MMAD) in the respirable range of 3.8 µm with geometric standard deviations of 3.2 and 2.2 µm, respectively [see Table 5.2.3-4].

Table 5.2.3-4: Acute inhalation toxicity in rats – particle size measurements

Sample	MMAD [µm]	Geometric standard deviation
1	3.8	3.2
2	3.8	2.2

III. CONCLUSION

Under the conditions of this study the 4-hour acute inhalation LC_{50} of BAS 750 F for male and female rats was estimated to be > 5.314 mg/L (MMAD 3.8 µm, GD 3.2). There were no mortalities. First clinical signs of toxicity (labored breathing) were observed after 2 hours of exposure. Decreased body weight was noted on day 1, afterwards rats recovered. No clinical signs were seen after day 11 of the study. There were no findings upon necroscopy of the animals.

CA 5.2.4 Skin irritation

Report:	CA 5.2.4/1 Remmele M., 2012 a Reg.No. 5834378 - EpiDerm skin corrosion / irritation test 2012/1367952
Guidelines:	OECD 431, OECD 439, Commission Regulation (EC) No 440/2008, (EC) No 761/2009
GLP:	no
Report:	CA 5.2.4/2 [redacted], 2013 a BAS 750 F - Acute dermal irritation / corrosion in rabbits 2013/1150122
Guidelines:	OECD 404 (2002), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, EPA 870.2500, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

EXECUTIVE SUMMARY

A primary skin irritation test was carried out with New Zealand White rabbits to determine the potential for BAS 750 F (Batch: COD-001740; Purity: 98.8%) to produce dermal irritation after single topical exposure.

0.5 g of BAS 750 F was minimally moistened with water and subsequently applied for four hours to the intact skin of three New Zealand White rabbits (stepwise procedure starting with one animal and supplementing two additional animals) using a patch of 2.5 cm x 2.5 cm, covered with semi-occlusive dressing. After removal of the patch, the application area was washed off. The cutaneous reactions were assessed immediately after removal of the patch and approximately 1, 24, 48 and 72 hours thereafter.

The only test item-related clinical observation was recorded immediately after removal of the patch and comprised very slight erythema (grade 1) in one out of three animals. This cutaneous reaction was reversible within one hour after removal of the patch.

Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0 and 0.0 for erythema and 0.0, 0.0 and 0.0 for edema.

Considering the described cutaneous reactions as well as the average scores for irritation, BAS 750 F does not show a skin irritating potential under the test conditions chosen.

According to classification criteria of UN-GHS and EU CLP Regulation 1272/2008, no classification is warranted as to skin irritation for BAS 750 F.

(DocID 2013/1150122)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: distilled water (test substance was minimally moistened with water immediately prior to application)

3. Test animals:

Species:	Rabbit
Strain:	New Zealand white Crl:KBL(NZW)
Sex:	female
Age (day 0):	ca. 7 months
Weight at dosing (mean):	4.02 - 4.98 kg
Source:	Charles River Wiga GmbH, Sulzfeld, Germany
Acclimation period:	At least 5 days
Diet:	STANRAB (P) SQC; SDS Special Diets Services, Altrip, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	single housing in stainless steel wire mesh cages with grating with shallowcage body; floor area: 4225 cm²; enmrichment: wooden gnawing blocks (KNH E-041), Abedd ® Lab. and Vet. Service GmbH Vienna, Austria

Environmental conditions:

Temperature:	20 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 03-Jun-2013 to 09-Jul-2013 [in-life phase: 03-Jun-2013 (treatment) to 13-Jun-2013 (day of last observation)]

2. *In vitro* pretest:

An *in vitro* study using the EpiDerm™ human skin model was performed with a comparable batch of BAS 750 F (L84-176). The test consists of a single topical exposure of 25 µL bulk volume (about 11 mg) of the test substance to the surface of a human reconstituted epidermis model for 3 minutes and 1 hour (corrosion test) or 1 hour with 42 hours post-incubation (irritation test) followed by a cell viability test. Cell viability is measured by dehydrogenase conversion of the yellow, water-soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), present in cell mitochondria, into a blue formazan salt that is measured quantitatively after isopropanol extraction from the tissues. The optical density of the extracts of test substance treated tissues is compared to negative control values from tissue treated with de-ionized water or PBS and is expressed as relative tissue viability.

3. Animal assignment and treatment:

Three female NZW rabbits with intact healthy skin were assigned to the study. 24 hours before application the fur was removed from the dorso-lateral part of the trunk by clipping. 0.5 g of the test material was minimally moistened with distilled water immediately prior to application to enhance the contact to skin. The test material was applied onto the flank under a patch of 2.5 cm x 2.5 cm, which was secured in position by means of a semi-occlusive dressing (Idealbinde, Pfaelzische Verbandstoff-Fabrik, Kaiserslautern and Fixomull® adhesive fleece, Beiersdorf AG, Germany). After removal of the patch the residuals of the test substance were washed off with Lutrol® E400 and Lutrol®/water (1:1).

The cutaneous reactions were assessed according to the quoted guidelines immediately after removal of the patch, approximately 1, 24, 48 and 72 hours, and then in weekly intervals up to day 14. Untreated skin sites of the animals served as negative control.

Body weights were determined just before application and after the last reading. A check for dead or moribund animals was performed at least once each work day.

II. RESULTS AND DISCUSSION

A. RESULTS

In vitro Epiderm corrosion / irritation test

The tissue viability values obtained for BAS 750 F were comparable to the negative control (100-102% of control values), regardless of the exposure conditions. The sensitivity of the assay was demonstrated by utilization of a positive control substance. It was concluded that BAS 750 F did not show a skin irritation potential in the EpiDerm skin corrosion/irritation test under the test conditions chosen.

In vivo Rabbit irritation test

Individual irritation scores after 4 hour dermal application of BAS 750 F are presented in Table 5.2.4-1. Slight erythema (grade 1) was observed in one of the three treated animals immediately after removal of the patch. No other cutaneous reactions were observed during the study. The cutaneous reaction was reversible within 1 hour after removal of the patch. Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0 and 0.0 for erythema as well as for edema.

Table 5.2.4-1: Skin irritation in rabbits – Individual irritation scores

Readings	Animal	Erythema	Edema	Additional findings
0 h	1	0	0	–
	2	0	0	–
	3	**1**	0	–
1 h	1	0	0	–
	2	0	0	–
	3	0	0	–
24 h	1	0	0	–
	2	0	0	–
	3	0	0	–
48 h	1	0	0	–
	2	0	0	–
	3	0	0	–
72 h	1	0	0	–
	2	0	0	–
	3	0	0	–
Individual 24-48-72 h means	1	0.0	0.0	
	2	0.0	0.0	
	3	0.0	0.0	

III. CONCLUSION

Based on the findings of this study, BAS 750 F does not show a skin irritating potential *in vitro* or *in vivo* under the test conditions chosen.

CA 5.2.5 Eye irritation

Report:	CA 5.2.5/1 Remmele M., 2012 b Reg.No. 5834378 - EpiOcular eye irritation test 2012/1367953
Guidelines:	<none>
GLP:	no

Report:	CA 5.2.5/2 Remmele M., 2012 c Reg.No. 5834378 - Bovine corneal opacity and permeability test (BCOP test) 2012/1367954
Guidelines:	OECD 437, Commission Regulation EU No. 1152/2010
GLP:	no

Report:	CA 5.2.5/3 [redacted] 2013 a BAS 750 F - Acute eye irritation in rabbits 2013/1150121
Guidelines:	OECD 405 (2002), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, JMAFF No 12 Nosan No 8147, EPA 870.2400
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

EXECUTIVE SUMMARY

In a primary eye irritation study, the eye irritation/corrosion potential of BAS 750 F (Batch: COD-001740; Purity: 98.8%) was determined by instillation of 0.1 ml bulk volume (ca. 38 mg) of the unchanged test substance into the conjunctival sac of the right eye of three New Zealand White rabbits. The application of the test substance was performed in a stepwise procedure starting with one animal and supplementing two additional animals. About 24 hours after application the eye was rinsed with tap water.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after administration of the test substance. An additional eye examination was performed 24 and 48 h after application with the instillation of a fluorescein solution. At the 24 and 48 hours reading reactions on cornea or iris were observed using a slit lamp and otoscope lamp.

There was no corneal opacity or signs of iritis during the study. Slight conjunctival redness was observed in all three animals during the study (at hour 1 and 24 for all animals, at hour 48 for only one animal); two of the three treated rabbits also showed slight conjunctival chemosis and one rabbit was found with conjunctival discharge, but only at the 1-hour reading time point. Additional findings like injected scleral vessels in a circumscribed area were noted until hour 24 at the latest. The ocular reactions were reversible in all animals within 72 hours after application.

The individual and overall mean eye irritation scores (24 to 72 hours) were 0.0 for corneal opacity, for iris, and for conjunctival chemosis. The corresponding mean conjunctival redness score for each of the three animals was 0.3, 0.3 and 0.7.

Based on the results of the study the test substance BAS 750 F is not an eye irritant under the test conditions chosen. Hence, BAS 750 F does not warrant classification as eye irritant according to classification criteria of UN-GHS and EU CLP Regulation 1272/2008.

(DocID 2013/1150121)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: The test substance was applied undiluted.

3. Test animals:

Species:	Rabbit
Strain:	New Zealand white Crl:KBL(NZW)
Sex:	female
Age (day 0):	ca. 3 months
Weight at dosing:	2.27 - 2.32 kg
Source:	Charles River Wiga GmbH, Sulzfeld, Germany
Acclimation period:	At least 5 days
Diet:	STANRAB (P) SQC; SDS Special Diets Services, Altrip, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	single housing in stainless steel wire mesh cages with grating with shallow cage body; floor area: 4225 cm^2; enrichment: wooden gnawing blocks (KNH E-041), Abedd ® Lab. and Vet. Service GmbH Vienna, Austria

Environmental conditions:

Temperature:	20 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 02-Sep-2013 to 17-Oct-2013 [in-life phase: 02-Sep-2013 (treatment) to 19-Sep-2013 (day of last observation)]

2. *In vitro* pretests:

Separate *in vitro* studies were performed prior to *in vivo* testing in order to evaluate the potential for corrosion / severe eye damage and eye irritation. A comparable batch of BAS 750 F (L84-76) was tested in the reconstructed human cornea model EpiOcular™ and in the Bovine Cornea Opacity test (BCOP) for this purpose.

EpiOcular™ test

Two EpiOcular™ tissue samples were incubated with 50 µL bulk volume (about 15 mg) of the test substance for 90 minutes followed by an 18-hours post-incubation period. Tissue destruction was determined by measuring the metabolic activity of the tissue after exposure/post-incubation using a colorimetric test. The reduction of mitochondrial dehydrogenase activity, measured by reduced formazan production after incubation with a tetrazolium salt (MTT) was chosen as endpoint. The formazan production of the test substance treated epidermal tissues is compared to that of negative control tissues (relative tissue viability).

Bovine Cornea Opacity test (BCOP)

The test method consists of a topical exposure of the test substance to the epithelial surface of isolated corneas from the eyes of freshly slaughtered cattle. Corneal opacity is measured quantitatively as the amount of light transmission through the cornea. Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea. Both measurements are used to calculate an In Vitro Irritancy Score (IVIS) of the test substance, which is used for prediction of serious eye damage. In addition H&E-stained cross sections were evaluated for pathohistological findings of the test substance. Histological evaluation was performed in addition to the assessment of opacity and permeability. The result may provide additional information on the potential corrosivity of a test substance. However currently, no guideline how to include histological findings in the overall BCOP evaluation is available. The pathohistological evaluation for all corneas treated with the test substance was summarized as no findings, minimal, mild, moderate or moderate / severe. Three corneas were treated with 750 µL of a 20% test-substance preparation in de-ionized water for an exposure period of 4 hours.

3. Animal assignment and treatment:

The potential of BAS 750 F to cause acute eye irritation was assessed by instillation of 0.1 ml bulk volume (ca. 38 mg) of the comminuted test substance into the conjunctival sac of the right eye. The untreated left eye served as the negative control. About 24 hours after application of the test substance the treated eye was rinsed with 3 to 6 ml of hand warm tap water for 1 to 2 minutes using a syringe.

The ocular reactions were assessed according to quoted guidelines approximately 1, 24, 48 and 72 hours after application and then in weekly intervals maximally up to day 28 after the administration of the test substance.

An additional eye examination was performed 24 and 48 h after application with the instillation of a fluorescein solution to verify the absence of corneal opacity. At the 24 and 48 hours reading, reactions on cornea or iris were observed using a slit lamp and otoscope lamp.

Body weights were measured just before application and after the last reading. The animals were checked for mortality, morbidity and clinical signs at least once each work day.

II. RESULTS AND DISCUSSION

A. RESULTS

***In vitro* eye irritation tests**

EpiOcular™ Assay:

The test result is considered as "irritant", if the mean relative tissue viability with a test material is less than or equal to 50%. Under the study conditions, BAS 750 F exposure gave viability values that corresponded to 81% viability of the control incubation. The positive control reduced the tissue viability to 20% of the control value. It was concluded that BAS 750 F does not show an eye irritation potential in the EpiOcular™ eye irritation test under the test conditions chosen.

BCOP Assay

Following the prediction model, IVIS scores higher than 55 would indicate a risk of serious damage to the eyes. BAS 750 F gave a mean IVIS value of -0.4 ± 2.1; the negative control value was 5.5 ± 2.5 and the positive control 118.3 ± 3.6. The pathohistological evaluation summarized for all corneas treated with BAS 750 F revealed no findings indicating eye damage. It was concluded that that BAS 750 F does not cause serious eye damage in the BCOP Test under the test conditions chosen.

Rabbit eye irritation test

All animals appeared healthy during the study. There were no signs of gross toxicity, adverse clinical signs or abnormal behavior following treatment of the rabbits with BAS 750 F.

Results of the eye irritation assessment are presented in Table 5.2.5-1.

Slight conjunctival redness (grade 1) was noted in all three animals at hour 1 and 24 after application and persisted in one animal up to hour 48.

Slight conjunctival chemosis (grade 1) was noted in one out of three animals 1 hour after application.

Slight discharge (grade 1) was noted in two out of three animals 1 hour after application.

Additional findings like injected scleral vessels in a circumscribed area were noted in all animals at hour 1 and persisted in two animals up to 24 hours.

No corneal lesions were detectable even after instillation of fluorescein performed 24 and 48 hours after application.

Mean scores calculated for each animal over 24, 48 and 72 hours were 0.0, 0.0 and 0.0 for corneal opacity, 0.0, 0.0 and 0.0 for iris lesions, 0.3, 0.3 and 0.7 for redness of the conjunctiva and 0.0, 0.0 and 0.0 for chemosis.

The ocular reactions were reversible in two animals within 48 hours and in one animal within 72 hours after application.

Table 5.2.5-1: Eye irritation in rabbits – Individual irritation scores

Readings	Animal	Cornea		Iris	Conjunctiva			Additional findings
		Area involved	Opacity		Redness	Chemosis	Discharge	
1 h	1	0	0	0	1	1	1	#48
	2	0	0	0	1	0	1	#48
	3	0	0	0	1	0	0	#48
24 h	1	0	0	0	1	0	0	FL neg; #48
	2	0	0	0	1	0	0	FL neg
	3	0	0	0	1	0	0	FL neg; #48
48 h	1	0	0	0	0	0	0	FL neg
	2	0	0	0	0	0	0	FL neg
	3	0	0	0	1	0	0	FL neg
72 h	1	0	0	0	0	0	0	–
	2	0	0	0	0	0	0	–
	3	0	0	0	0	0	0	–
Individual 24-48-72 h means	1		0.0	0.0	0.3	0.0		
	2		0.0	0.0	0.3	0.0		
	3		0.0	0.0	0.7	0.0		

FL neg. = fluorescein-negative (= no evidence of corneal damage)
48 = scleral vessels injected, circumscribed area, central ingrown

III. CONCLUSION

Based on the findings of this study, BAS 750 F is not an eye irritant under the test conditions chosen.

CA 5.2.6 Skin sensitisation

Report:	CA 5.2.6/1 [redacted] 2013 a BAS 750 F - Test for skin sensitization using the guinea pig maximization test (GPMT) 2013/1150123
Guidelines:	OECD 406 (1992), EPA 870.2600, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.6 No. L 142, EPA 712-C-03-197
GLP:	yes (certified by Land Brandenburg Ministerium fuer Umwelt, Gesundheit und Verbraucherschutz, Potsdam, Germany)
Report:	CA 5.2.6/2 Grauert E.,Kamp H., 2014 a Analytical report - BAS 750 F - Homogeneity and concentration control analyses in paraffinum subliquidum 2014/1116448
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Report:	CA 5.2.6/3 Stinchcombe S., 2016 a Assessment of the skin sensitization potential of BAS 750 F - Consideration of the appropriate sub-category for GHS classification as skin sensitizer 2016/1028946
Guidelines:	<none>
GLP:	no

EXECUTIVE SUMMARY

The potential of BAS 750 F (Batch: COD-001740; Purity: 98.8%) for skin sensitization was investigated in the Guinea Pig Maximization Test (GPMT) performed according to the method of Magnusson and Kligman, using a control and a treated group of 5 and 10 female Dunkin-Hartley (Crl:HA) Guinea pigs, respectively.

The test-substance concentrations for the main test were selected based on the results of the pretests. The intradermal induction was performed with a 5% test substance preparation in the vehicle Paraffin subliquidum or a 5% test substance preparation in Freund's complete adjuvant / 0.9% aqueous NaCl-solution (1:1). The epicutaneous induction was conducted with a 60% test substance preparation in Paraffin subliquidum (maximum homogeneously producible and applicable concentration). For the challenge a 50% test substance preparation in Paraffinum subliquidum was chosen.

After the intradermal induction, all animals of the control and test groups showed graduated skin reactions in form of slight to moderate erythema (grade 1 and 2) initially on the different injection sites. On the day prior and after the epicutaneous induction, necrosis, additionally with slight to moderate erythema, was observed on the area of the injections sites with adjuvant involvement.

After the challenge, discrete or patchy erythema was noted in 6 of 10 test group animals 24 and/or 48 hours after removal of the patch; papules were additionally noted in two of the test group animals. The test fields treated with the vehicle were free of any reactions in the test group. None animal of the control group responded with skin reactions to the challenge treatment neither with the test item nor with the vehicle.

Under the test conditions chosen and applying the evaluation criteria, the test substance BAS 750 F showed skin sensitizing effects in the guinea pig maximization test.

Results of Local lymph node assays with formulations containing BAS 750 F at concentrations of up to 400 g/L, and LLNA data available for structurally closely related BAS 750 F precursors [see KCA 5.2.6/3 2016/1028946] support the conclusion that BAS 750 F has a weak skin sensitizing potential. Therefore, the following classification of BAS 750 F is proposed according to CLP and UN-GHS classification criteria:

Skin Sens 1B; H317

“May cause an allergic skin reaction”

(DocID 2013/1150123)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.
2. Vehicle:	Paraffin oil (Paraffinum subliquidum)
Positive control:	alpha-hexylcinnamaldehyde techn. 85% (separate investigation by the test facility)

3. Test animals:

Species:	Guinea Pig
Strain:	Dunkin-Hartley SPF (albino guinea pigs)
Sex:	female
Age at dosing (day 0):	no data
Weight (day -1):	291 – 320 g
Source:	Harlan Laboratories GmbH, Venray, Netherlands
Acclimation period:	At least 5 days
Diet:	Altromin 3123 pellets (Altromin, Lage/Lippe, Germany), ad libitum
Water:	Tap water enriched with vitamin C and acidified to pH 2.5 with hydrochloric acid to prevent microbiological growth, ad libitum
Housing:	2-3 guinea pigs housed/cage in Macrolon cages; floor area: 2280 cm^2; bedding: "Lignocel-Granulat" or "Lignocel-Fasern" (Altromin, Lage/Lippe, Germany) enrichment: hay bricks "Raufutter rolls" (Provimi Kliba, Kaiseraugst, Switzerland)
Environmental conditions:	
Temperature:	20 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 26-Jun-2013 to 4-Nov-2013 [in-life phase main study: 08-Oct-2013 (1^{st} treatment) to 04-Nov-2013 (final recording of body weights)]

2. Animal assignment and treatment

The skin sensitizing potential of BAS 750 F was assessed using the Guinea Pig Maximization Test. This test consists of pretests in order to identify an appropriate vehicle for intradermal induction and for determination of suitable induction and challenge concentrations of the test substance, and the Maximization test itself.

For the Maximization test (=main experiment), female Guinea pigs were randomly allocated into two groups: a vehicle control group of 5 animals, and test group consisting of 10 animals.

For the intradermal and epicutaneous occlusive route of application, animal fur was clipped 1 day before each application at the appropriate treatment sites.

Possible signs of illness being discernible during the experimental period were recorded. Individual body weights were determined on study day -1 and on the last day of observation (day 27).

Evaluations of the skin reactions were performed according to the grading scale of Magnusson and Kligman (The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test. J. Invest. Dermatol. 52, 268 - 276 (1969)).

3. Preliminary tests

In pre-tests with different test substance / vehicle suspensions, paraffin oil showed satisfactory results in intradermal injection pre-tests. 5% (w/w) BAS 750 F suspension was the maximum injectable concentration. The irritation potential of 5% and 2.5% BAS 750 F in paraffin oil was investigated in two groups of 2 guinea pigs.

Six intradermal injections per animal and test substance concentration were performed in groups of two (see below) at the neck region in order to determine a test substance concentration that is well-tolerated locally and systemically for the intradermal induction treatment in the main test. The evaluation of the skin reactions was performed 24 h and 48 h after application.

Intradermal injections:

A)	front row:	2 injections each of 0.1 ml Freund's complete adjuvant / 0.9% aqueous NaCl-solution (1:1 w/w) without the test substance
B)	middle row:	2 injections each of 0.1 ml of a test substance preparation in an appropriate vehicle at the selected concentration
C)	back row:	2 injections each of 0.1 ml Freund's complete adjuvant / 0.9% aqueous NaCl-solution (1:1 w/w) with test substance at the selected concentration

Additionally, for the dermal induction treatment the lowest concentration of the test substance that causes slight to moderate irritation and for the challenge the maximum non-irritant concentration was determined. Two different vehicles were tested, 1% aqueous carboxymethylcellulose and paraffin oil. For each vehicle/test substance combination, 24-hour and 48-h skin reactions were determined following 24-hour dermal exposure of two Guinea pigs to 0.5 mL of four different BAS 750 F concentrations (60%, 50%, 25%, 10%), which were applied under occlusive patches at four different flank positions (left anterior, left posterior, right anterior and right posterior flank).

The patch consisted of filter paper (2 x 4 cm) coated with impermeable tape (Blenderm®) on one side. This patch was placed on the skin and fixed by wrapping the body of the animals with Gothaplast® tape (2.5 cm and 5 cm width).

4. Main experiment – intradermal and topical induction

On Day 0, the intradermal induction was carried out, which consisted of 6 intradermal injections in groups of two into the shoulder region of the animals according to the scheme given above (see section “preliminary tests”).

On Days 1 and 6, the intradermal injection sites were assessed for skin reactions.

On Day 7 (one week after intradermal treatment), the topical induction was performed. Each animal of the control group was administered 0.5 ml of the vehicle under occlusive conditions (for details see section “preliminary tests”). Test group animals received 0.5 g of the test substance-vehicle preparation at the concentration that was selected in the pre-test. The dressing and patch were removed after 48 hours.

5. Main experiment –topical challenge

On Day 21 (two weeks after topical induction treatment), the challenge treatment was carried out. An occlusive patch, consisting of multi-layered gauze (2 x 2 cm) and coated with impermeable tape (Blenderm®) on one side, was saturated with an amount of 0.5 ml of test item / vehicle preparation, representing the maximum non-irritating concentration. This patch was placed on the right posterior flank of all animals of the control and test groups. An additional patch was saturated in the same manner with 0.5 ml of the vehicle and was placed on the left posterior flank of all animals. The body of the animals was wrapped with Gothaplast® tape (2.5 cm x 5 cm) in order to fix the patches.

On Day 22 (24 hours after application), the dressing and patches were removed and the skin was cleaned with water. Residues of glue from the dressing were additionally removed from the skin with a 1:1-mixture of PEG (polyethylene glycol) and water if necessary.

On Days 23 and 24 (i.e. 24 and 48 hours after the end of exposure), skin reactions were recorded. In order to facilitate the evaluation hair was removed by an electric razor from the posterior flanks on both sides about three hours before the first observation.

6. Positive controls

A positive control (reliability check) with a known sensitizer was not performed in this study. However, separate positive control studies are performed twice a year in the laboratory. A positive control study with alpha-hexylcinnamaldehyde techn. was performed from July to October 2013 and showed that the test system is able to detect sensitizing compounds under the laboratory conditions chosen.

7. Evaluation of results

The number of animals with skin findings at 24 and/or 48 hours after removal of the patch was taken into account for the determination of the sensitization rate. The evaluation "sensitizing" resulted if at least 30% of the test animals exhibit skin reactions in this adjuvant test. This evaluation was based on the classification criteria of the Globally Harmonized System (GHS) and Regulation (EC) No. 1272/2008.

II. RESULTS AND DISCUSSION

A. PRE-TESTS

1. Test concentration for intradermal induction

Paraffin oil ("paraffin subliqu.") was selected as vehicle for intradermal injection of BAS 750 F, after preliminary investigations showed that alternative vehicles (1% aqueous CMC or PEG 400 (polyethylene glycol) were unsuitable. Two test item concentrations (5.0 and 2.5% (w/w) in paraffin subliqu.) were tested to determine the minimal irritating concentration. A sufficient amount of test item in the vehicle or in the adjuvant mixture could be successfully injected. Although the solid particles impeded the spread in the dermis and due to back pressure caused a partial reflux of liquid (approx. 50%), the appearance of skin reactions indicated the intradermal placement of sufficient depot of suspension (see Table 5.2.6-1). Based on the results of this pre-test, a concentration of 5% (w/w) test item was selected for intradermal induction.

Table 5.2.6-1: GPMT pre-test – skin reactions after intradermal injection

	Skin reactions							
	Animal no. VII				Animal no. VIII			
	left	right	left	right	left	right	left	right
Pair of injections	24h		48h		24h		48h	
(1) 1:1 mixture FCA + 0.9% NaCl	2	2	2	2n	2	2	2	2
(2) **5.0% BAS 750 F** in paraffin subliqu.	1	1	1	2	1	1	1	1
(3) **5.0% BAS 750 F** in adjuvant mixture (1)	2	2	2	2	2	2	2	2
	Animal no. IX				Animal no. X			
	left	right	left	right	left	right	left	right
Pair of injections	24h		48h		24h		48h	
(1) 1:1 mixture FCA + 0.9% NaCl	2	2	2	2	2	2	2	2
(2) **2.5% BAS 750 F** in paraffin subliqu.	1	1	1	1	1	1	1	1
(3) **2.5% BAS 750 F** in adjuvant mixture (1)	2	2	2	2	2	2	2	2

n = beginning necrosis

2. Test concentrations for topical induction and challenge treatment

BAS 750 F in the vehicle paraffin oil ("paraffin subliqu.") was tested in a dermal preliminary investigation. Two animals pre-treated with the adjuvant were used in order to determine the minimal irritating concentration of BAS 750 F for the topical induction and the maximum non-irritating concentration of BAS 750 F for the challenge application. Based on the results of this pre-test, a concentration of 60% (w/w) test item was selected for topical induction and a concentration of 50% (w/w) test item for topical challenge (see Table 5.2.6-2).

Table 5.2.6-2: GPMT pre-test – skin reactions after topical treatment

BAS 750 F concentration (w/w)	Patch position	Skin reactions							
		Animal no. XI				Animal no. XII			
		24h*		48h*		24h		48h*	
60%	Left anterior flank	**1**	0	0	0	0	0	0	0
50%	Left posterior flank	0	0	0	0	0	0	0	0
25%	Left anterior flank	0	0	0	0	0	0	0	0
10%	Left posterior flank	0	0	0	0	0	0	0	0

* = after termination of treatment

B. MAXIMIZATION TEST

1. Induction

The intradermal injections with 5% (w/w) BAS 750 F in the adjuvant-mixture (FCA) involvement caused skin irritations (grade 1 to 2) and necrosis in the test and control groups. The sites of injections with only BAS 750 F or vehicle showed none or a slight erythema only. Necrosis was also observed on the cranial and caudal injection sites in both groups during the topical induction phase. The injection sites of the animals of the control group and test group without FCA-involvement had no discernible erythema during the phases of topical induction with 60% (w/w) BAS 750 F. Detailed findings are summarized in Table 5.2.6-3.

Table 5.2.6-3: GPMT main-test – skin reactions after induction treatment

Group	Animal no.	Skin findings of the injection and application sites on …																	
		Day 1 (intradermal induction)						Day 6						Day 9 (topical induction)					
		(1)		(2)		(3)		(1)		(2)		(3)		(1)		(2)		(3)	
		le.	ri.	le.	ri.	le.	ri.	le.	ri.	le.	ri.	le.	ri.	le.	ri.	le.	ri.	le.	ri.
Control group	1	2	2	0	0	2	2	2n	2n	0	0	2n	1n	2 N	2 N	0	0	2n	1n
	2	2	2	0	0	2	2	2n	2n	0	0	1n	1n	2 N	2 N	0	0	1n	1n
	3	2	2	0	0	1	1	2n	2n	0	0	1n	1n	2 N	2 N	0	0	1n	1n
	4	2	2	0	0	1	1	2n	2n	0	0	1n	1n	2 N	2 N	0	0	1n	1n
	5	2	2	0	0	2	2	2n	2n	0	0	1n	1n	2 N	2 N	0	0	1n	1n
Test group	6	2	2	1	1	1	1	2n	2n	0	0	1n	1	2 N	2 N	0	0	1 N	1 N
	7	2	2	1	1	1	1	2n	2n	0	0	1	1	2 N	2 N	0	0	1 N	1 N
	8	2	2	1	1	1	1	2n	2n	0	0	1	1	2 N	2 N	0	0	1n	1n
	9	2	2	1	1	2	2	2n	2n	0	0	2	1	2 N	2 N	0	0	1n	1 N
	10	2	2	0	0	1	2	2n	2n	0	0	1	1	2 N	2 N	0	0	1 N	1 N
	11	2	2	1	1	1	2	2n	2n	0	0	1	1	2 N	2 N	0	0	1 N	1 N
	12	1	2	1	1	1	2	2n	2n	0	0	2	1	2 N	2 N	0	0	1 N	1 N
	13	2	2	1	1	2	2	2n	2n	0	0	2	1	2 N	2 N	0	0	1n	1 N
	14	2	2	0	0	2	2	2n	2n	0	0	1	1	2 N	2 N	0	0	1 N	1 N
	15	2	2	0	0	2	2	2n	2n	0	0	2	2	2 N	2 N	0	0	1 N	1 N

(1) = 1st pair of injections (only adjuvant mixture)
(2) = 2nd pair of injections (5% test item in vehicle)
(3) = 3rd pair of injections (5% test item in adjuvant mixture)

le. / ri. = left / right injection site
n = necrosis
N = open necrosis

2. Challenge

The frequency of skin findings after the challenge treatment with the test item is summarized in Table 5.2.6-4. The numerical scores for the individual animals elicited by the challenge application are shown in Table 5.2.6-5.

None animal of the control group responded with skin reactions to the treatment with the 50% (w/w) test item or the vehicle paraffin subliqu. during the challenge.

In the test group, 6 of 10 animals showed skin reactions in form of discrete or patchy erythema (grade 1), in two animals additionally with papules 24 and/or 48 hours after the challenge treatment with the 50% (w/w) test item. The challenge treatment with the vehicle paraffin subliqu. did not cause skin reactions in any animals of the test group.

The frequency of positive skin reactions (60 %) in the test group in comparison to the reaction-free control group represented sufficient evidence for a skin sensitizing effect of the test item.

Table 5.2.6-4: GPMT main-test – summary of skin reaction scores after challenge

Skin findings		Control group			Test group		
		24 h	**48 h**	**24 or 48 h**	**24 h**	**48 h**	**24 or 48 h**
Grade 0	No visible change	5 / 5	5 / 5	5 / 5	8 / 10	4 / 10	4 / 10
Grade 1	Discrete or patchy erythema	0 / 5	0 / 5	0 / 5	**2 / 10**	**6 / 10**	**6 / 10**
Grade 2	Moderate or confluent erythema	0 / 5	0 / 5	0 / 5	0 / 10	0 / 10	0 / 10
Grade 3	Intense erythema and swelling	0 / 5	0 / 5	0 / 5	0 / 10	0 / 10	0 / 10

Table 5.2.6-5: GPMT main-test – individual skin reactions after challenge treatment

Group	Animal no.	24-hour observation		48-hour observation		Body weight [g]	
		Left posterior	Right posterior	Left posterior	Right posterior	Day -1	Day 27
		Vehicle	BAS 750 F	Vehicle	BAS 750 F		
Control group	1	0	0	0	0	312	436
	2	0	0	0	0	310	462
	3	0	0	0	0	308	498
	4	0	0	0	0	291	436
	5	0	0	0	0	316	478
Test group	6	0	0	0	**1**	302	466
	7	0	**1**	0	**1**	302	468
	8	0	**1**	0	**1**	306	449
	9	0	0	0	0	310	458
	10	0	0	0	**1***	300	481
	11	0	0	0	**1**	302	471
	12	0	0	0	**1***	291	512
	13	0	0	0	0	305	479
	14	0	0	0	0	311	493
	15	0	0	0	0	320	513

* Papules

3. Observations

The animals showed no signs of illness.

4. Body weights

The animals had a normal development of body weight during the study period. The body weights on day -1 and day 27 are listed in Table 5.2.6-5.

C. POSITIVE CONTROL

The sensitivity and reliability of the experimental technique and test systems used were verified. The last positive control test with the reference material α-hexylcinnamaldehyde, technical grade, 85 % was performed at the test facility from July to October 2013. The sensitization potential of the positive reference item has been shown sufficiently by the response of 7 of 10 guinea pigs (70 %) of the test group with positive skin reactions (erythema grade 1 and 2). A summary of the results is presented Table 5.2.6-6.

Table 5.2.6-6: Positive control data: GPMT main-test with α-hexylcinnamaldehyde

Study code no. 03671 Experimental phase: 07/2013 – 10/2013		**Vehicle (olive oil)**			**10% (w/w) α-HCA 85%**		
Study type	Group	**24 h**	**48 h**	**24 or 48 h**	**24 h**	**48 h**	**24 or 48 h**
GPMT (OECD 406)	Control group	0 / 5	0 / 5	0 / 5	1 / 5	0 / 5	1 / 5
	Test group	0 / 10	0 / 10	0 / 10	5 / 10	6 / 10	7* / 10

* One animal showed erythema only after 24 hours and two animals only after 48 hours

III. CONCLUSION

BAS 750 F showed a sensitizing potential to the skin in the guinea pig maximization test (GPMT).

CA 5.2.7 Phototoxicity

In accordance with the requirements of Commission Regulation (EU) No. 283/2013, an *in vitro* NRU phototoxicity study in Balb/c 3T3 cells was performed since BAS 750 F's extent of absorption in the wavelength range of natural sunlight (290-790 nm) exceeded the ultraviolet/visible molar extinction/absorption coefficient threshold of $10\ L \times mol^{-1} \times cm^{-1}$ specified in the Regulation, roughly between light wavelengths of 290-665 nm (see Kroehl T, DocID 2015/1183750).

Report:	CA 5.2.7/1 Cetto V.,Landsiedel R., 2015 a BAS 750 F - In vitro 3T3 NRU phototoxicity test 2015/1117503
Guidelines:	OECD 432 (2004) In vitro 3T3 NRU Phototoxicity test, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.41 No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (batch COD-001880; purity 98.6%) was tested for its ability to induce phototoxic effects in Balb/c 3T3 cells in vitro according to OECD Test guideline 432 (2004). The photo-cytotoxicity was estimated by the means of the Neutral Red Uptake (NRU) method. Following an initial range-finding phototoxicity test, BAS 750 F was tested at concentration of 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4 and 100 µg/mL. The highest test substance concentrations were cytotoxic with and without UV/VIS irradiation.

On the basis from the results of the present study, the test substance was predicted to have no phototoxic potential (PIF=1.1) indicated by Neutral Red Uptake method. The positive control chlorpromazine led to the expected cytotoxicity both with and without UV/VIS irradiation (PIF = 102.1), thus, demonstrating the sensitivity of the test system.

Under the experimental conditions of this study, BAS 750 F is considered not to be a phototoxic substance in the in vitro 3T3 NRU phototoxicity test using Balb/c 3T3 cells.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001880
Purity / content:	98.6% (tolerance ± 1.0%)
Stability of test compound:	The stability was guaranteed for the duration of the study.
Solvent used:	Dimethyl sulfoxide (DMSO)

2. Control Materials:

Negative control:	A negative control was not employed in this study
Solvent control:	1% (v/v) DMSO in culture medium
Positive control:	Chlorpromazine (absorption peak at 309 nm) Without irradiation: 1.9, 3.8, 7.5, 15, 30, 60, 90, and 180 µg/mL With irradiation: 0.03, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µg/mL

3. Test organism: Balb/c 3T3, clone A31 cells

The cell line was isolated from the muscle tissue of mouse embryo. This fibroblast cell line has a high proliferation rate and a high plating efficiency of untreated cells both necessary for the appropriate performance of the study. The Balb/c 3T3 cell line was obtained from the "European Collection of Cell Cultures" Salisbury, UK.

4. Culture media and reagents

Culture medium:	Dulbecco's Modified Eagle's Medium (DMEM) supplemented with - 10% (v/v) newborn calf serum (NCBS) - 4 mM L-glutamine - 100 IU penicillin - 100 µg/mL streptomycin
Neutral red solution:	0.4 g Neutral Red powder in 100 mL deionized water
Neutral red medium:	1 mL Neutral Red solution in 79 mL culture medium, incubated overnight at 37° C with 5% CO_2 and filtered with a 0.22 µm filter prior to use).

Other solutions / reagents: - Phosphate buffered saline (PBS) without Ca/Mg
- Trypsin/EDTA solution (0.05%; 0.02%)
- Neutral Red desorb solution (1 mL acetic acid, 50 mL ethanol, 49 mL deionized water)

5. Irradiation source: Sol 500 solar simulator (Dr. Hönle AG, 82166 Gräfelfing, Germany). Following the requirements of the current OECD Guideline 432 the filter H1 was used in combination with Sol 500 to obtain a wavelength spectrum as closely as possible comparable to that of natural sunlight, with a spectral distribution covering wavelengths in the UVB, UVA and the visible light range.

6. Test concentrations:

Preliminary toxicity assay: Eight concentrations ranging from 4.6 to 1000 µg/mL (corresponding to a max. concentration of ca. 2.5 mM)

Main assay: With and without UV/VIS irradiation:

- Experiment 1 and 2*: 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4, 100.0 µg/mL

* In the 1st Experiment the mean OD_{550} value of the vehicle controls was below 0.3 (data not shown) and thus did not fulfill the acceptance criteria and therefore has to be judged as not valid. Based on this a repeat experiment was carried out (Experiment 2).

B. TEST PERFORMANCE:

1. Dates of experimental work: 21-Apr-2015 to 20-May-2015

2. Cytotoxicity test for dose selection

Following the requirements of the current international guidelines a test substance should be tested up to a maximum concentration of 1000 µg/mL or 10 mM, whichever is the lowest. In the pretest for toxicity, based on the purity of the test substance 1000 µg/mL was used as top concentration both with and without irradiation. The pretest was performed following the method described for the main experiment (see below). In the pretest various additional parameters (pH value, osmolarity and solubility) were checked or determined for all or at least some selected concentrations.

On the basis of the findings from the pretest (cytotoxicity) and taking into account the current guideline or recommended criteria 100 µg/mL was selected as the top dose without and with UV/VIS irradiation. Moreover, seven further doses with a diluting factor of $3\sqrt{10}$ were selected to detect a possible dose-response relationship. The concentrations are given as rounded values.

3. Seeding and treatment of cells

About 24 hours before start of treatment 100 µL of 1.5 x 10^5 cells/mL were seeded per well in 96-weIl plates (except the peripheral wells, in which 100 µL culture medium was added only). 6 replicate wells were used per test substance concentration. Two plates per substance (test substance or positive control) were prepared. All incubations were performed at 37 °C with a relative humidity of ≥90% in a 5% (v/v) CO_2 atmosphere. After an attachment period of about 24 hours the cells were washed once with PBS and treated with the respective substance (8 concentrations each with 6 replicates) and the vehicle control in parallel for 1 hour in the dark. Then, one microtiter plate per substance was irradiated for 50 min at room temperature using a Sol 500 simulator (with monitoring to obtain UVA intensities of 1.5–2.1 mW/cm^2 (equal to 5 J/cm^2) underneath the lid. The respective reference plate was kept in the dark at room temperature for the same time period. After test substance removal the cells were washed with 100 µL PBS and incubated in culture medium overnight.

4. Determination of Neutral Red Uptake (NRU)

24 hours after start of treatment the culture medium was removed. 100 µL medium containing 50 µg/mL Neutral Red was added. The plates were incubated for another 3 hours to allow uptake of the vital dye into the lysosomes of viable cells. Thereafter, the cells were washed, the dye was extracted by Neutral Red desorb solution. Cytotoxicity was determined by measuring the Neutral Red Uptake using a microplate reader (Perkin Elmer, Waltham, Massachusetts, US; Wallac 1420 multilabel counter) equipped with a 550 nm filter to read the absorption of the extracted dye. The absorption shows a linear relationship with the number of surviving cells.

C. EVALUATION / ASSESSMENT

For the assessment of the phototoxic potential of a compound two prediction models are currently available. The choice of the adequate model depends on the test conditions.

- The **Photo-Irritancy-Factor** prediction model for substances, which allow the comparison of two equi-effective concentrations (EC_{50}) in concurrently performed experiments in the presence and absence of UV light. This model includes the special case of absence of cytotoxicity in the presence and absence of light for substances obviously showing no phototoxic potential.
- The **Mean Photo Effect** prediction model is used if no equi-effective concentrations (EC_{50}) are obtained in the absence and presence of UV light.

1. Cytotoxicity

The mean absorbance values obtained for each test group of every plate were used to calculate the percentage of cell viability relative to the respective vehicle control, which is arbitrarily set at 100%.

$$\text{Cytotoxicity [\%]} = \frac{\text{Mean absorbance of the test group}}{\text{Mean absorbance of the vehicle control}} \times 100$$

In case of outlier values (having half or double the value of the respective mean), at least 4 single values per test group were sufficient for calculating reliable mean values.

In case of cytotoxicity, an EC_{50} value (effective concentration, calculated to result in 50% viability relative to the respective vehicle control) was calculated by a linear interpolation method (linear dose-response curve). Therefore, two toxicity values were needed: one between 100% and 50% cytotoxicity and one between 50% and 0% cytotoxicity. From these two points the concentration that inhibits the Neutral red uptake down to 50% of the respective control was calculated.

2. Photo-Irritancy-Factor

For substances which induce a 50% cytotoxicity (EC_{50}) in the presence and absence of light the Photo-Irritancy-Factor (PIF) is calculated based on comparison of the EC_{50} values in the absence and presence of UV/VIS irradiation.

$$\text{PIF} = \frac{EC_{50}\ (-\text{UV/VIS})}{EC_{50}\ (+\text{UV/VIS})}$$

The resulting classification rules are:

PIF ≥ 5:	phototoxic potential predicted
2 < PIF < 5:	probable phototoxic potential predicted
PIF ≤ 2:	no phototoxic potential predicted

If cytotoxicity occurs only after irradiation (UV/VIS), the PIF cannot be calculated. Instead, the "C PIF" value is determined by considering the highest test concentration (C_{max}) applied in the experimental part in the absence of UV light (-UVA) in a modified formula as follows:

$$\text{C PIF} = \frac{C_{max}\ (-\text{UV/VIS})}{EC_{50}\ (+\text{UV/VIS})}$$

The resulting classification rules are:

PIF > 1:	Probably phototoxic potential predicted
PIF ≤ 1:	no phototoxic potential predicted

If no cytotoxicity occurs in the concurrently performed experiments in the absence and presence of irradiation up to the highest applied test concentration it has to be considered that the test substance has no phototoxic potential. In this case, a formal PIF = *1 is used to characterize the result:

$$\text{PIF} = *1 = \frac{C_{max}\ (-\text{UV/VIS})}{C_{max}\ (+\text{UV/VIS})}$$

The resulting classification rules is:

PIF = *1:	No phototoxic potential predicted

3. Mean photo effect

A major limitation of the Photo-Irritancy-Factor prediction model is the fact that the PIF is based on the comparison of two equi-effective concentrations (EC_{50}) in the concurrently performed experiments in the absence and presence of irradiation, which cannot be determined in every case. To overcome this limitation, the mean photo effect (MPE) can be used as measure for the phototoxic potential of chemicals. The Mean Photo Effect is calculated based on a comparison of the +UV/VIS and –UV/VIS concentration response curves on a grid of concentrations ci (i=1,..., N) chosen from the common concentration range of the (−UV/VIS) and (+UV/VIS) experiments. The photo effect (PE_i) at concentration ci is calculated as the product of the concentration effect (CE_i) and the response effect (REi). The mean photo effect (MPE) is defined as a weighted averaging across all PE_i values, with a weighting factor defined by the highest response value.

The resulting classification rules are:

MPE ≥ 0.1:	phototoxic potential predicted
MPE ≤ 0.1:	no phototoxic potential predicted

4. Check or determination of further parameters:

- The **pH** was measured at least for the top concentration and the vehicle controls with and without UV/VIS irradiation.
- **Osmolarity** was measured, at least for the top dose and for the vehicle controls with and without UV/VIS irradiation.
- **Solubility**: Test substance precipitation was checked immediately after treatment of the test cultures and at the end of treatment.
- **Cell morphology**: The test cultures of all test groups were examined microscopically before staining with NRU, which allows conclusions to be drawn about the attachment of the cells.

5. Statistics

No special statistical tests were performed.

D. ACCEPTANCE CRITERIA

The assay is considered valid if the following criteria are met:

- The mean OD_{550} value of the vehicle controls (with and without UV/VIS irradiation) should be ≥ 0.3.
- After irradiation with a UVA dose of ~ 5 J/cm2 the cell viability of the vehicle control should be at least 80% of the concurrent non-irradiated vehicle control.
- The standard deviation of the mean values of both vehicle control rows should not exceed ± 15%
- For the positive control chlorpromazine the EC_{50} value should be in the following ranges:

  With irradiation (+UV/VIS): 0.1 - 2.0 µg/mL

  Without irradiation (-UV/VIS): 7.0 - 90.0 µg/mL

  The PIF should be at least 6

II. RESULTS

In the 1st Experiment the mean OD_{550} value of the vehicle controls was below 0.3 (data not shown) and thus did not fulfill the acceptance criteria and therefore has to be judged as not valid. Based on this a repeat experiment, designated 2nd Experiment was carried out.

A. TREATMENT CONDITIONS AND CELL MORPHOLOGY

Osmolarity and pH values were not influenced by test substance treatment.

In this study, in the absence and presence of UV/VIS irradiation, test substance precipitation in culture medium was observed at the end of treatment at 100 µg/mL.

In addition, changes in cell morphology were observed at the end of exposure period at 100 µg/mL with and without irradiation.

B. CYTOTOXICITY WITH AND WITHOUT UV/VIS IRRADIATION

1. BAS 750 F

After treatment with the test substance BAS 750 F clear cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the 2nd Experiment in the absence and the presence of UV/VIS irradiation at least in the highest applied concentrations.

In detail, without UV/VIS irradiation, there was a decrease in the cell number at 100 µg/mL (EC_{50}: 73.6 µg/mL).

In addition, with UV/VIS irradiation, there was a decrease in the cell number at 100 µg/mL (EC_{50}: 69.8 µg/mL) in the 2nd Experiment.

Based on the EC_{50} values a PIF of 1.1 (no phototoxic potential) was obtained in the 2nd Experiment.

Table 5.2.7-1: Mean cytotoxicity of BAS 750 F

Test groups	Without UV/VIS irradiation			With UV/VIS irradiation		
		Relative cytotoxicity			Relative cytotoxicity	
	Mean OD_{corr}*	Mean [% of ctrl]	SD [%]	Mean OD_{corr}*	Mean [% of ctrl]	SD [%]
Vehicle [1]	0.423	100.0	4.2	0.421	100.0	6.9
0.5 µg/mL	0.415	98.2	2.2	0.443	105.2	10.2
1.0 µg/mL	0.444	105.0	4.7	0.430	101.9	10.5
2.2 µg/mL	0.416	98.5	4.3	0.434	103.1	11.9
4.6 µg/mL	0.446	105.5	7.3	0.421	99.9	16.2
10.0 µg/mL	0.424	100.3	3.5	0.402	95.4	15.6
21.5 µg/mL	0.421	99.7	4.9	0.417	98.9	15.4
46.4 µg/mL	0.428	101.3	6.9	0.372	88.4	13.6
100.0 µg/mL	0.001	0.3	0.2	0.001	0.3	0.2

[1] = DMSO 1% (v/v)

* = Mean OD_{corr}: Mean absorbance (test group) minus mean absorbance (blank)

2. Positive control

After treatment with the positive control chlorpromazine clear cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the 2nd Experiment in the absence and the presence of UV/VIS irradiation at least in the highest applied concentrations.

Table 5.2.7-2: Mean cytotoxicity of positive control (chlorpromazine)

Without UV/VIS irradiation				With UV/VIS irradiation			
		Relative cytotoxicity				Relative cytotoxicity	
Test groups	Mean OD_{corr}*	Mean [% of ctrl]	SD [%]	Test groups	Mean OD_{corr}*	Mean [% of ctrl]	SD [%]
Vehicle [1]	0.437	100.0	8.3	Vehicle [1]	0.426	100.0	3.8
1.9 µg/mL	0.430	98.2	5.2	0.03 µg/mL	0.425	99.7	2.2
3.8 µg/mL	0.439	100.5	4.6	0.05 µg/mL	0.414	97.3	3.4
7.5 µg/mL	0.479	109.5	6.3	0.10 µg/mL	0.416	97.7	3.2
15.0 µg/mL	0.499	114.2	4.4	0.20 µg/mL	0.386	90.7	3.4
30.0 µg/mL	0.261	59.6	8.1	0.40 µg/mL	0.141	33.2	3.2
60.0 µg/mL	0.003	0.6	0.7	0.80 µg/mL	0.009	2.0	1.4
90.0 µg/mL	0.006	1.4	2.7	1.60 µg/mL	0.004	0.8	0.6
180.0 µg/mL	0.005	1.2	0.8	3.20 µg/mL	0.006	1.4	0.3

[1] = DMSO 1% (v/v)

* = Mean OD_{corr}: Mean absorbance (test group) minus mean absorbance (blank)

In detail, without UV/VIS irradiation, there was a decrease in the cell number from 60 µg/mL (EC_{50}: 34.9 µg/mL) onward.

In addition, with UV/VIS irradiation, there was a decrease in the cell number at 0.4 µg/mL (EC_{50}: 0.3 µg/mL) and above in the 2nd Experiment.

Based on the EC_{50} values a PIF of 102.1 (phototoxic potential) was obtained in the 2nd Experiment.

III. DISCUSSION

According to the results of the present in vitro study, the test substance BAS 750 F did not lead to a relative reduction in viability of cells exposed in the presence versus absence of light. Cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed both in the absence and the presence of UV/VIS irradiation.

Due to the clearly negative results both in the 2nd Experiment and the Pretest no additional testing of the test substance is required.

The UV/VIS absorption spectrum of the test substance BAS 750 F shows that absorption occurs mainly in the UVC wavelength range, and to decreasing extents at wavelengths in the UVB, UVA and visible ranges; the molar absorption/extinction coefficient ε of BAS 750 F is between 10 - 1516 L x mol^{-1} x cm^{-1} at wavelengths in the range of about 475 - 290 nm (occasional marginal exceedance of the threshold are seen at higher wavelengths (ε range between 475-665 nm is -2.6 to 21.5 L x mol^{-1} x cm^{-1}; see Kroehl T., 2015; BASF DocID 2015/1183750). For phototoxicity, the relevant spectrum of the natural sunlight ranges from 290 to 700 nm with a power distribution, which can be closely reproduced using an artificial light source such as the H1-filtered Sol 500 simulator. This irradiation source emits light mainly in the UVA and visible range (which is usually associated with phototoxicity *in vivo*), and to a lesser extent in the UVB range (which is associated with high direct cytotoxicity and regarded to be of less relevance in the context of substance-induced phototoxicity [OECD Guideline 432]). However, during the validation of the test method, the experimental setting was demonstrated to be sufficient of detecting phototoxic effects also of chemicals typically absorbing in the UVB range, e.g. Chlorpromazine (absorption peak at 309 nm, used as positive control in this assay) or Amiodarone (18; in-house validation of the test method). Therefore, the experimental conditions - required by OECD Test guideline 432 (2004) and fully employed in the study reported here - were suitable to investigate the phototoxic potential of the test substance BAS 750 F.

The absorbance values of the vehicle controls were in the expected range with the accepted variability and, thus, fulfilled the acceptance criteria of this study.

The concentration-dependent decrease in the absorbance values induced by the positive control chlorpromazine and the calculated EC_{50} values as well as the PIF value clearly demonstrated the sensitivity of the test method with and without UV/VIS irradiation. The values were in the expected range with the accepted variability and, thus, fulfilled the acceptance criteria of this study.

IV CONCLUSION

Under the experimental conditions chosen, BAS 750 F has no phototoxic potential in the *in vitro* 3T3 NRU phototoxicity test after UV/VIS irradiation under in vitro conditions.

CA 5.3 Short-Term Toxicity

The short-term oral toxicity was investigated in Wistar rats and C57BL/6 J Rj mice (28-day and 90-day dietary) and in Beagle dogs (28-day and 90-day via oral capsules). In addition, BAS 750 F was tested in 4-wk dermal toxicity study in Wistar rats. No short-term inhalation toxicity studies were performed because BAS 750 F is not volatile (vapour pressure at 20 °C: 3.2 x 10^{-6} Pa; at 25°C: 6.5 x 10^{-6} Pa; at 55 °C: 3.1 x 10^{-4} Pa) and is not used as a fumigant or aerosol.

In rat studies, adverse findings were seen at the top dose levels tested in the 28-day and 90-day studies (4000 and 3600 ppm, corresponding to dose intakes in the range of 256 – 388 mg/kg bw/d). Body weight and body weight gain were reduced, and the liver was identified as target organ on the basis of slight or marginal changes in clinical chemistry parameters (decreased serum albumin and increased cholesterol, and after 90-day treatment also increased alkaline phosphatase (ALP)), increased liver weights associated with liver cell hypertrophy. Although there were no signs of liver cell degeneration, collectively the findings at the top dose level were considered to be adverse. The NOAEL of the 28-day study in Wistar rats was 1500 ppm (135 mg/kg bw/d in males and 138 mg/kg bw/d in females). The NOAEL of the 90-day study in Wistar rats was 1200 ppm (76 mg/kg bw/d in males and 91 mg/kg bw/d in females).

In C57BL/6 J Rj mice of the 28-day study, relative liver weights of female mice were increased (+33%) and associated with liver cell hypertrophy at 300 ppm (61 mg/kg bw/d). At the next higher dose level of 1000 ppm, food consumption and body weight gains were decreased in both sexes, and clinical parameter changes indicating impaired liver function were observed (increased alanine aminotransferase, decreased cholesterol in males, decreased albumin and glucose in females). Liver weights were markedly increased in both sexes and associated with liver cell hypertrophy, liver cell necrosis and hyperplasia of oval cells and of the bile duct. The NOAEL was 100 ppm (18.5 mg/kg bw/d) in females and 300 ppm (47.9 mg/kg bw/d in males. In the 90 day mouse study, signs of hemoconcentration (e.g. slightly increased red blood cell parameters in combination with increased platelet counts) were noted in males at the LOAEL of 250 ppm (58 mg/kg bw/d); however, there were no indication of hemoconcentration in any other mouse study. At this dose level, serum cholesterol decreases were seen in both sexes; moreover decreased albumin/protein ratios were noted in females at 250 ppm and in both sexes at 750 ppm. In male mice at and above 50 ppm and in female mice at and above 250 ppm dose-related increases in liver weights with liver cell hypertrophy were observed. Degenerative liver cell changes (liver cell necrosis and cytoplasmic alterations) were seen in males at 250 ppm and in both sexes at 750 ppm. The NOAEL in the 90-day mouse study was 50 ppm, corresponding to intakes of 11 mg/kg bw/d in males and 15 mg/kg bw/d in female mice.

The liver was also identified as target organ in oral capsule studies with Beagle dogs. In a 28-day range-finding study, dose levels of 250 mg/kg bw/d or higher were not tolerated by the dogs, which reacted to treatment with vomiting, reductions or significant delays in food intake and general clinical signs of impaired health. In the 90-day dog study, reduced or delayed food intake and isolated occurrences of vomiting occurred in both male and female dogs at the top dose level of 180 mg/kg bw/d. At this dose level, which was considered to be the LOAEL, body weight development was significantly reduced, clinical chemistry changes associated with altered liver function were noted (increased ALP and decreased total protein in both sexes, transient decrease of creatinine in female dogs) and liver weights were slightly increased. The NOAEL was 90 mg/kg bw/d.

In a 4-week dermal toxicity study in Wistar rats, no adverse signs of systemic toxicity or skin lesions were induced by BAS 750 F following semi-occlusive exposure of the skin for 6 hours/day, 5 days/week up to the limit dose level of 1000 mg/kg bw/d.

Table 5.3-1: Summary of short-term toxicity studies with BAS 750 F

Study **Batch / purity** **Dose levels (ppm)** **Intakes (mg/kg bw/d)**	**NOAEL (mg/kg bw/d)**	**Main adverse effect**	**Reference (BASF DocID)**
28-day diet **Wistar rat** Batch: L84-176 / 97.7% 0 – 500 – 1500 – 4000 ppm ♂: 0 – 47 – **135** – 388 mg/kg bw/d ♀: 0 – 47 – **138** – 334 mg/kg bw/d	♂: **135** ♀: **138**	4000 ppm: ↓ bw & bw gain (♂+♀) and food intake (♀) ↓ albumin (♀), ↑ cholesterol (♀) ↑ rel. liver wt (♀) ↑ Liver cell hypertrophy (♂+♀)	[redacted] 2015a (2014/1170747) [redacted] 2015a (2015/1249664)
28-day diet **C57BL/6 Rj mouse** Batch: COD-001662 / 95.5% 0 – 30 – 100 – 300 – 1000 ppm ♂: 0 – 4.8 – 15.5 – **47.9** – 128 mg/kg bw/d ♀: 0 – 5.8 – **18.5** – 61.0 – 145 mg/kg bw/d	♂: **47.9** ♀: **18.5**	≥300 ppm: ↑ rel. liver wt (+33%) & liver cell hypertrophy (♀) 1000 ppm: ↓ body weight gain & food intake (♂+♀) ↑ ALT (♂), ↓ cholesterol (♂),↓ glucose (♀). ↓ albumin (♀); ↑ marked liver wt (>+70%), hypertrophy, liver cell necrosis, oval cell & bile duct hyperplasia (♂+♀)	[redacted] 2014a (2013/1110704)
28-day oral capsule **Beagle dog (RF study)** Batch: COD-001880 / 98.6% ♂: d1-2: 300 or 1000 mg/kg bw/d d7-35/36: 125 or 250 mg/kg bw/d ♀: d1: 300 or 500 mg/kg bw/d d3-29/30: 125 or 250 mg/kg bw/d	No NOAEL	≥ 300 mg/kg bw/d: Severe clinical signs in all dogs (♂+♀) 250 (←1000 /500) mg/kg bw/d (♂/♀) 2-3 dogs/sex with delayed food intake; isolated vomiting, single occurrence of unsteady gait and poor general condition ↓ bw gain, ↑ cholesterol 1♀ with ↑ AST & ALT, ↓ terminal bw (-12%) ↑ liver wt (> +25%) with hypertrophy and eosinophilic change of hepatocytes 125 (← 300) mg/kg bw/d (♂+♀) 1♀ with delayed food intake 1♂ with vomit on 3 days ↓ bw gain (♂),↑ cholesterol ↓ terminal bw (-5 to -7%) ↑ liver wt (> +25%) with hypertrophy and eosinophilic change of hepatocytes	[redacted] 2015a (2014/1170748
90-day diet **Wistar rat** Batch: COD-001662 / 95.5% 0 – 400 – **1200** – 3600 ppm ♂: 0 – 27 – **76** – 256 mg/kg bw/d ♀: 0 – 30 – **91** – 314 mg/kg bw/d	♂: **76** ♀: **91**	3600 ppm: ↓ bw gain (♂: -11%, ♀: -20%) ↑ ALP (♂+♀),↑ cholesterol + ↓ albumin (♀) ↑ rel. liver wt (♂: +11%, ♀: +13%) ↑ min. hepatocellular hypertrophy (♂+♀)	[redacted] 2015b (2015/1198721)

Table 5.3-1: Summary of short-term toxicity studies with BAS 750 F

Study **Batch / purity** **Dose levels (ppm)** **Intakes (mg/kg bw/d)**	**NOAEL (mg/kg bw/d)**	**Main adverse effect**	**Reference (BASF DocID)**
90-day diet **C57BL/6 Rj mouse** Batch: COD-001740 / 98.8% 0 – 10 – 50 – 250 – 750 ppm ♂: 0 – 2 – **11** – 58 – 174 mg/kg bw/d ♀: 0 – 3 – **15** – 67 – 211 mg/kg bw/d	♂: **11** ♀: **15**	<u>≥ 250 ppm:</u> ↑ ♂ Hb, Ht, MCH, RBC & platelet counts (evidence of hemoconcentration) ↓ ♂+♀ CHOL ↑ liver wt (♂: +38%, ♀: +26%) & hypertrophy ↑ ♂ liver cell necrosis (gr. 1) in 2/10 males, & cytoplasmic alteration (gr.1) in 4/10 males <u>750 ppm:</u> ↓ bw gain (♂ consistent, ♀ transient) ↑ ♀ platelet ↓ ♀ rel. eosinophil counts ↓ ♂+ ♀ ALB/GLOB ratio ↑ liver wt (♂: +87%, ♀: +67%) ↑ ♂+♀ liver cell necrosis + cytopl. alteration	[redacted], 2015a (2014/1046542)
90-day oral capsule **Beagle dog** Batch: COD-001880 / 98.6% 0 – 15 – 90 – 180 mg/kg bw/d	♂: **90** ♀: **90**	<u>180 mg/kg bw/d</u> 1♂ and 3♀ with vomit and delayed food intake ↓ food intake (♀) (max. -7%, day 7) ↓ bw gain (d0-91: ♂: -49.4%, ♀: -59.6%), ↑ Alkaline phosphatase (3-mo, ♂+♀) ↓ protein (♂: 6-wk, ♀: 6-wk + 3-mo); ↓ creatinine (♀: 6-wk) ↑ Rel. liver wt (♂: +20%)	[redacted] 2015a (2015/1000530)
28-day dermal **Wistar rat** Batch: COD-001880 / 98.6% 0 – 100 – 300 – 1000 mg/kg bw/d	♂+♀ **1000**	<u>No adverse effects</u>	[redacted] 2015b (2014/1170751)

Comparison with CLP criteria:

Target organ toxicity, repeated exposure

Rats: In the 90-day rat study, treatment-related adverse effects occur after dietary exposure to 3600 ppm (corresponding to intakes of 256 mg/kg bw/d in males and 314 mg/kg bw/d in females), indicating the liver as the only target organ at the highest dose level tested. Treatment-related effects in male rats at 256 mg/kg bw/d were confined to slightly increased alkaline phosphatase (ALP); females with mean BAS 750 F intakes of 314 mg/kg bw/d had additional clinical-chemistry parameters marginally changed (increased cholesterol, reduced albumin). Slightly increased liver weight and liver cell hypertrophy of minimal severity were seen in both sexes. Overall, the observed clinical chemistry changes seen at 256 / 314 mg/kg bw/d might be considered statistically significant; however, they are marginal (just outside HCR) and are not considered to be severe effects by any means. By comparison, guidance values for STOT RE 2 classification for 3-mo treatment are 100 mg/kg bw/d, a much lower dose. On the basis of expert judgement and consideration of classification practice, the effects on the rat liver are not regarded to constitute serious toxicity and, therefore, do not warrant STOT RE classification.

In a 28-day dermal toxicity study, there were no adverse effects observed in Wistar rats that were exposed to the limit dose level of 1000 mg/kg bw/d. No STOT RE classification is required for dermal route exposure based on the results of this rat study.

Mouse: In a 28-day range finding study with C57BL/6JRj mice, clinical chemistry changes, liver weight increases with hypertrophy plus signs of liver cell degeneration were noted at 1000 ppm in both male and female animals (corresponding to intakes of 128 and 145 mg/kg bw/d, respectively). At the next lower dose level of 300 ppm (47.9 / 61 mg/kg bw/d in males/females), liver weight was increased in females (rel. bw. +33%) with associated minimal/slight hypertrophy but in the absence of clinical chemistry changes.

In the 90-day mouse study at 50 ppm, mild effects were noted in males (slightly increased liver weight associated with minimal grade hypertrophy), which were considered to reflect adaptation rather than adverse changes. In male mice at a dietary concentration of 250 ppm (corresponding to intakes of 58 mg/kg bw/d), some blood parameters were altered indicating hemoconcentration (RBC, MCH and platelets), but there were no corroborative histopathological changes in the spleen or the bone marrow that would indicate specific toxicity (also, there were no changes in hematological parameters in the 28-day mouse study at a 4-fold higher dose level). Serum cholesterol was reduced in both sexes. Liver weights were moderately increased (rel. bw ♂: +38%, ♀+26%), while marked liver weight increases were observed at the top-dose level of 750 ppm (rel. bw, ♂: +87%, ♀+67%). Hepatocellular hypertrophy was observed in males at and above 50 ppm and in females at and above 250 ppm. The hypertrophy correlated in both sexes with the observed weight increases. In males at 250 ppm, two of 10 male mice with single cell necrosis of minimal severity and four further male mice with cytoplasmic alteration of liver cells also of minimal severity were noted. The cytoplasmic alteration was seen in hypertrophied hepatocytes and was characterized by numerous hyaline inclusions and concentric whorls of cell organelles, and interpreted as an early degenerative change, potentially resulting in necrosis by continued exposure. At 750 ppm (174 mg/kg bw/d in males and 211 mg/kg bw/d), liver cell necrosis and/or cytoplasmic change of minimal-slight severity occurred in 8/10 males and 8/10 females.

Overall, some findings were observed in the 28-day and 90-day mouse study at dose levels below the respective guidance values. Even though some of the observed findings were severe findings (such as necrosis or findings interpreted to be early degenerative changes in males after 90 days at 250 ppm), importantly, they were not observed after longer treatment (18 months) at dose levels below the guidance values. Therefore, it is proposed not to classify BAS 750 F with STOT RE 2 (“May cause damage to organs through prolonged or repeated exposure”).

Dog: Beagle dogs were treated with BAS 750 F by oral capsule administration for 90 days at dose levels up to 180 mg/kg bw/d. At this dose level, which was considered to be the LOAEL, body weight development was significantly reduced, and clinical chemistry changes associated with altered liver function were noted (increased ALP and decreased total protein in both sexes, transient decrease of creatinine in female dogs) and liver weights were slightly increased without corresponding hypertrophy. The NOAEL was 90 mg/kg bw/d. The observed effects do not indicate the need for STOT RE classification.

CA 5.3.1 Oral 28-day study

Report: CA 5.3.1/1
[redacted], 2015 a
BAS 750 F - Repeated dose 28-day toxicity study in Wistar rats - Administration via the diet
2014/1170747

Guidelines: EPA 870.3050, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.7 No. L 142, OECD 407 (2008)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report: CA 5.3.1/2
[redacted] 2015 a
Amendment No. 1 to the report - BAS 750 F - Repeated dose 28-day toxicity study in Wistar rats - Administration via the diet
2015/1249664

Guidelines: EPA 870.3050, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.7 No. L 142, OECD 407 (2008)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Note: in the supplement section of the original report, the provided historical control data for the clinical chemistry parameter “cholesterol” was not up-to-date. The amendment contains an updated version of this data.

EXECUTIVE SUMMARY

In a dose-range finding study, BAS 750 F (batch L84-176; purity 97.7%) was administered via the diet to groups of 5 male and 5 female Wistar rats over a period of 4 weeks at concentrations of 0, 500, 1500 and 4000 ppm (corresponding to intakes in males/females of 0/0, 47/47, 135/138 and 388/334 mg/kg bw/d, respectively). Food consumption and body weights were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. In addition, the animals were daily examined for any clinically abnormal signs. Moreover, detailed clinical examinations in an open field were conducted prior to the start of the application period and weekly thereafter. Clinicochemical and hematological examinations were also performed towards the end of the application period. After the application period all animals were sacrificed and assessed by gross pathology. Organ weights were determined followed by histopathological examinations.
Dietary administration of BAS 750 F to Wistar rats for 28 days resulted in reduced body weight gain of about -30% in both male and females given 4000 ppm, resulting in body weights that were about 15% and 9% lower than the respective male and female control group mean weight at the end of the study. Females of the high dose group also should a consistent reduction of feed intake. The liver was identified as target organ. Female rats showed slightly decreased serum albumin and increased serum cholesterol levels.

Slight increases in relative liver weight were noted in females at 4000 ppm (+23%). Histopathological examination revealed the occurrence of centrilobular hepatocellular hypertrophy of minimal severity in these groups, reflecting the slight increases in liver weight. As there were no signs of adverse changes in histopathology in the liver, and the associated clinical pathology parameters were only slightly altered, effects on the liver can be considered borderline between an adaptive and an adverse response. The NOAEL of the study was considered to be 1500 ppm (corresponding to intakes of 135 / 138 mg/kg bw/d in male / female rats), based on reduced body weight gain in both sexes and reduced feed intake in female rats, and borderline effects suggesting slight impairment of liver function at the LOAEL of 4000 ppm (388 / 334 mg/kg bw/d in male / female rats).

(DocID 2014/1170747)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	L84-176
Purity / content:	97.7 ± 1%
Stability of test compound:	The test substance was stable over the study period (expiry date 01-Apr-2014).
2. Vehicle:	Rodent diet
3. Test animals:	
Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Sex:	male and female
Age:	34 ± 1 days at delivery; 42 ± 1 days at start of dosing
Weight at dosing (mean):	♂: ca. 158.6 ± 4.8 g; ♀: ca. 130.8 ± 6.1 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimatization period:	at least 8 days
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Group housing (5 animals/cage) housed in H-Temp (PSU) cages (TECNIPLAST Deutschland GmbH, Hohenpeißenberg, Germany, floor area about 2065 cm² with dust free wooden bedding.; wooden gnawing blocks as enrichment ((Typ NGM E-022), Abedd®, Lab. and Vet. Service GmbH, Vienna, Austria)

Environmental conditions:

Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 05-Jun-2012 to 12-Nov-2012 [in life phase: 13-Jun-2012 (start of treatment) to 12-Jul-2012 (necropsy)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 5 male and 5 female rats at dietary concentrations of 0, 500 (low dose), 1500 (intermediate dose), and 4000 ppm (top dose) for at least 28 days. The animals were assigned to the treatment groups by randomization on the basis of their body weights.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diet preparations were performed weekly.

Stability analyses performed in parallel to the study revealed that the test substance was stable in the diet for at least 35 days.

Concentration control analyses of all dose levels were determined at the beginning of the study. The homogeneity of the low dose and of the high dose was determined at the beginning of the study. No test article was determined in control diets. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC analysis with an external standard.

Table 5.3.1-1: Analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [ppm]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [ppm]	% of nominal concentration	Mean ± RSD
500 ppm	13-Jun-2012	27-Sep-2012	3	533.209	106.6	104.3 ± 2.5
			4	507.219	101.4	
			5	523.985	104.8	
1500 ppm	13-Jun-2012	27-Sep-2012	6	1587.884	105.9	
4000 ppm	13-Jun-2012	27-Sep-2012	7	3967.616	99.2	101.5 ± 4.8
			8	3931.210	98.3	
			9	4286.188	107.2	

Relative standard deviations of maximum 4.8% indicated the homogenous distribution of BAS 750 F in the diet preparations. The actual nominal test-item concentrations were in the range of 98.3 to 107.2% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Blood parameters with uni-directional changes	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided)
Urinalysis parameters (except pH, urine volume, specific gravity, color and turbidity	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians
Urine pH, volume, specific gravity, color and turbidity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians *Urine color and turbidity are not evaluated statistically.*
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly over a period of 1 day and calculated as mean food consumption in grams per animal and day.

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and food consumption, according to the following equation:

$$\text{Substance intake for } Day_x = \frac{FC_x \times C}{BW_x}$$

with FC_x as the mean daily food consumption (in g/day) on Day_x, C as the dose in ppm and BW_x as body weight on Dayx of the study (in g).

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any changes in volume.

5. Ophthalmoscopy

Not performed in this study

6. Functional observation battery (FOB)

Not performed in this study.

7. Motor activity measurement

Not performed in this study.

8. Hematology and clinical chemistry

On the day of necropsy (Day 29), blood was withdrawn in the morning from fasted, isoflurane-anesthetized animals from the retro-orbital plexus. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
✓	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis

For urinalysis on Day 27, the individual animals were transferred to metabolism cages (withdrawal of food and water) and urine was collected overnight. Urine samples were evaluated in a randomized sequence.

Urinalysis					
	Quantitative parameters		*Semi-quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilinogen
		✓	Glucose	✓	Sediment (microscop. examination)
		✓	Ketones		

10. Plasma concentration analysis

Blood samples (about 200 µL) were taken in the morning on Study Day 15 from all animals (non-fasted) by puncturing the retro-orbital venous plexus under isoflurane anesthesia. After addition of EDTA-K3 and centrifugation for preparation of plasma, the plasma samples were stored at -80 °C prior to LC-MS analysis for determination of the BAS 750 F concentration.

11. Sacrifice and pathology

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	#	adrenals	✓			jejunum (w. Peyer's patches)	✓			rectum
✓			aorta	✓	✓	#	kidneys	✓			salivary glands*
✓			bone marrow[§]	✓			lachrymal glands[%]	✓			seminal vesicles
✓	✓		brain	✓			larynx	✓			skin
✓			caecum	✓	✓	#	liver	✓			spinal cord (3 levels)[@]
✓			colon	✓			lung	✓	✓	#	spleen
✓			duodenum	✓			lymph nodes[#]	✓			sternum w. marrow
✓	✓		epididymides	✓			mammary gland (♀)	✓			stomach (fore- & glandular)
✓			esophagus	✓			muscle, skeletal	✓	✓		testes
✓			eyes (with optic nerve)	✓			nerve, peripheral (sciatic n.)	✓	✓		thymus
✓			femur (with joint)	✓			nose/nasal cavity[‡]	✓	✓	#	thyroid/parathyroid
			gall bladder	✓	✓		ovaries and oviduct**	✓			trachea
✓		✓	gross lesions	✓			pancreas	✓			urinary bladder
✓			Harderian gland	✓			pharynx	✓	✓		uterus
✓	✓		heart	✓			pituitary	✓			vagina
✓			ileum	✓			prostate				

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.

[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

One male rat (#018) from the high dose group (4000 ppm) died spontaneously during the blood sampling procedure on Day 29. The finding was not considered to be related to the test substance administration; pathological examination of the animal at necropsy and histopathological evaluation of examined organs did not reveal findings of concern. The reason of the premature death could not be determined.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals. Male rat #016 from the high dose group (4000 ppm) was found with “penis prolapse” during days 7-12 and subsequently recovered. Due to the transient occurrence, the finding was assessed to be spontaneous in nature and not related to treatment.

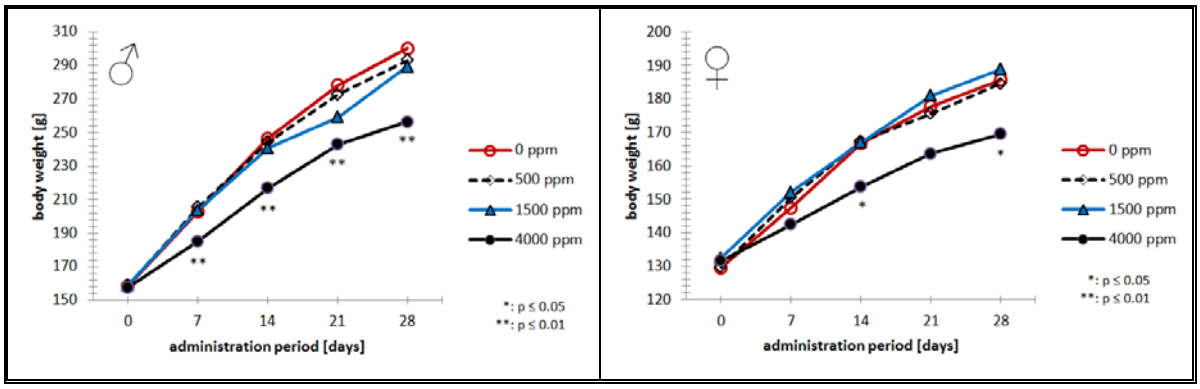
C. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight [see Figure 5.3.1-1]

Mean body weights were consistently lower in high-dose male animals (4000 ppm) throughout the treatment period; the body weight decrease attained statistical significance from study day 7 until study day 28 with a maximum of -15% on study day 28. In female animals administered 4000 ppm, mean body weights were significantly lower on Study Days 14 and 28 with a maximum of -8.7% on study day 28. The changes were regarded to be related to treatment and adverse.

No significant effects on mean body weights were observed in male and female animals of the low- and mid-dose groups (500 and 1500 ppm).

Figure 5.3.1-1: Body weight development



2. Body weight gain [see Table 5.3.1-2**]**

Mean body weight change values were significantly lower in high-dose male animals from study day 7 until study day 28 with a maximum by -38% on Study Day 7. At the end of the administration period, the mean body weight change value of the 4000 ppm male rats was 30% below the control group value. In female animals given 4000 ppm, the mean body weight change values were significantly lower from Study Days 14-28 onwards with a maximum by -40.6% on study day 14. At the end of the administration period, the mean body weight change value of high-dose females was 33% lower than the control group. Again, the changes at 4000 ppm were assessed as being related to treatment and adverse.

No obvious effects on body weight development were observable in male and female rats of the low- and mid-dose groups (500 and 1500 ppm).

Table 5.3.1-2: Body weight development

	Males				Females			
Dose level [ppm]	**0**	**500**	**1500**	**4000**	**0**	**500**	**1500**	**4000**
Body weight [g]								
- Day 0	158.8	158.9	159.1	157.8	129.5	129.8	132.5	131.6
- Day 28	300.2	293.0	289.3	**265.5****	185.6	184.5	188.8	**169.4***
Δ% (compared to control[#]		-2.4	-3.6	**-14.6**		-0.6	1.7	**-8.7**
Overall body weight gain (g)	141.4	134.1	130.2	**98.7****	56.1	24.8	56.3	**37.8****
Δ% (compared to control[#]		-5.2	-7.9	**-30.2**		-2.4	0.4	**-32.6**

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

Due to group housing with 5 rats/cage, data are available from only one cage per dose group and sex. In females, the relative changes of food intake were consistently decreased at 4000 ppm (-27%, -16%, -5.4% and -31% on Study Days 7, 14, 21 and 28). The parameter was neither affected in male animals of the same test group nor in male and female animals of low- and mid-dose test groups (500 and 1500 ppm).

2. Water consumption

No test substance-related, overt changes in water consumption were observed.

3. Test substance intake

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following table:

Table 5.3.1-3: Test substance intake

	Males				Females			
Dose level [ppm]	**0**	**500**	**1500**	**4000**	**0**	**500**	**1500**	**4000**
BAS 750 F (mg/kg bw/d)	-	47	135	388	-	47	138	334

E. BLOOD ANALYSES

1. Hematology

No statistically significant or treatment-related changes of hematological parameters were observed.

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related:

Females

- ↓ Albumin at 4000 ppm
- ↓ Total bilirubin (is thought to reflect increased metabolism and excretion of bilirubin secondary to induction of xenobiotic metabolizing enzymes, not adverse)
- ↑ Cholesterol at 1500 and 4000 ppm (marginally above the historical control range (HCR) at 4000 ppm)

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

- ↑ Total bile acids at 500 and 1500 ppm (no dose-response, within HCR)

Table 5.3.1-4: Clinical chemistry parameters (selected)

		Males				Females			
Dose level [ppm]		**0**	**500**	**1500**	**4000**	**0**	**500**	**1500**	**4000**
ALP	[µkat/l]	2.09	2.28	2.13	2.34	1.17	1.17	1.56	1.17
AST	[µkat/l]	1.62	1.74	1.62	1.70	1.75	1.42	1.81	1.67
GGT	[nkat/l]	0	0	0	0	0	0	1	3
Cholesterol	[mmol/l]	1.72	1.74	1.96	2.08	1.05	1.07	1.37*	**1.84****
						Historical control range: 0.95 - 1.81			
Albumin	[g/l]	37.77	37.32	37.38	37.77	39.19	39.36	38.37	**36.31****
Total protein	[g/l]	59.95	59.24	59.32	59.31	60.69	60.22	60.08	58.33
Urea	[mmol/l]	5.41	6.13	5.88	6.57	6.68	5.80	5.94	6.77
Calcium	[mmol/l]	2.60	2.61	2.62	2.58	2.55	2.53	2.55	2,50
Total bile acids	[µmol/l]	21.1	**44.9***	**38.7***	13.2	28.5	13.7	12.9	9.4
Total bilirubin	[µmol/l]	1.58	1.31	1.06	0.93	1.14	1.02	0.91	**0.39***

Historical control data from the test facility: 49 studies 28-day treatment of Wistar rats (Dec-2007 - Jul-2012)

3. Test substance concentration in blood plasma

BAS 750 F was detected in 4 control group samples each from the male and female control group, from which 2 values were comparable to the quantification limit of 5 ng/ml (♂#04, ♀#24). Higher BAS 750 F concentrations were found in samples ♂#03 (21.51 ng/ml) and ♀#21 (11.06 ng/ml). No quantifiable amounts of BAS 750 F were detectable in the remaining 6 control samples. Overall, the concentrations found in control samples are minute and of no relevance. According to the study director, their occurrence is most likely due to a system carry-over during the HLPC analysis of samples, resulting from the "sticky" behavior of BAS 750 F.

Plasma concentrations from male and from female rats were comparable at the low dose of 500 ppm, but were about 2.5-fold higher in females at 1500 ppm and 2-fold higher in females at 4000 ppm.

A three-fold increase in dietary concentration / test substance intake from the low dose to the mid dose (i.e. from 500 to 1500 ppm) corresponded to a roughly five-fold increase of plasma concentration in males and almost to a 12-fold increase in females. The about 2.7-fold dietary concentration increase from 1500 ppm to 4000 ppm corresponded to a 3.5-fold / 2.8-fold increase in male and females plasma concentration of BAS 750 F, respectively.

Table 5.3.1-5: BAS 750 F plasma concentration

Dose level [ppm]	500	1500	4000	500	1500	4000
Sampling on …	BAS 750 F plasma concentration [ng/ml]					
	Males			Females		
Day 15	182± 48	889 ± 458	3131 ± 1250	197 ± 66	2264 ± 594	6298 ± 1487

F. URINALYSIS

No statistically significant or treatment-related changes of urinalysis parameters were observed. Specifically, there were no change in pH, or increased incidence of animals with urinary casts or urinary transitional epithelial cells.

Table 5.3.1-6: Urinalyses (selected)

	Males				Females			
Dose level [ppm]	0	500	1500	4000	0	500	1500	4000
pH (Day 27)	6.3	6.2	6.3	5.7	6.0	5.7	6.1	5.8
Transitional epithelial cells	1	1	1	1	1	1	1	1
Casts	0	0	0	0	0	0	0	0

Statistics: Wilcoxon test (1-sided), * $p \leq 0.05$; * $p \leq 0.01$; mean severity: 1=few; 2=many

G. NECROPSY AND PATHOLOGY

1. Organ weight

Statistically significant changes of organ weights comprised reduced absolute kidney weights in high-dose group males (-12%**) and increased relative liver weights in high-dose group females (+23%**).

The following findings were considered to be treatment-related:

Terminal body weight: The mean body weight of males and females given 4000 ppm was slightly decreased (-11% and -7%), but the differences to the respective control group values did not attain statistical significance.

Liver: In female rats, relative liver weights were slightly increased at 4000 ppm (+23%, statistically significant), compared to mean control group values.

The following statistically significant organ weight findings were not considered to be treatment-related:

Kidney: The mean absolute kidney weight of male rats given 4000 ppm was slightly, albeit significantly decreased (-12%). This weight reduction was considered to be unspecific and related to the general body weight decrease observed in high-dose group males (-11%). The mean relative kidney weight of these animals was comparable to the control group value (0.752 vs. 0.761% rel. bw, corresponding to a difference of -1%).

Table 5.3.1-7: Organ weight findings (statistically significant)

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	272.54				168.94			
	500	266.64	(-2)			169.08	(±0)		
	1500	265.34	(-3)			172.96	(+2)		
	4000	241.65	(-11)			157.92	(-7)		
Kidneys (g)	0	2.072		0.761		1.326		0.785	
	500	2.002	(-3)	0.752	(-1)	1.416	(+7)	0.837	(+7)
	1500	2.052	(-1)	0.773	(+2)	1.408*	(+6)	0.814	(+4)
	4000	**1.815***	**(-12)**	0.752	(-1)	1.308	(-3)	0.827	(+5)
Liver (g)	0	7.350		2.693		4.460		2.644	
	500	7.304	(-1)	2.736	(+1)	4.822	(+8)	2.840	(+7)
	1500	7.492	(+2)	2.818	(+5)	4.936	(+11)	2.849	(+8)
	4000	6.875	(-6)	2.844	(+6)	5.116	(+15)	**3.241****	**(+23)**

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

2. Gross pathology

All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

Histopathology of control group and 4000 ppm group tissues was carried out for the adrenal glands, the kidneys, the liver, the spleen and the thyroid glands. Additionally, histopathology was performed on all animals with gross lesions.

The following findings were considered to be treatment-related:

Liver: At the high-dose level of 4000 ppm, centrilobular hypertrophy of minimal severity (grade 1) was diagnosed in all of the 5 female rats examined, which corresponded to increases in relative liver weights. There were also 2 male rats with liver cell hypertrophy of minimal grade severity, but there was no association to a significant liver weight increase or to clinical chemistry changes; therefore the treatment-relationship of the observed hypertrophy in male rats is questionable.

No histopathological findings at all were detected upon examination of the adrenal cortex, the adrenal medulla, the spleen or the thyroid glands.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 5.3.1-8: Histopathology findings

	Males				Females			
Dose level [ppm]	**0**	**500**	**1500**	**4000**	**0**	**500**	**1500**	**4000**
No. of animals	5	5	5	5	5	5	5	5
LIVER								
examined	5			5	5		1	5
Infiltration, lymphoid	5			5	5			5
Constriction, focal[1]							1	
Hypertrophy, centrilobular				2				5
Grade 1				2				5
KIDNEYS								
examined	5	1	1	5	5			5
Tubules, basophilic	3		1	3	5			4
Cyst(s) [1]		1	1					
Mineralization, medulla					5			5
Dilation, renal pelvis								1
EPIDIDYMIDES								
examined	1			1				
Granuloma, spermatogenic[1]	1			1				

[1] Gross necropsy correlates, epididymides: focus; kidney: cyst; liver: focal constriction

III. CONCLUSION

Dietary administration of BAS 750 F to Wistar rats for 28 days resulted in reduced body weight gain of about -30% in both male and females given 4000 ppm, resulting in body weights that were about 15% and 9% lower than the respective male and female control group mean weights at the end of the study. Females of the high dose group also showed a consistent reduction of feed intake. The liver was identified as target organ. Female rats given 4000 ppm showed slightly decreased serum albumin and increased serum cholesterol levels. Slight increases in relative liver weight were noted in females at 4000 ppm (+23%). Histopathological examination revealed the occurrence of centrilobular hepatocellular hypertrophy of minimal severity in all females of the high-dose group and in two male rats. As there were no signs of adverse changes in histopathology in the liver, and the associated clinical pathology parameters were slightly altered only in females, effects on the liver can be considered borderline between an adaptive and an adverse response in females and non-adverse in males. The NOAEL of the study was considered to be 1500 ppm (corresponding to intakes of 135 / 138 mg/kg bw/d in male / female rats), based on reduced body weight gain in both sexes and reduced feed intake in female rats, and borderline effects suggesting slight impairment of liver function at the LOAEL of 4000 ppm (388 / 334 mg/kg bw/d in male / female rats).

Report:	CA 5.3.1/3 [redacted] 2014 a BAS 750 F - Repeated-dose 28-day toxicity study in C57BL/6 Rj mice - Administration via the diet 2013/1110704
Guidelines:	OECD 407 (2008), EPA 870.3050, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.7 No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (batch COD-001662, purity: 95.5%) was administered via the diet to groups of 5 male and 5 female C57BL/6 J Rj mice at concentrations of 0, 30, 100, 300 and 1000 ppm over a period of 4 weeks (corresponding to intakes in males/females of 0/0, 4.8/5.8, 15.5/18.5, 47.9/61.0 and 128/145 mg/kg bw/d, respectively) with the aim to determine appropriate dose levels for a subsequent 13-week dietary study. Dietary administration of BAS 750 F to C57BL/6 J Rj mice for 4 weeks caused reduced food intake, reduced body weight and body weight gain at 1000 ppm in both sexes. There were no treatment-related effects on hematology parameters. Treatment-related, adverse changes in clinical chemistry parameters were observed at 1000 ppm and comprised increased ALT and decreased serum cholesterol serum levels in males and reduced serum glucose and albumin levels in females. Body weights were decreased by 13% in male and by 6% in female mice given 1000 ppm at the end of the study. Liver weight increases associated with hepatocellular hypertrophy noted in males at concentrations of 30, 100 and 300 ppm were considered to be adaptive changes. In females, the extent of the liver weight increase at 300 ppm (+33%) was considered to be potentially adverse; however, it was only associated with hypertrophy (no clinical chemistry changes or liver histopathology). At 1000 ppm liver weight increases were marked in both sexes (>70%) and not only correlated with hypertrophy but also associated with clear hepatotoxicity: liver cell necrosis and hyperplasia of oval cells and bile ducts in both sexes. Under the study conditions, a NOAEL of 300 ppm (47.9 mg/kg bw/d) is derived for male mice and a NOAEL of 100 ppm (18.5 mg/kg bw/d) is derived for female mice.

(DocID 2013/1110704)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	COD-001662
Purity / content:	95.5%
Stability of test compound:	The test substance was stable over the study period (expiry date 31-Aug-2013).
2. Vehicle:	Rodent diet
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6 J Rj
Sex:	male and female
Age:	42 - 44 days at delivery; 48 - 50 days at start of dosing
Weight at dosing (mean):	♂: ca. 21.1 ± 0.7 g; ♀: ca. 17.9 ± 0.7 g
Source:	Janvier Labs SAS, Le Genest Saint Isle, France
Acclimatization period:	6 days
Diet:	Ground Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Group housing (5 animals/cage) housed in polycarbonate cages type MIII with mesh wire tops (Becker & Co, Castrop-Rauxel, Germany), dust free wooden bedding; enrichment: mouse tunnel and nest building material (Plexx B.V., Elst, Netherlands)
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 15-Jan-2013 to 07-Jun-2013 [in life phase: 21-Jan-2013 (start of treatment) to 19-Feb-2013 (necropsy)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 5 male and 5 female C57BL/6 J Rj mice at dietary concentrations of 0, 30 (low dose), 100 (low-intermediate dose), 300 (high-intermediate dose) and 1000 ppm (top dose) for at least 28 days. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer.

Analyses performed prior to the start of the administration period revealed that the test-substance was stable in the diet for at least 32 days.

Concentration control analyses of all dose levels were determined at the beginning of the study. The homogeneity of the low dose and of the high dose was determined at the beginning of the study. No test article was determined in control diets. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC analysis with an external standard.

Table 5.3.1-9: Analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [ppm]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [ppm]	% of nominal concentration	Mean ± RSD
30 ppm	18-Jan-2013	24-Jan-2013	3	90.6	90.6	93.3 ± 3.5
			4	96.9	96.9	
			5	92.4	92.4	
100 ppm	18-Jan-2013	24-Jan-2013	6	97.188	97.2	–
300 ppm	18-Jan-2013	24-Jan-2013	7	293.502	97.8	–
1000 ppm	18-Jan-2013	24-Jan-2013	8	987.211	98.7	98.0 ± 0.9
			9	970.202	97.0	
			10	982.241	98.2	

Relative standard deviations of maximum 3.5% indicated the homogenous distribution of BAS 750 F in the diet preparations. The actual nominal test-item concentrations were in the range of 90.6 to 98.7% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Blood parameters with uni-directional changes	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly over a period of 1 day and calculated as mean food consumption in grams per animal and day.

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and food consumption, according to the following equation:

$$\text{Substance intake for Day}_x = \frac{FC_x \times C}{BW_x}$$

with FCx as the mean daily food consumption (in g/day) on Day_x, C as the dose in ppm and BWx as body weight on Day_x of the study (in g).

4. Water consumption

Drinking water consumption was determined on study days 7, 14, 21, and 28 and calculated as mean water consumption in grams per mouse and day.

5. Ophthalmoscopy

Not performed in this study.

6. Functional observation battery (FOB)

Not performed in this study.

7. Motor activity measurement

Not performed in this study.

8. Hematology and clinical chemistry

Blood was withdrawn in the morning from fasted, isoflurane-anesthetized animals from the retro-orbital plexus (for hematology) or after decapitation (for clinical chemistry). The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)		Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (GGT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis

Not performed in this study.

10. Plasma concentration analysis

Not performed in this study.

11. Sacrifice and pathology

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	✓	adrenals	✓			jejunum (w. Peyer's patches)	✓			rectum
✓			aorta	✓	✓	#	kidneys	✓			salivary glands*
✓			bone marrow[§]	✓			lachrymal glands[%]	✓			seminal vesicles
✓	✓		brain	✓			larynx	✓			skin
✓			caecum	✓	✓	✓	liver	✓			spinal cord (3 levels)[@]
✓			colon	✓			lung	✓	✓	#	spleen
✓			duodenum	✓			lymph nodes[#]	✓			sternum w. marrow
✓	✓		epididymides	✓			mammary gland (♀+♂)	✓			stomach (fore- & glandular)
✓			esophagus	✓			muscle, skeletal	✓	✓		testes
✓			eyes (with optic nerve)	✓			nerve, peripheral (sciatic n.)	✓	✓		thymus
✓			femur (with joint)	✓			nose/nasal cavity[‡]	✓		#	thyroid/parathyroid
✓			gall bladder	✓	✓		ovaries and oviduct**	✓			trachea
✓		✓	gross lesions	✓			pancreas	✓			urinary bladder
✓			Harderian gland	✓			pharynx	✓	✓		uterus (weight incl. cervix)
✓	✓		heart	✓			pituitary	✓			vagina
✓			ileum	✓			Prostate[&]				

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.

[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III, [&]with coagulating gland

The spleen was additionally evaluated according to the following parameters:

- Changes of the cellularity of PALS, lymphoid follicles, marginal zone, red pulp
- Altered cellular composition of follicles
- Altered number of germinal centers

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

No animal died prematurely in the study.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Compared to controls, mean body weight of males in test group 4 (1000 ppm) was significantly decreased on study days 7 (-12.7%), 14 (-7.4%), 21 (-10.5%) and on study day 28 (-13.1%). The mean body weight of females in test group 4 (1000 ppm) was significantly decreased on study day 7 (-13.8%), on study day 14 (-6.6%) and on study day 28 (-6.2%).

In males of test group 3 (300 ppm) the mean body weight was significantly increased on study day 7 (+6.5%). In females of test group 2 (100 ppm), the mean body weight was significantly increased on study day 28 (+5.4%). Both differences to control values were considered incidental. There were no other significant or relevant body weight effects in male or female treatment groups 1-3 (30, 100 or 300 ppm).

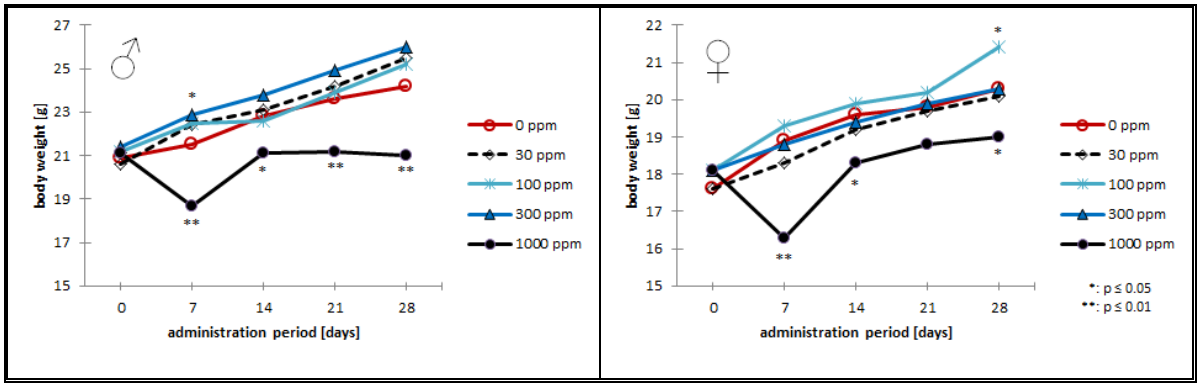
Table 5.3.1-10: Body weight and body weight gain

	Males					Females				
Dose level [ppm]	**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
Body weight [g]										
- Day 0	20.9	20.6	21.2	21.4	21.1	17.6	17.6	18.1	18.1	18.1
- Day 28	24.2	25.5	25.2	26.0	**21.0****	20.3	20.1	21.4*	20.3	**19.0***
Δ% (compared to control[#]		5.5	4.3	7.4	**-13.1**		-0.9	5.4	0.4	**-6.2**
Overall body weight gain (g)	3.3	4.9	4.0	4.6	**-0.1****	2.6	2.5	3.2	2.2	**0.9****
Δ% (compared to control[#]		47	21	37	**-103**		-6	22	-15	**-65**

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided);# values are rounded

Male and female mice of test group 4 (1000 ppm) lost weight during the first treatment week, which they could regain in treatment week 2. Thereafter, body weight development stagnated in males, while weight gain in females was comparable to that of the control group. At the end of the 4-week treatment period, the mean weight of males given 1000 ppm was more or less the same as on day 0, while the mean weight of the control group males had increased by 16% during this time. Females given 1000 ppm had increased their initial weight by 5% while control group females had gained 10% weight within 4 weeks. The weight changes of the top-dose group males and females were considered treatment-related and potentially adverse, but could also partly have been caused by reduced palatability of the feed during the first treatment week.

Figure 5.3.1-2: Body weight development



D. FOOD AND WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

Food consumption was consistently decreased in male and female animals given 1000 ppm throughout the entire study period, with maximum decreases seen on day 7 in males (-41.9%) and on study day 14 in females (-45%), compared to the control group; the decreased food intake at 1000 ppm was assessed as treatment-related. In males of test groups 30, 100 or 300 ppm, food consumption was increased to a similar extent on days 7, 21 and 28, while a decrease of similar degree was seen in these test groups on day 14. In females administered 30, 100 or 300 ppm, feed intake was usually slightly lower than the control value, but still within the normal range. No dose-related change was apparent in both sexes up to 300 ppm. Therefore, the changes in food consumption observed in male and female test groups 1-3 were assessed as incidental.

Table 5.3.1-11: Food consumption

	Males				Females			
(ppm)	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
0	3.1 g	4.4 g	3.0 g	3.8 g	3.8 g	4.0 g	3.9 g	4.7 g
30	3.7 g	3.7 g	3.8 g	4.2 g	3.5 g	3.7 g	3.5 g	4.1 g
100	3.7 g	3.3 g	3.5 g	4.2 g	3.5 g	3.6 g	3.7 g	4.2 g
300	3.7 g	4.1 g	3.7 g	4.1 g	3.8 g	3.8 g	3.9 g	4.5 g
1000	**1.8 g**	3.6 g	**2.3 g**	**2.8 g**	**2.7 g**	**2.2 g**	**2.3 g**	**3.2 g**

2. Water consumption

Water consumption fluctuated during the study period in all groups including controls. No clear test substance-related, adverse change was observed.

Table 5.3.1-12: Water consumption

	Males				Females			
(ppm)	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
0	3.2 g	4.6 g	3.9 g	3.9 g	3.1 g	3.8 g	3.8 g	3.6 g
30	4.2 g	3.6 g	4.1 g	3.5 g	3.5 g	3.7 g	3.7 g	3.3 g
100	3.5 g	2.7 g	3.6 g	3.8 g	3.5 g	3.5 g	4.3 g	3.7 g
300	3.6 g	3.6 g	3.6 g	3.6 g	3.4 g	3.2 g	2.8 g	3.3 g
1000	2.9 g	3.6 g	3.3 g	2.8 g	2.7 g	4.1 g	3.3 g	2.9 g

3. Test substance intake

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following table:

Table 5.3.1-13: Test substance intake

	Males				**Females**			
Dose level [ppm]	**30**	**100**	**300**	**1000**	**30**	**100**	**300**	**1000**
BAS 750 F (mg/kg bw/d)	4.8	15.5	47.9	128.2	5.8	18.5	61.0	145.2

E. BLOOD ANALYSES

1. Hematology

Red blood cell parameters: Statistically significant changes among the red blood cell parameters were found at 1000 ppm and comprised a decrease of relative reticulocytes in males, which was within the historical control range (HCR), and altered *calculated* RBC values (decreased mean corpuscular volume MCV in both sexes and increased mean corpuscular hemoglobin concentration MCHC in males), without any change of the *measured* parameters (red blood cell count, hemoglobin and hematocrit). Therefore, the alterations of the red blood cell parameters were assessed as incidental and not treatment related.

White blood cell parameters: In males given 300 ppm, total white blood cell (WBC) counts were higher compared to controls, but values were not dose-dependently changed. Increased absolute monocyte counts (at and above 300 ppm) and increased relative monocyte counts (at and above 100 ppm) were all within HCR, apart from the relative monocyte count value in males given 1000 ppm which was marginally above this range. Absolute and relative monocyte counts were also higher compared to controls in females of all dose groups, but the values were not dose-dependently changed. In females given 300 ppm relative basophil counts were increased, but again this parameter was not dose-dependently changed. In females of test group 4 (1000 ppm) relative neutrophil counts were decreased. Although this relative parameter was below the HCR, corresponding absolute neutrophil counts in this test group were not significantly changed and within the HCR. Overall, all mentioned parameters of the white blood cell counts were regarded as incidental and not treatment-related.

Table 5.3.1-14: Hematology parameters – 28-day mouse study

	Males					Females				
Dose level [ppm]	**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
Red blood cell count [tera/l]	11.22	10.60	10.43	10.21	11.06	10.47	10.28	10.22	10.43	10.73
Hemoglobin [mmol/l]	9.8	9.5	9.4	9.1	9.9	9.4	9.4	9.3	9.4	9.6
Hematocrit [l/l]	0.496	0.473	0.470	0.461	0.474	0.466	0.454	0.459	0.465	0.464
MCV [fl]	44.2	44.6	45.0	45.2	42.8**	44.5	44.2	44.9	44.6	43.3**
MCHC [mmol/l]	19.72	20.06	19.87	19.81	20.88**	20.21	20.64	20.37	20.36	20.51
Reticulocytes [%]	3.0	3.0	2.8	3.1	2.1**	2.5	2.5	3.0	2.3	2.3
	Historical control range: 0.6 – 2.9%									
Platelets [giga/l]	1536	1522	1470	1414	1566	1294	1296	1257	1428	1344
Total WBC [giga/l]	5.78	4.29	4.51	7.96*	4.83	3.72	3.75	5.29	3.75	5.67
Neutrophil counts abs. [giga/l]	0.43	0.32	0.43	0.56	0.30	0.42	0.52	0.44	0.35	0.34
						Historical control range:0.31 – 1.34				
Monocytes abs. [giga/l]	0.02	0.02	0.07	0.09**	0.11*	0.02	0.07**	0.18**	0.15**	0.17**
	Historical control range: 0.02 – 0.13									
Eosinophil counts abs. [giga/l]	0.06	0.03	0.04	0.09	0.10	0.04	0.06	0.06	0.04	0.06
Basophil counts abs. [giga/l]	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Monocytes rel. [%	0.3	0.5	1.4**	1.1**	2.6*	0.5	1.8*	4.4**	3.9**	3.5**
	Historical control range: 0.4 – 2.5									
Basophil counts rel. [%]	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2*	0.1
Neutrophil counts rel. [%]	7.4	7.7	9.0	7.2	7.3	11.9	14.2	8.6	9.2	6.0**
						Historical control range: 14.1 – 30.4				

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon (two-sided)

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related (see Table 5.3.1-15):

Males

↑ Alanine aminotransferase at 1000 ppm (**adverse**).

↓ Cholesterol 30, 100 and 300 ppm (non-adverse) and 1000 ppm (**adverse**)

Decreased cholesterol in males at 30, 100 and 300 ppm was the only clinical-chemistry change considered to be treatment-related, therefore this change was not considered to be adverse at these dose levels.

Females

↓ Albumin at 100 and 300 ppm (non-adverse) and 1000 ppm (**adverse**)

↓ Glucose at 1000 ppm (**adverse**)

Decreased albumin in females at 100 and 300 ppm was the only clinical-chemistry change considered to be treatment-related; therefore this change was not considered to be adverse at these dose levels.

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

↑ Alkaline phosphatase at 100, 300 and 1000 ppm (within HCR)

↓ Total protein, albumin, and globulin at 100 and 300 ppm (not dose-dependent)

↓ Triglycerides at 100 ppm (not dose-dependent)

↓ Total bilirubin at 30, 100, 300 and 1000 ppm (not dose-dependent)

Females

↓ Total protein and globulin at 100, 300 and 1000 ppm (not dose-dependent)

↓ Cholesterol 30, 100, 300 ppm and 1000 ppm (not dose-dependent)

↓ Triglycerides at 30, 100, 300 ppm and 1000 ppm (not dose-dependent)

Table 5.3.1-15: Clinical chemistry parameters – 28-day mouse study

		Males					Females				
Dose level [ppm]		**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
ALT		0.83	0.81	0.97	1.14	**2.08***					
AST	[U/l]	5.18	4.40	4.83	5.11	5.72	5.70	5.86	5.48	4.87	6.46
ALP	[µkat/l]	1.56	1.70	1.82*	1.87*	2.07*	2.28	2.07	2.11	1.98	2.53
		Historical control range: 1.12 - 2.29									
Cholesterol	[mmol/l]	2.53	2.01*	1.51**	1.24**	**0.69****	2.04	1.32**	0.87**	0.68**	0.83**
Triglycerides	[mmol/l]	0.98	0.85	0.50*	0.68	0.66	0.82	0.48**	0.39**	0.30**	0.63*
Total protein	[g/l]	49.55	49.89	45.88*	44.56**	49.67	48.29	46.13	43.41*	42.71*	43.22*
Albumin	[g/l]	31.17	31.33	28.63**	27.87**	31.31	31.85	30.64	28.67*	28.16*	**27.69***
Globulin	[g/l]	18.38	18.56	17.25**	16.69**	18.37	16.44	15.48	14.74*	14.55**	15.53
Urea	[mmol/l]	12.22	12.44	14.15	12.28	13.14	11.32	11.61	10.23	10.94	10.05
Total bilirubin	[µmol/l]	1.46	0.91*	0.52**	0.63**	0.67	0.88	0.95	0.85	0.47	0.64
Glucose	[mmol/l]	7.54	7.21	6.96	7.29	5.70	7.79	7.90	6.83	5.95	**5.25****
Potassium	[mmol/l]	6.94	6.01	6.88	7.14	6.21	6.21	5.94	5.72	5.48	6.57
Calcium	[mmol/l]	2.42	2.36	2.35	2.34	2.38	2.29	2.26	2.27	2.26	2.35
Inorg. Phos.	[mmol/l]	2.82	2.97	3.25	2.89	3.10	2.83	2.92	3.02	3.01	3.19

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)

3. Test substance concentration in blood plasma

Plasma kinetics were not determined in this study.

F. NECROPSY AND PATHOLOGY

1. Organ weight

The following statistically significant alterations of organ weight parameters were considered treatment-related and organ-specific (not secondary to body weight change):

Males

- ↓ Terminal body weight 1000 ppm (-12%*); **adverse**
- ↑ Liver weight 30 (rel. +12%), 100 (rel. +18%), 300 ppm (rel. +22%) - outside HCR at and above 100 ppm and correlated with hepatocellular hypertrophy; **adaptive**
- ↑ Liver weight 1000 ppm (+71%) - correlated with hepatocellular hypertrophy and liver cell necrosis; **adverse**

Females

- ↓ Terminal body weight at 1000 ppm (-5%*); **adverse**
- ↑ Liver weight 300 ppm (+33%) - above HCR and correlated with hepatocellular hypertrophy without clinical chemistry changes; **potentially adverse**
- ↑ Liver weight 1000 ppm (+72%) - correlated with hepatocellular hypertrophy and liver cell necrosis; **adverse**
- ↑ Thymus weight at 1000 ppm - above HCR (control was below HCR); **adverse**

The following statistically significant changes of organ weight parameters were assessed to be incidental, i.e. not considered treatment-related, or treatment-related but secondary to body weight reductions:

Males

- ↑ Brain weight, abs. at 300 ppm, rel. at 1000 ppm (no dose-related change at 300 ppm; effect at 1000 ppm secondary to body weight decrease)
- ↓ Heart weight at 1000 ppm (abs: secondary to body weight decrease; rel.: no significant change)
- ↓ Kidney weight at 1000 ppm (abs: secondary to body weight decrease; rel.: no significant change)
- ↓ Spleen weight at 1000 ppm (abs: secondary to body weight decrease; rel.: no significant change; no clear dose-response)

Females

- ↑ Liver weight 100 ppm (relative weight not significantly increased, within HCR and not correlated with hepatocellular hypertrophy)
- ↑ Thymus weight at 100 and 300 ppm (within HCR)
- ↓ Adrenal weight at 1000 ppm (abs: secondary to body weight decrease; rel.: no significant change)
- ↓ Ovary weight at 1000 ppm (no clear dose-response)

Table 5.3.1-16: Organ weights

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	20.875				17.56			
	30	21.48	(+3)			17.68	(+1)		
	100	21.54	(+3)			18.22	(+4)		
	300	22.37	(+7)			17.44	(-1)		
	1000	**18.32***	**(-12)**			**16.66***	**(-5)**		
Adrenal glands (mg)	0	4.22		0.020		6.98		0.040	
	30	4.04	(-4)	0.019	(-4)	6.48	(-7)	0.037	(-8)
	100	4.52	(+7)	0.021	(+8)	6.56	(-6)	0.036	(-9)
	300	3.96	(-6)	0.018	(-10)	6.74	(-3)	0.039	(-3)
	1000	4.90	(+16)	0.027	(+38	**6.04***	**(-13)**	0.036	(-9)
Brain (mg)	0	438.8		2.093		451.6		2.573	
	30	459.8	(+5)	2.143	(+2)	454.4	(+1)	2.571	(±0)
	100	451.0	(+3)	2.095	(±0)	449.0	(-1)	2.462	(+-4)
	300	**459.8***	**(+5)**	2.056	(-2)	450.0	(±0)	2.580	(±0)
	1000	428.8	(-2)	**2.342***	**(+12)**	435.6	(-4)	2.615	(+2)
Epididymides (mg)	0	48.6		0.230		13.36		0.076	
	30	55.0	(+13)	0.256	(+11)	11.78	(-12)	0.067	(-13)
Ovaries (mg)	100	50.4	(+4)	0.233	(+1)	9.88	(-26)	0.054	(-28)
	300	53.0	(+9)	0.237	(+3)	12.08	(-10)	0.069	(-9)
	1000	46.6	(-4)	0.255	(+11)	**9.62***	**(-28)**	**0.058***	**(-24)**
Heart (mg)	0	137.4		0.666		117.2		0.669	
	30	142.2	(+3)	0.662	(-1)	122.8	(+5)	0.693	(+4)
	100	137.8	(±0)	0.639	(-4)	136.4	(+16)	0.751	(+12)
	300	150.6	(+10)	0.674	(+1)	121.2	(+3)	0.694	(+4)
	1000	**108.4****	**(-21)**	0.593	(-11)	112.6	(-4)	0.675	(+1)
Kidneys (mg)	0	273.8		1.294		238.8		1.360	
	30	287.8	(+5)	1.338	(+3)	236.8	(-1)	1.340	(-1)
	100	283.8	(+4)	1.319	(+2)	250.5	(+5)	1.357	(±0)
	300	300.2	(+10)	1.342	(+4)	244.0	(+2)	1.399	(±0)
	1000	**228.8***	**(-16)**	1.250	(-3)	223.2	(-7)	1.340	(-1)
Liver (mg)	0	864.6		4.067		784.8		4.469	
	30	**978.8***	**(+13)**	**4.552***	**(+12)**	827.4	(+5)	**4.680****	**(+5)**
	100	**1037.0****	**(+20)**	**4.818***	**(+18)**	**944.6***	**(+20)**	5.185	(+16)
	300	**1110.6***	**(+28)**	**4.958***	**(+22)**	**1035.8****	**(+32)**	**5.939****	**(+33)**
	1000	**1276.2****	**(+48)**	**6.959***	**(+71)**	**1282.2****	**(+63)**	**7.696****	**(+72)**
	HCR	*854 - 1006*		*3.716 – 4.603*		685.0 – 856.2		4.172 – 5.447	
Spleen (mg)	0	48.8		0.222		49.8		0.283	
	30	45.4	(-7)	0.211	(-5)	48.0	(-4)	0.272	(-4)
	100	43.0	(-12)	0.199	(-10)	54.6	(+10)	0.300	(+6)
	300	63.6	(+30)	0.284	(+28)	47.2	(-5)	0.271	(-4)
	1000	**33.2***	**(-32)**	0.181	(-19)	41.4	(-17)	0.249	(-12)
Thymus (mg)	0	32.84		0.157		36.40		0.207	
	30	38.12	(+16)	0.178	(+13)	43.88	(+21)	0.249	(+20)
	100	29.72	(-10)	0.138	(-12)	**48.22****	**(+32)**	**0.265***	**(+28)**
	300	40.34	(+23)	0.181	(+15)	**49.04****	**(+35)**	**0.281****	**(+36)**
	1000	31.52	(-4)	0.171	(+9)	**58.88****	**(+62)**	**0.354****	**(+71)**
	HCR					39.2 – 55.0		0.224 – 0.315	

Table 5.3.1-16: Organ weights

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Testes (mg)	0	175.4		0.828		115.8		0.665	
/	30	190.2	(+8)	0.886	(+7)	88.2	(-24)	0.501	(-25)
Uterus (mg)	100	188.6	(+8)	0.874	(+6)	87.4	(-25)	0.49	(+28)
	300	185.6	(+6)	0.829	(±0)	93.0	(-20)	0.534	(-20)
	1000	173.4	(-1)	0.948	(+14)	69.4	(-40)	0.417	(-37)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

2. Gross pathology

The kidney of 1 female given 100 ppm was found with a cyst, which was associated with hydronephrosis in this animal; the finding was assessed as incidental. A liver focus was found in 1 male given 300 ppm, and in 2 males and females each given 1000 ppm.

3. Histopathology

The **liver** was identified as target organ. Hepatocellular hypertrophy was observed in all treated males in a centrilobular pattern and more diffuse in all females of test groups 3 and 4 (300 and 1000 ppm, respectively). The hypertrophy correlated in both sexes with the observed weight increases. At 1000 ppm, the hypertrophy was associated with multifocal hepatocellular necrosis in 4/5 male and 3/5 female animals correlating with foci observed macroscopically on the liver. Additionally oval cell proliferation was noted in 2 males and 4 females, and bile duct hyperplasia was found in 1 male and all 5 females at 1000 ppm.

Findings in the liver in males and females given 1000 ppm were regarded as adverse as the weight increase was above historical controls, there were correlating clinical pathology findings and histopathologically observed necrosis. At 300 ppm in females, the liver weight increase was above 130% and outside of the historical-control range. However, clear signs of hepatotoxicity were lacking at this dose, since clinical pathology findings were absent and histology showed hypertrophy only. The centrilobular hepatocellular hypertrophy observed in males given 30, 100 or 300 ppm were regarded as adaptive.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

There were no histopathological findings upon examination of adrenals (cortex and medulla), spleen, thymus, and thyroid in any of the control or high-dose group animals.

Table 5.3.1-17: Histopathology

	Males					Females				
Dose level [ppm]	**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
No. of animals	5	5	5	5	5	5	5	5	5	5
LIVER										
examined	5	5	5	5	5	5	5	5	5	5
Infiltration, lymphoid, Grade 1	5	5	5	5	5	1	3	5	3	5
Hypertrophy, centrilobular		5	5	5	5					
Grade 1		5	1							
Grade 2			4	1						
Grade 3				4	5					
Hypertrophy, diffuse									5	5
Grade 1									4	
Grade 2									1	5
Necrosis, (multi)focal					4					3
Grade 1					3					2
Grade 2					1					1
Fatty change, peripheral Grade 3				1						
Fatty change, (multi)focal										1
Oval cell proliferation Grade 1					2			2		4
Bile duct hyperplasia Grade 1					1			1		5
KIDNEYS										
examined	5				5	5		1		5
Tubules, basophilic Grade 1	1					2				1
Hydronephrosis Grade 5								1		

III. CONCLUSION

Dietary administration of BAS 750 F to C57BL/6 J Rj mice for 4 weeks caused test-substance related adverse signs of systemic toxicity (reductions in body weight gain and feed intake, evidence of liver toxicity from clinical chemistry and pathology findings) in males at concentrations of 1000 ppm (128 mg/kg bw/d) and in females at 300 ppm (61 mg/kg bw/d) and 1000 ppm (145 mg/kg bw/d). Under the study conditions, a NOAEL of 300 ppm (47.9 mg/kg bw/d) was established in male mice and a NOAEL of 100 ppm (18.5 mg/kg bw/d) was derived in female mice.

Report:	CA 5.3.1/4 [redacted] 2015 a BAS 750 F - Repeated-dose 28-day oral toxicity study in beagle dogs - Oral administration (capsule) 2014/1170748
Guidelines:	OECD 407 (2008), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, JMAFF No 12 Nosan No 8147 (2-1-9 2000), OECD 409 (1998)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a dose-range finding 28-day study with BAS 750 F (batch: COD-001880; purity: 98.6%), groups of 3 male dogs received daily dose levels of 300 or 1000 mg/kg bw/d by oral capsule (control groups received empty capsules). After two days, treatment was interrupted for 5 days due to severe clinical signs in both dose groups (vomitus, impaired general condition, unsteady gait, reduced food intake) and then continued at lower dose levels of 125 and 250 mg/kg bw/d until sacrifice on study day 35/36. Subsequently, groups of 3 female dogs received the test substance by daily oral capsule at dose levels of 300 and 500 mg/kg bw. Due to clinical signs seen after the first dosing similar to males, treatment of the females was interrupted for two days and continued on study day 3 at lower dose levels of 125 and 250 mg/kg bw/d until sacrifice on study day 29/30. Body weight, food consumption and clinical signs were investigated, hematology, clinical chemistry and urinalysis parameters were assessed in fasted animals after 4-wk treatment. On study day 23, blood samples were taken before and after dosing of all treatment group dogs at several time intervals to determine BAS 750 F plasma concentrations. At sacrifice a comprehensive set of organs were weighed and assessed by gross necropsy. Histopathology of the liver was performed for all dogs, the remaining tissues were histopathologically assessed in case of gross necropsy findings and for dogs of the control and high-dose group dogs.

After treatment continued with reduced dose levels, clinical findings at the low dose level of 125 mg/kg bw/d were confined to one female dog with delayed food intake on some treatment days, and to one male dog with vomitus occurring on 3 of 28 days. At the high dose level of 250 mg/kg bw/d, two of three dogs from each sex consistently showed delayed food intake, additionally there were isolated occurrences of vomiting and single findings of unsteady gait and poor general condition. Body weight development was clearly affected in the high dose groups (both sexes). At the low dose level body weight gain was slightly reduced in male dogs but comparable to control development in female dogs. Cholesterol values were decreased in both sexes at both dose levels, but only in one high-dose female dog serum activities of AST and ALT were increased, which indicated an adverse effect on liver cells in this animal. At necropsy, the terminal body weight of high-dose group dogs was decreased by 12%, and slightly reduced at the low dose level (by 5-7%).

Liver weights were clearly increased at both dose levels in both sexes (≥25%), histopathologically reflected by centrilobular hypertrophy of minimal severity in 5 of 6 dogs (one high-dose male with grade-2 (slight) hypertrophy). Additionally the cytoplasm of hepatocytes from treated dogs were eosinophilic (minimal-slight in females and minimal in males), and did not show the extent of vacuolation seen in control animals. In conclusion, a NOAEL could not be derived in this study, mainly due to adverse clinical findings in the low dose group (300 mg/kg bw/d reduced to 125 mg/kg bw/d) in both sexes.

(DocID 2014/1170748)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F
Description:	solid / white
Batch #:	COD-001880
Purity / content:	98.6%
Stability of test compound:	The test substance was stable over the study period (expiry date 30-Nov-2015).
2. Vehicle:	Hard gelatine capsules, size 11 (volume: 7 mL), Torpac Inc., Fairfield, NJ, USA
3. Test animals:	
Species:	Dog
Strain:	purebred Beagle
Sex:	male and female
Age (Day 0):	♂: 7.3 – 7.8 months; ♀: 7.8 – 8.9 months
Weight at dosing (range):	♂: 10.6 – 13.1 kg; ♀: 9.3 – 12.1 kg
Source:	BASF; Ludwigshafen/Rhein; Germany Fed.Rep.
Acclimatization period:	at least 13 days
Diet:	Dog maintenance KLIBA laboratory diet, Provimi Kliba SA, Kaiseraugst, Switzerland A daily food ration of about 400 g (males) and 350 g (females) were offered to each animal for a maximum of 2 hours. For animal welfare reasons, when retarded food consumption was observed, the feeding period was extended up to the morning of the subsequent study day.
Water:	Drinking water ad libitum from automatic watering device; in metabolism cage (during urine collection): 500 ml drinking water (from bottle).
Housing:	Single housing in kennels of ~ 2.7 m² indoor and ~ 2.7 m² outdoor; the animals had free access to the outdoor kennel 24h/day.

Environmental conditions:

Temperature:	ambient
Humidity:	not measured
Air changes:	Forced ventilation, approx. 8 air changes/hour
Photo period:	Natural day/night rhythm with additional artificial light as required during working hours

B. STUDY DESIGN

1. Dates of work: 10-Apr-2014 to 12-Feb-2015 [administration period:
♂: 28-Apr-2014 to 03-Jun-2014;
♀: 16-Jun-2014 to 16-Jul-2014]

2. Animal assignment and treatment

Before the start of the acclimatization period, the animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

BAS 750 F was orally administered in capsules once daily in the morning, immediately before the feed ration (portions of 400 g for males and 350 g for females) was offered to the animals. Control group animals received the same number of gelatin capsules as the animals of the high dose group (however empty).

Initially groups of 3 male dogs of test groups 0, 1 and 2 were given BAS 750 F at dose levels of 0, 300 or 1000 mg/kg bw/d, respectively. After two dose administrations, however, the treatment was interrupted for 5 days due to severe clinical findings in both dose groups. On day 7, daily treatment was continued with lowered dose levels of 125 and 250 mg/kg bw/d, administered to animals of test groups 1 and 2, respectively. The daily administration was continued until sacrifice of the animals on day 35 or 36.

Subsequently, female animals were treated at dose levels of 0 (control), 300 (test group 1) and 500 mg/kg bw/d (test group 2). Due to severe clinical findings in test groups 1 and 2, administration was interrupted between study days 1 and 2. Application was continued at dose levels of 125 mg/kg bw/d (test group 1) and 250 mg/kg bw/d (test group 2) from study day 3 onwards until sacrifice on day 29 or 30.

3. Test substance preparation and analysis

On each day immediately prior to administration, gelatin capsules were filled with appropriate amounts of test substance, which were determined on the basis of each individual animal’s weekly body weight. The weights of representative capsules containing the test substance were checked against empty capsules at the start of the study, to ensure dose correctness. The results were documented in the raw data. Homogeneity and concentration control analyses of the test substance in the gelatin capsules were not considered necessary and therefore not carried out.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change and food consumption	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means

For clinical pathology (hematology and clinical chemistry) and pathological examinations (organ weights), means and/or medians and standard deviations of each test group were calculated. However, because only three individuals per group and sex were used, no statistical significant differences can be expected among groups, due to lack of statistical power. Therefore no statistical analysis was performed and instead individual values were compared.

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Each animal was examined once a day for any evident signs of toxicity before and after treatment and, if signs occurred, several times daily.

Detailed clinical observations were performed were performed at weekly intervals, starting at the beginning of the administration period (study day 0). The findings were ranked according to the degree of severity. The chosen housing conditions with kennels of appropriate size including areas inside and outside the building allowed the animals to move freely. Therefore, deviations in motor activity and altered behavior could be determined if evident. The scope of examinations and the scoring of the findings included but was not limited to the following parameters listed:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Mucosal membranes
6. Salivation
7. Respiration
8. Activity/arousal level
9. Tremors
10. Convulsions
11. Abnormal movements
12. Impairment of gait
13. Lacrimation
14. Visible swellings masses
15. Feces (appearance/consistency)
16. Urine (volume color)
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals, on study day -7, at the beginning of the administration period (day 0) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption

The extent of food intake was monitored several times daily. Food consumption of the animals was quantified at weekly intervals, starting one week before the start of test substance administration (day -7). For this purpose, the extent of food intake was measured within a two-hour period immediately following the capsule administration.

4. Ophthalmoscopy

Not performed in this study.

5. Hematology and clinical chemistry

For hematology and clinical chemistry investigation, blood was taken in the morning from the *vena cephalica antebrachii* from fasted animals (on day 30 in males and on day 28 in females). The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to ensure reliable test results.

The results of clinical pathology examinations were expressed in International System (SI) units. The following examinations were carried out in all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Quick's test)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)	✓	Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

6. Urinalysis

For urinalysis (performed on day 29 in males and on day 25 in females), food was withdrawn in the afternoon and the animals were transferred to individual metabolism cages for overnight collection of urine. During this time, the dogs had access to about 500 ml of water, but to no food. Urine samples were evaluated in a randomized sequence.

Urinalysis					
	Quantitative parameters		*Semi-quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilinogen
		✓	Glucose	✓	Sediment (microscop. examination)
		✓	Ketones		

7. Plasma concentration analysis

On the morning of day 23 before, and 0.5, 1, 2, 4 and 8 hours after capsule administration, approx. 200 µl blood samples were taken from the *vena cephalica antebrachii of* all (non-fasted) animals. After addition of EDTA-K3 and centrifugation for preparation of plasma, the plasma samples were stored at -80 °C prior to LC-MS analysis for determination of the BAS 750 F concentration.

8. Sacrifice and pathology

The animals were anesthetized and sacrificed by exsanguination from the cervical and brachial vessels. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:											
S	**W**	**H**		**S**	**W**	**H**		**S**	**W**	**H**	
✓	✓	#	adrenals	✓		#	jejunum (w. Peyer's patches)	✓		#	rectum
✓		#	aorta	✓	✓	#	kidneys	✓		#	salivary glands*
✓		#	bone marrow[§]	✓			lachrymal glands[%]				seminal vesicles
✓	✓	#	brain	✓		#	larynx	✓		#	skin
✓		#	caecum	✓	✓	✓	liver	✓		#	spinal cord (3 levels)[@]
✓		#	colon	✓		#	lung	✓	✓	#	spleen
✓		#	duodenum	✓		#	lymph nodes[#]	✓		#	sternum w. marrow
✓	✓	#	epididymides	✓		#	mammary gland (♀ + ♂)	✓		#	stomach
✓		#	esophagus	✓		#	muscle, skeletal	✓	✓	#	testes
✓		#	eyes (with optic nerve)	✓		#	nerve, peripheral (sciatic n.)	✓	✓	#	thymus
✓			femur (with joint)	✓		#	nose/nasal cavity[‡]	✓	✓	#	thyroid/parathyroid
✓			gall bladder	✓	✓	#	ovaries and oviduct**	✓		#	trachea
✓		✓	gross lesions	✓		#	pancreas	✓		#	urinary bladder
			Harderian gland	✓		#	pharynx	✓	✓	#	uterus
✓	✓	#	heart	✓	✓	#	pituitary	✓		#	vagina
✓		#	ileum	✓	✓	#	prostate				
S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.											
[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III											

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

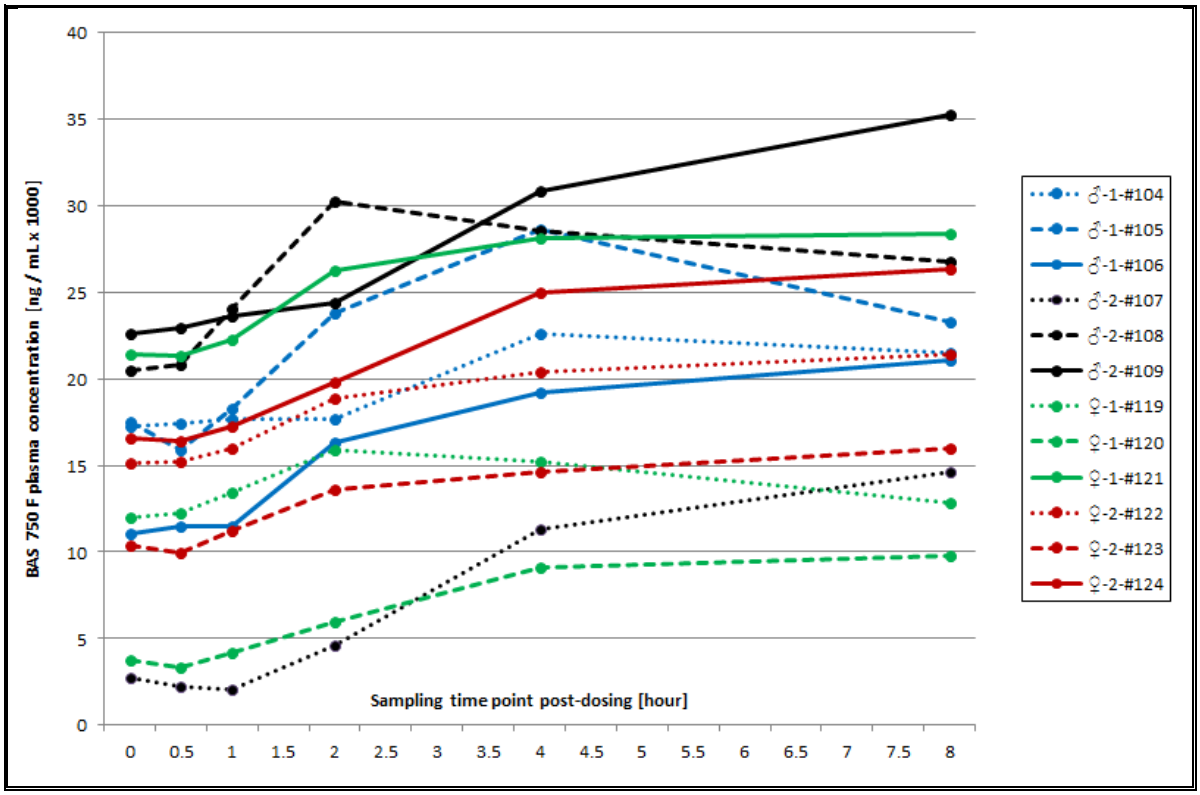
Test substance analyses (stability, homogeneity and concentration control analyses in the test preparation) were not required, because the test substance was not mixed with any vehicle but administered unchanged via gelatin capsules.

1. Test substance concentration in blood plasma

Six blood samples were taken from each dog on day 23 of the study, at the time points 0, 0.5, 1, 2, 4 and 8 hours post dosing. The plasma from the t=0 sampling time point were taken immediately before the administration of the daily capsule and therefore the corresponding plasma concentrations reflect the residual test substance burden from the previous administration period (day 0-22).

Results are presented in Figure 5.3.1-3 and Table 5.3.1-18.

Figure 5.3.1-3: BAS 750 F plasma concentrations in dogs (day 23)



Although intragroup variability was high, the mean plasma concentrations of BAS 750 F were very similar between dose groups; male dogs appeared to have slightly higher BAS 750 F plasma levels than females. Plasma concentrations started to increase about 1-2 hours after dosing. By 4-8 h, the plasma concentration levels had usually reached a plateau or started to decrease (except for two dogs of the high-dose group that still showed clearly increased plasma levels at the last sampling time point of 8 hours post dosing. The overall mean plasma level increase (= maximum – minimum plasma concentration) was slightly higher in males than in females (ca. 10531 vs. 6608 ng/mL), which might reflect higher extent of absorption and/or lower extent of metabolisation/elimination in male compared to female animals. Based on these results, it was decided to select blood sampling time-points of t=0 h and t=6 h for the definitive 90-day dog study.

Table 5.3.1-18: BAS 750 F plasma concentration in dogs (day 23)

		BAS 750 F plasma concentration [ng/ml]			
		Males		Females	
Test group		1	2	1	2
Dose level [mg/kg bw/d]		300 → 125	1000 → 250	300 → 125	500 → 250
t = 0 h	1	17274.68	2708.22	12016.15	15111.33
	2	17533.77	20447.42	3735.22	10405.38
	3	11025.17	22563.64	21394.35	16607.77
	Mean	**15278 ± 3685**	**15240 ± 10904**	**12382 ± 8835**	**14041 ± 3237**
t = 0.5 h	1	17457.85	2207.40	12231.33	15202.37
	2	15897.74	20785.76	3323.13	9966.15
	3	11517.84	22911.74	21328.73	16430.73
	Mean	**14958 ± 3080**	**15302 ± 11390**	**12294 ± 9003**	**13866 ± 3433**
t = 1 h	1	17641.30	2033.35	13403.58	16008.53
	2	18241.84	24019.92	4179.04	11185.82
	3	11484.26	23601.75	22293.77	17220.58
	Mean	**15789 ± 3740**	**16552 ± 12575**	**13292 ± 9058**	**14085 ± 3192**
t = 2 h	1	17693.30	4619.14	15893.66	18885.81
	2	23745.42	30264.94	5950.32	13641.34
	3	16276.15	24403.89	26245.36	19800.09
	Mean	**19238 ± 3967**	**19763 ± 13438**	**16030 ± 10148**	**17442 ± 3323**
t = 4 h	1	22578.84	11279.87	15225.33	20423.62
	2	28619.79	28539.94	9116.59	14625.19
	3	19166.75	30842.63	28145.31	24937.32
	Mean	**23455 ± 4787**	**23554 ± 10692**	**17496 ± 9715**	**19995 ± 5169**
t = 8 h	1	21460.60	14653.21	12858.82	21380.03
	2	23271.86	26746.11	9769.25	16014.73
	3	21075.46	35236.55	28402.83	26366.13
	Mean	**21936 ± 1173**	**25515 ± 10391**	**17010 ± 9986**	**21254 ± 5177**

B. OBSERVATIONS

1. Mortality

No animal died prematurely in the study.

2. Clinical signs of toxicity

The following findings were observed on study days 0 and 1 (males) or study day 0 (females) before reduction of the dose levels:

On study days 0 and 1, dose levels of 300 mg/kg bw/d (test group 1) and 1000 mg/kg bw/d (test group 2) elicited vomitus in all males. On study day 0, vomitus was also observed in two of three females at the dose levels of 300 mg/kg bw/d (test group 1) and in all three females administered 500 mg/kg bw/d. Poor general condition occurred in all male animals given 300 mg/kg bw/d (test group 1) and in two males administered 1000 mg/kg bw/d (test group 2). Poor general condition was also observed in one female administered 500 mg/kg bw/d (test group 2), but not in any female at the dose level of 300 mg/kg bw/d (test group 1). Reduced food consumption occurred in all males of test group 2 (1000 mg/kg bw/d), in two females of test group 2 (500 mg/kg bw/d) and in one female of test group 1 (300 mg/kg bw/d). In one male animal of each test group 1 and 2, only, unsteady gait was observed. The observed findings were regarded as treatment-related.

Due to these severe clinical findings, the treatment of male dogs was interrupted from days 2-6. Similarly, female dosing was not carried out on study days 1-2. Subsequently, treatment of the dogs was continued until the end of the administration period, but with reduced dose levels of 125 mg/kg bw/d (test groups 1) and 250 mg/kg bw/d (test groups 2).

After the reduction of the dose levels and, also, after administration of the test substance, further findings were observed in male and female animals. Reduced food consumption occurred in all animals on individual study days. On study days 11 and 14, vomitus was observed in one male of test group 1 (125 mg/kg bw/d). The finding also occurred in two males of test group 2 (250 mg/kg bw/d) on study days 8, 9 and 11 and in one male on study day 10. The above mentioned clinical observations were regarded as treatment-related.

Soft feces were seen in one male of test group 1 (twice, on days 6 and 16) and in two females of test group 2 (once each, on day 0 or 1). Due to the isolated occurrence, the finding was not considered to be related to treatment.

C. FOOD CONSUMPTION

As part of the daily detailed clinical observations, the intake of food was inspected visually. Usually a daily food ration (pellets) of about 400 g is offered to males, and 350 g to females, for a maximum of two hours in the morning. However, when retarded food consumption was observed, the feeding period was extended up to the morning of the subsequent study day. In the test group 2 **males**, reduced food consumption was observed in dog #107 repeatedly on days 8-10, 12, 14-15, 17-18, 21-31, 33 and 34. Dog #109 exhibited reduced food intake on days 10-12, 14-18, 21-23, 25-26, and 31-32. The third male dog #108 of test group 2 was found with reduced food intake only on days 9 and 15. No episodes of reduced food intake were seen in male dogs of group 1. In **females** of group 2, reduced food intake was observable in two dogs throughout most of the study period: female #122 on days 0 and 3-27, and female #123 from days 4-27 and on day 29.

The third female dog from group 2 (#124) ate normally. In the group 1, there was only one female dog (#120) with sporadically reduced food intake (on study days 3, 10, 21-23, 26-27 and 29).

Results from the weekly quantification of the food intake (see Table 5.3.1-19) were in line with the findings of the daily clinical observations.

Two male dogs from test group 2 showed sporadic incomplete food intake (intake of dog #107 was 64% and 45% of the normal ration on days 14 and 34; the intake of dog #109 was 26% on day 14 and 87% on day 21). In contrast, two female dogs from test group 2 showed marked reductions of feed intake. Female #122 consumed only about 11-15% of the normal ration on days 7, 14 and 21. Female #123 ate even less, only 5% on day 7, practically nothing on days 14 and 21, and about 10% on day 28. In group 1, complete food intake was observed in all male dogs, while female dog #120 showed a transient reduction of feed intake of on day 14 (61%) and day 21 (38%). The reduced food intakes of male and female dogs of group 2 and of the female dog of group 1 were considered to be treatment related.

Table 5.3.1-19: Food consumption

Test Group **Dose level** **[mg/kg bw/d]** (animal no.)	**Food consumption (g /day)**					
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 34**
Males (#)						
Group 0 **[0]** (101/102/103)	400/399/400	398/398/399	400/400/398	399/400/400	400/400/399	400/400/400
Group 1 **300** (Day 0-1) **125** (Day 7-35) (104/105/106)	401/401/399	398/398/399	399/399/398	398/399/399	399/399/400	399/400/399
Group 2 **1000** (Day 0-1) **250** (Day 7-35) (107/108/109)	401/399/400	401/401/398	**255**/398/**102**	398/398/**349**	399/399/400	**180**/398/399
Females (#)						
Group 0 **[0]** (116/117/118)	350/350/350	350/351/350	351/351/349	348/348/346	348/348/346	---
Group 1 **300** (Day 0) **125** (Day 2-28) (119/120/121)	350/350/349	350/349/350	350/**213**/349	345/**134**/348	349/349/350	---
Group 2 **500** (Day 0) **250** (Day 2-28) (122/123/124)	197/349/350	**053/ 19** /351	**40 / 1** /351	**40 / -1** /348	**47 / 32** /350	---

C. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight

The mean body weights of male and female dogs were reduced in both test groups 1 and 2 throughout the period of administration of 125 and 250 mg/kg bw/d, respectively (see Table 5.3.1-20).

Table 5.3.1-20: Mean body weight

Dose level [mg/kg bw/d]	Mean body weight (kg) [% of ctrl]						
MALES	**Day -7**	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
0	12.1	11.9	12.1	12.4	12.5	12.7	12.9
Test group 1 **300** (Day 0-1) **125** (Day 7-35)	11.7 [96]	11.8 [100]	12.0 [99]	11.6 [94]	11.9 [95]	12.0 [95]	12.1 [94]
Test group 2 **1000** (Day 0-1) **250** (Day 7-35)	11.4 [94]	11.6 [97]	11.6 [96]	11.1 [89]	11.3 [90]	11.4 [90]	**11.3*** **[87]**
FEMALES	**Day -7**	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	
0	10.7	10.8	11.0	11.1	11.4	11.3	
Test group 1 **300** (Day 0) **125** (Day 2-28)	10.2 [96]	10.4 [96]	10.4 [95]	10.2 **[92]**	10.4 **[91]**	10.3 **[91]**	
Test group 2 **500** (Day 0) **250** (Day 2-28)	10.7 [100]	10.7 [98]	10.5 [95]	9.8 **[88]**	10.1 **[89]**	10.0 **[88]**	

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

In males of test group 2, mean body weights were reduced up to -12.7% on day 35; in males of test group 1, body weight reductions of up to -6.5% were determined on study days 14 and 35. In females of test group 2, maximum reduction of body weight was noted on day 14 with -11.7%. In females of test group 1, mean weights were reduced up to -9.1% on study days 21 and 28.

2. Body weight gain

Mean Body weight gain data is summarized in Table 5.3.1-21, the mean body weight development is presented in Figure 5.3.1-4. Male control group dogs showed a steady weight increase during the study period, while dogs from test groups 1 and 2 lost weight during day 7-14 (more pronounced in test group 2). Thereafter, the dogs gained weight again, but the weight increase was generally lower than that of the control group dogs.

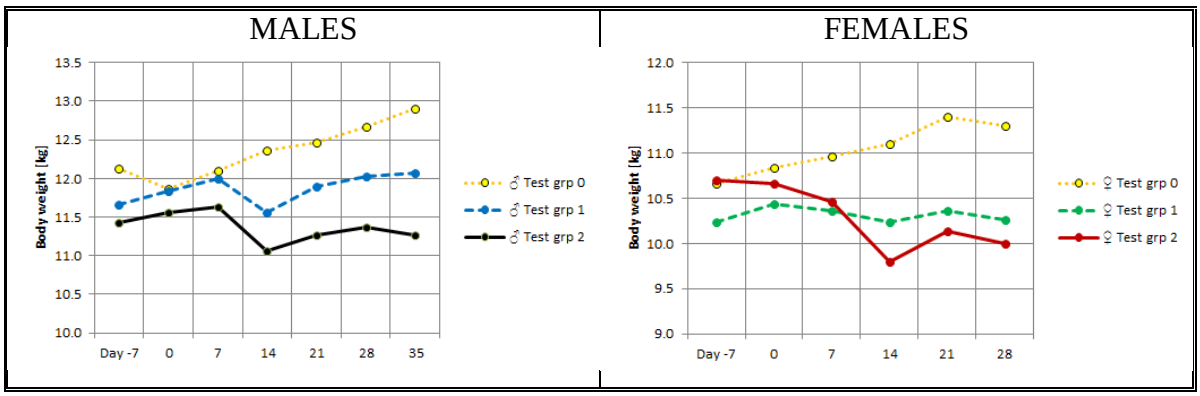
Table 5.3.1-21: Body weight development

	Males			Females		
Test group	**0**	**1**	**2**	**0**	**1**	**2**
Dose level [mg/kg bw/d]	**0**	**300 ➔ 125**	**1000 ➔ 250**	**0**	**300 ➔ 125**	**500 ➔ 250**
Day 0 – 7	0.2	0.2	0.1	0.1	-0.1	**-0.2****
Day 0 – 14	0.5	-0.3*	**-0.5****	0.3	-0.2	**-0.9***
Day 0 – 21	0.6	0.1	-0.3	0.6	-0.1	**-0.5***
Day 0 – 28	0.8	0.2	**-0.2***	0.5	-0.2*	**-0.7****
Day 0 – 35	1.0	0.2	**-0.3***			

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

In control group females, the body weight of one dog increased steadily, while the two other females increased only marginally during the 4-week study period. Two of three female dogs from test group 2 lost weight between Day 0-14 and regained some of the weight in the second half of the study. The weight of the other female from group 2 and of the three group 1 females did not change significantly throughout the study period.

Figure 5.3.1-4: Body weight development



E. OPHTHALMOSCOPY

Not performed in this range-finding study.

F. BLOOD ANALYSES

1. Hematology

No treatment-related changes of hematological parameters were observed.

It was noted that in females of test groups 1 and 2, counts of total white blood cells, neutrophils and eosinophils were decreased, and the proportion of lymphocytes increased compared to control group values (all statistically not significant). However, the individual values in these groups were all within the historical control range (HCR), except for one group 1 female with a neutrophil count that was marginally outside of the HCR (4.71 giga/L, HCR: 4.74 – 11.30 giga/L).

Table 5.3.1-22: Hematology parameters

	Males (Day 30)			Females (Day 28)		
Test group	**0**	**1**	**2**	**0**	**1**	**2**
Dose level [mg/kg bw/d]	**0**	**300 → 125**	**1000 → 250**	**0**	**300 → 125**	**500 → 250**
WBC [giga/L]	13.47	9.84	10.42	16.23	9.97	10.55
Abs. neutrophil count [giga/L]	7.94	5.75	6.40	10.03	5.31	5.57
Abs. eosinophil count [giga/L]	0.35	0.31	0.52	0.69	0.46	0.25
Rel. lymphocyte count [%]	33.4	33.4	27.9	26.7	37.4	39.6

2. Clinical chemistry

The following changes of clinical-chemistry parameters were considered treatment-related:

Males

- ↓ **Cholesterol** in group 2 (1000 → 250 mg/kg bw/d) and in group 1 (300 → 125 mg/kg bw/d), outside HCR; the only liver parameter changed at these dose levels, therefore considered not adverse

Females

- ↑ **Alanine aminotransferase** in group 2 (500 → 250 mg/kg bw/d), in one female dog associated with ↑ Aspartate aminotransferase, both ALT and AST values of this dog were outside of HCR; adverse effect
- ↓ **Cholesterol** in group 2 (500 → 250 mg/kg bw/d), outside of HCR; adverse effect
- ↓ **Cholesterol** in group 1 (300 → 125 mg/kg bw/d), outside of HCR; the only liver parameter changed at this dose, therefore considered not adverse

Table 5.3.1-23: Clinical chemistry parameters (selected)

	Males (Day 30)			Females (Day 28)		
Test group	**0**	**1**	**2**	**0**	**1**	**2**
Dose level [mg/kg bw/d]	**0**	**300 → 125**	**1000 → 250**	**0**	**300 → 125**	**500 → 250**
ALAT [µkat/L]	0.58	0.78	1.02	1.05	0.99	**1.20**
ASAT [µkat/L]	0.58	0.55	0.48	0.57	0.56	0.82
Cholesterol [mmol/L]	4.57	2.59	2.58	4.16	2.40	**2.30**
Urea [mmol/L]	3.61	4.30	4.19	3.98	4.89	5.04
Creatinine [mmol/L]	60.8	48.6	49.5	58.4	53.6	53.2
Inorganic phosphate [mmol/L]	1.80	1.85	1.81	1.68	1.58	1.65

The following changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

- ↑ **Alanine aminotransferase** in group 1 (300 → 125 mg/kg bw/d) and group 2 (1000 → 250 mg/kg bw/d) (with one exception within the HCR (0.34-1.16 µkat/L); one dog from group 2 with ALT value of 1.55 µkat/L (slightly outside HCR =1.3-fold of max. historical control); however AST value within the HCR. Elevated ALT values were considered to be incidental.
- ↑ **Urea** in group 1 (300 → 125 mg/kg bw/d) and group 2 (1000 → 250 mg/kg bw/d), but individual values were all within the HCR.

Females

- ↑ **Alanine aminotransferase** in group 1 (300 → 125 mg/kg bw/d), outside of HCR in two dogs; the increase over the upper limit of the HCR was marginal (1.47 and 0.88 µkat/L, range 0.36–0.80 µkat/L); one female control ALT value was 1.66 µkat/L and thus also outside of the HCR; the AST values of these animals were within a normal range, therefore overall the slightly elevated ALT values were considered incidental
- ↑ **Urea** in group 1(300 → 125 mg/kg bw/d) and group 2 (1000 → 250 mg/kg bw/d), but individual values were all within the HCR, except for one female with an urea value of 6.08 mmol/L, which marginally outside of the HCR (2.63-5.97 mmol/L). No other kidney parameter (creatinine, inorganic phosphate) was increased. Therefore the higher urea levels were considered to be incidental.

G. URINALYSIS

No treatment-related adverse changes among urinalysis parameters were observed. In females of test group 2, urine volume was lower and specific gravity of the urine was higher compared to controls. The observation is potentially related to vomiting episodes and reduced food or water uptake of the dogs in this group, and are therefore considered to be treatment-related; the changes were regarded to result from physiological renal function, there were no changes indicative of kidney toxicity.

Table 5.3.1-24: Urinalyses parameters (selected)

	Males (Day 30)			Females (Day 28)		
Test group	**0**	**1**	**2**	**0**	**1**	**2**
Dose level [mg/kg bw/d]	**0**	**300 → 125**	**1000 → 250**	**0**	**300 → 125**	**500 → 250**
Urine volume [mL]	486.7	403.3	303.3	413.3	290.0	113.3
Specific gravity [g/L]	1.017	1.017	1.030	1.016	1.036	1.034

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)

H. NECROPSY AND PATHOLOGY

1. Organ weight

Terminal body weight: The decreased mean body weights of males and females from group 1 and 2 were considered to be treatment-related.

Liver: The absolute and relative liver weights in both treatment groups were above the maximal historical control values for males (absolute/relative: 425.8 g / 3.185%) and females (absolute/relative: 391.0 g / 3.072%) and therefore assessed as treatment-related.

Table 5.3.1-25: Organ weights

Sex		Males (n=3)				Females (n=3)			
Organ weight	**Group**	**Absolute weight**	**Δ%**	**Relative weight [% of bw]**	**Δ% #**	**Absolute weight**	**Δ%**	**Relative weight [% of bw]**	**Δ% #**
Terminal weight [kg]	0	12.90				11.40			
	1	12.00	(-7)			10.40	(-9)		
	2	**11.33**	**(-12)**			**10.03**	**(-12)**		
Adrenal glands (g)	0	1.323		0.010		1.150		0.010	
	1	1.307	(-1)	0.011	(+6)	1.107	(-4)	0.011	(+5)
	2	1.223	(-8)	0.011	(+6)	1.257	(+9)	**0.012**	**(+23)**
Brain (g)	0	89.34		0.692		81.61		0.717	
	1	87.21	(-1)	0.727	(+5)	75.83	(-7)	0.733	(+2)
	2	91.47	(+2)	**0.810**	**(+17)**	78.64	(-4)	**0.797**	**(+11)**
Epididymides (g)	0	2.883		0.022					
	1	2.697	(-6)	0.022	(±0)				
	2	**2.360**	**(-18)**	0.021	(-6)				
Heart (g)	0	97.7		0.757		87.79		0.771	
	1	98.8	(+1)	0.820	(+8)	88.50	(+1)	0.852	(+10)
	2	101.6	(+3)	**0.896**	**(+18)**	85.13	(-3)	0.848	(+10)
Kidneys (g)	0	59.64		0.461		49.39		0.436	
	1	57.01	(-4)	0.475	(+3)	46.34	(-6)	0.449	(+3)
	2	53.66	(-10)	0.475	(+3)	45.26	(-8)	0.453	(+4)
Liver (g)	0	388.6		3.014		347.3		3.056	
	1	**503.2**	**(+29)**	**4.195**	**(+39)**	**400.4**	**(+15)**	**3.833**	**(+25)**
	2	**465.6**	**(+20)**	**4.098**	**(+36)**	**403.4**	**(+16)**	**3.989**	**(+31)**
Ovaries (g)	0					0.833		0.007	
	1					0.760	(-9)	0.007	(-1)
	2					0.820	(-2)	0.008	(+9)
Pituitary (g)	0	92.00		0.001		67.33		0.001	
	1	85.67	(-7)	0.001	(±0)	63.00	(-6)	0.001	(+1)
	2	**79.33**	**(-14)**	0.001	(-1)	74.67	(+11)	**0.001**	**(+23)**
Prostate (g)	0	2.433		0.019					
	1	1.920	(-21)	0.016	(-16)				
	2	2.317	(-5)	**0.021**	**(+12)**				
Spleen (g)	0	24.27		0.188		23.82		0.209	
	1	21.88	(-10)	0.182	(-3)	23.61	(-1)	0.223	(+7)
	2	**20.34**	**(-16)**	0.179	(-5)	21.90	(-8)	0.219	(+5)
Testes (g)	0	16.89		0.131					
	1	15.81	(-6)	0.132	(+1)				
	2	**14.27**	**(-16)**	0.126	(-3)				
Thymus (g)	0	13.81				10.19		0.087	
	1	12.67	(-8)		(+2)	4.753	**(-53)**	**0.045**	**(-49)**
	2	**5.257**	**(-62)**		**(-57)**	4.833	**(-53)**	**0.049**	**(-44)**
Thyroid (g)	0	0.793		0.006		0.720		0.006	
	1	**1.173**	**(+48)**	0.010	**(+58)**	0.837	**(+16)**	**0.008**	**(+29)**
	2	**0.990**	**(+25)**	0.009	**(+42)**	0.807	**(+12)**	**0.008**	**(+28)**
Uterus (g)	0					6.123		0.056	
	1					1.803	**(-71)**	**0.018**	**(-68)**
	2					1.077	**(-82)**	**0.011**	**(-81)**

Testes and epididymides: The absolute decrease of the testes and epididymides weights in males of test group 2 was due to a single male (No. 108), showing lower weights of genital organs, including the prostate. These findings most probably reflected incomplete sexual maturity as revealed in the histopathological pattern of testes (immature spermatids, slight presence of multinucleated giant cells and luminal debris, associated with slight multifocal degeneration of seminiferous tubules), epididymides (luminal debris and oligospermia) and prostate (immaturity, characterized by small size of acini and abundant interstitial stroma). Sexual maturity in male Beagle dogs has been estimated to occur between 7 and 8 months of age. However, spontaneous inter-individual variations in the age are expected to occur. Although the sexual maturity of animal No. 108 was further behind the control and remaining animals of test group 2, it was considered to be within the physiological range of inter-individual variation. In the same sense, the relative prostate weight of males in test group 2 was *increased* due to one upwards outlier animal (No. 107), with an absolute prostate weight that almost doubled the individual prostate weights of control animals. This finding was also considered to be within expected biological variations.

Uterus: Female Beagle dogs reach their first estrus between 8 and 14 month of age. The absolute and relative uterus weight in females of test group 1 and 2 was remarkably decreased. Histopathological examination showed that neither control nor treated females of test group 2 had reached the first estrus (lack of functional or old degraded corpora lutea). Furthermore, mammary glands were immature (characterized by undifferentiated ducts in the dermis of the nipples). In spite of this, a great range of variability in sexual organ development was noted when examining ovaries, vagina, uterus and mammary gland of each female animal. Therefore, the decreased uterus weight in females of test group 1 and 2 was attributed to sexual immaturity which was ascribed to the age-related variations in reaching the first estrus and sexual maturity, and not to treatment.

Brain: The relative brain weight increase in males and females of test group 2 and the relative heart weight increase in males were regarded to be related to the terminal body weight decrease observed at the high dose.

Pituitary gland, spleen, thyroid and adrenal glands: No histopathological changes were seen in the pituitary gland, spleen, thyroid and adrenal glands that could explain their weight deviations which were therefore regarded as incidental and not treatment-related.

Thymus: Whereas the absolute thymus weight in males of test group 2 (5.257 g) was below the minimum historical control (6.600 g), the relative thymus weight of males (0.045%) in test group 2 was above the minimal HCR (0.043%) and had no histopathological correlate to the weight decrease. In females, the thymus weights in females of test group 1 (absolute/relative: 4.753 g / 0.045%) and 2 (absolute/relative: 4.833 g / 0.049%) were below the minimum historical control values (absolute / relative: 7.098 g / 0.057%). These findings were interpreted to be secondary to the reduced terminal body weight but also due to the high inter-individual variability of this organ, since no histopathological correlate could be noted.

2. Gross pathology

Findings noted in one male (red foci in the liver) and one female (focal red discoloration in the jejunum) of the high dose had no histopathological correlates in the male or correlated with focal hyperemia in the female. These findings were considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

Treatment-related centrilobular hepatocellular hypertrophy was observed in the liver of all male dogs and in 1 female dog from each dose group. In group 2, there was one male dog with hypertrophy of slight severity (grade 2); the other 4 affected dogs had livers with hypertrophy of minimal severity (grade 1). Additionally, eosinophilic change of minimal or slight severity (the latter only in 1 female dog each of group 1 and 2) was diagnosed in the livers of all treatment group male and female dogs. The eosinophilic change was characterized by a different cytoplasm in the treated animals, compared to the more vacuolated appearance of control animal hepatocytes that are characteristic for the cytoplasmic storage of glycogen.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 5.3.1-26: Histopathology

	Males (Day 35)			Females (Day 29)		
Test group	**0**	**1**	**2**	**0**	**1**	**2**
Dose level [mg/kg bw/d]	**0**	**300 → 125**	**1000 → 250**	**0**	**300 → 125**	**500 → 250**
No. of animals	3	3	3	3	3	3
Liver						
examined	3	3	3	3	3	3
Hypertrophy, hepatocellular	0	3	3	0	1	1
Grade 1		3	2		1	1
Grade 2			1			
Eosinophilic change	0	3	3	0	3	3
Grade 1		3	3		2	2
Grade 2					1	1

III. CONCLUSION

Under the study conditions, dogs orally administered 300 mg/bw/d or higher dose levels by capsule showed severe clinical signs after one or two treatments, which required the interruption of treatment and reduction of the high dose level from 1000/500 mg/kg bw/d (♂/♀) to 250 and the low dose level from 300 mg/kg bw/d (♂+♀) to 125 mg/kg bw/d.

After treatment continued with reduced dose levels, clinical findings at the low dose level of 125 mg/kg bw/d were confined to one female dog with delayed food intake on some treatment days, and to one male dog with vomitus occurring on 3 of 28 days. At the high dose level of 250 mg/kg bw/d, two of three dogs from each sex consistently showed delayed food intake, additionally there were isolated occurrences of vomiting and single findings of unsteady gait and poor general condition. Body weight development was clearly affected in the high dose groups (both sexes). At the low dose level body weight gain was slightly reduced in male dogs but comparable to control development in female dogs. Cholesterol values were decreased in both sexes at both dose levels, but only in one high-dose female dog serum activities of AST and ALT were slightly increased, which indicated an adverse effect on liver cells in this animal. At necropsy, the terminal body weight of high-dose group dogs was decreased by 12%, and slightly reduced at the low dose level (by 5-7%). Liver weights were clearly increased at both dose levels in both sexes (≥25%), histopathologically reflected by centrilobular hypertrophy of minimal severity in 5 of 6 dogs (one high-dose male with grade-2 (slight) hypertrophy). Additionally the cytoplasm of hepatocytes from treated dogs were eosinophilic (minimal-slight in females and minimal in males), and did not show the extent of vacuolation seen in control animals.

In conclusion, a NOAEL could not be derived in this study, mainly due to adverse clinical findings in the low dose group (300 mg/kg bw/d reduced to 125 mg/kg bw/d) in both sexes.

CA 5.3.2 Oral 90-day study

Report:	CA 5.3.2/1 [redacted] 2015 b Final amended report - BAS 750 F - Repeated dose 90-day oral toxicity study in Wistar rats - Administration via the diet 2015/1198721
Guidelines:	OECD 408, EPA 870.3100, Commission Regulation (EC) No 440/2008, JMAFF No 12 Nosan No 8147 (2-1-9 2000)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (batch: COD-001662, purity: 95.5%) was administered to groups of 10 male and 10 female rats at dietary concentrations of 0, 400 (low dose), 1200 (intermediate dose), and 3600 ppm (top dose) for at least 90 days. The doses corresponded to time-weighted mean intakes of 27, 76 and 256 mg/kg bw/d in males and 30, 91, and 314 mg/kg bw/d in females. The scope of investigations was in compliance with OECD Guideline 408. Treatment with 3600 ppm caused reductions in body weight and body weight gain. At the end of the in-life period, males and females given 3600 ppm weighed 6.5% and 8.9% less than controls, respectively. The overall body weight gain during the administration period of high-dose males and females was about 11% and 20% less than controls, respectively. No clinical findings or functional changes or effects on motor activity were noted at any dose level. In clinical chemistry and pathological examinations, the liver was identified as target organ of BAS 750 F. Males and females given 3600 ppm had increased levels of serum alkaline phosphatase activities (slightly outside HCR), high-dose females additionally were found with slightly (outside HCR) increased cholesterol and decreased albumin levels. Relative liver weights were slightly increased at 3600 ppm in both sexes (+11% in males and +13% in females), which correlated with minimal centrilobular hepatocellular hypertrophy in 8 of 10 males and 3 of 10 females; relative liver weights were marginally increased also in male rats at 1200 ppm (+6%), and associated with minimal centrilobular hypertrophy in 3 of 10 males, which was considered an adaptive response and not adverse. The NOAEL was established at 1200 ppm (76 mg/kg bw/d in males and 91 mg/kg bw/d in females), based on liver weight increases with minimal grade hypertrophy in combination with changes of clinical chemistry parameters indicating slight dysregulation of liver cell function at the LOAEL of 3600 ppm.

(DocID 2015/1198721)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F (Reg.No. 5834378)

Description:	solid / white
Batch #:	COD-001662
Purity / content:	95.5%
Stability of test compound:	The test substance was stable over the study period (expiry date 31-Aug-2013).

2. Vehicle: Rodent diet

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Sex:	male and female
Age:	32 ± 1 days at delivery; 42 ± 1 days at start of dosing
Weight at dosing (mean):	♂: ca. 167.3 ± 6.6 g; ♀: ca. 127.3 ± 5.8 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimatization period:	at least 8 days
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Group housing (5 animals/cage) housed in H-Temp (PSU) cages (TECNIPLAST Deutschland GmBH, Hohenpeißenberg, Germany, floor area about 2065 cm² with dust free wooden bedding.; wooden gnawing blocks as enrichment ((Typ NGM E-022), Abedd®, Lab. and Vet. Service GmbH, Vienna, Austria)

Environmental conditions:

Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 21-Aug-2012 to 26-Nov-2014 [in life phase: 31-Aug-2012 (start of treatment) to 04-Dec-2012 (necropsy)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 10 male and 10 female rats at dietary concentrations of 0, 400 (low dose), 1200 (intermediate dose), and 3600 ppm (top dose) for at least 90 days. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diet preparations were performed approximately every 4 weeks.

Analyses performed prior to the start of the administration period revealed that the test-substance was stable in the diet for at least 35 days.

Concentration control analyses of all dose levels, and the homogeneity of the low and of the high dose levels were determined at the beginning of the study. No test article was determined in control diets. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC analysis with an external standard.

Table 5.3.2-1: Analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [ppm]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [ppm]	% of nominal concentration	Mean ± RSD
400 ppm	29-Aug-2012	27-Sep-2012	3	433.366	108.3	106.7 ± 2.5
			4	432.737	108.2	
			5	414.276	103.6	
1200 ppm	29-Aug-2012	27-Sep-2012	6	1169.080	97.4	
3600 ppm	29-Aug-2012	27-Sep-2012	7	3566.635	99.1	104.4 ± 5.2
			8	3958.220	110.0	
			9	3748.736	104.1	

Relative standard deviations of maximum 5.2% indicated the homogenous distribution of BAS 750 F in the diet preparations. The actual nominal test-item concentrations were in the range of 97.4 to 110% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
feces, rearing, grip strength forelimbs and hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Blood parameters with uni-directional changes	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians
Urinalysis parameters (except pH, urine volume, specific gravity, color and turbidity	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians
Urine pH, volume, specific gravity, color and turbidity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians *Urine color and turbidity are not evaluated statistically.*

Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly over a period of 1 day and calculated as mean food consumption in grams per animal and day.

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and food consumption, according to the following equation:

$$\text{Substance intake for Day}_x = \frac{FC_x \times C}{BW_x}$$

with FC_x as the mean daily food consumption (in g/day) on Day_x, C as the dose in ppm and BW_x as body weight on Day_x of the study (in g).

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any changes in volume.

5. Ophthalmoscopy

Prior to the start of the administration period the eyes of all animals, and on day 91 the eyes of the control and high dose animals were examined for any changes using an ophthalmoscope after administration of a mydriatic agent.

6. Functional observation battery (FOB)

A functional observational battery was performed in all animals at the end of the administration period starting at about 10.00 a.m. For this purpose, the animals were placed individually in polycarbonate cages during the time of the examination. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The findings were ranked according to the degree of severity, if applicable (for details see Part III of the report).

Home cage observations:

During the home cage observation, special attention was paid to posture, tremors, convulsions, abnormal movements and impairment of gait.

Open field observations:

For open field observation, the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm high) and observed for at least 2 minutes. The following parameters were assessed:

1. Behavior on removal from the cage
2. Fur
3. Skin
4. Salivation
5. Nasal discharge
6. Lacrimation
7. Eyes / pupil size
8. Posture
9. Palpebral closure
10. Respiration
11. Tremors
12. Convulsions
13. Abnormal movements / stereotypes
14. Gait abnormalities
15. Activity / arousal level
16. Feces excreted within 2 min (number of fecal pellets / appearance / consistency)
17. Urine excreted within 2 min (amount / color)
18. Number of rearings within 2 minutes

Sensimotor tests / reflexes:

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. Approach response (reaction to an object being moved towards the face)
2. Touch response (touch sensitivity)
3. Visual placing response (vision)
4. Pupillary reflex
5. Pinna reflex
6. Auditory startle response
7. Righting response (coordination of movements)
8. Behavior during handling
9. Vocalisation
10. Tail pinch (pain perception)
11. Forelimb grip strength
12. Hindlimb grip strength
13. Landing foot-splay test
14. Other findings

7. Motor activity measurement

Motor activity was measured on the same day as the FOB was performed, from 14:00 h onwards. The examinations were performed using the TSE Labmaster System (TSE Systems GmbH, Bad Homburg, Germany). For this purpose, the animals were placed in new clean polycarbonate cages with a small amount of bedding for the duration of the measurement. Eighteen beams were allocated per cage. The number of beam interrupts was counted over 12 intervals for 5 minutes per interval. The sequence in which the animals were placed in the cages was selected at random. On account of the time needed to place the animals in the cages, the starting time was "staggered" for each animal. The measurement period began when the first beam was interrupted and finished exactly 1 hour later. No food or water was offered to the animals during these measurements and the measurement room was darkened after the transfer of the last animal.

8. Hematology and clinical chemistry

Blood was withdrawn in the morning from fasted, isoflurane-anesthetized animals from the retro-orbital plexus. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
✓	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis

For urinalysis the individual animals were transferred to metabolism cages (withdrawal of food and water) and urine was collected overnight. Urine samples were evaluated in a randomized sequence.

Urinalysis					
	Quantitative parameters		*Semi-quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilirubin
		✓	Glucose	✓	Sediment (microscop. examination)
		✓	Ketones		

10. Plasma concentration analysis

Blood samples (about 200 µL) were taken at [TIME OF DAY] on study days 24, 45 and 66 from all animals (non-fasted) by puncturing the retrobulbar venous plexus under isoflurane anesthesia. After addition of EDTA-K3 and centrifugation for preparation of plasma, the plasma samples were stored at -80 °C prior to LC-MS analysis for determination of the BAS 750 F concentration.

11. Sacrifice and pathology

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:											
S	**W**	**H**		**S**	**W**	**H**		**S**	**W**	**H**	
✓	✓	✓	adrenals	✓		#	jejunum (w. Peyer's patches)	✓		#	rectum
✓		#	aorta	✓	✓	✓	kidneys	✓		#	salivary glands*
✓		#	bone marrow[§]	✓			lachrymal glands[%]	✓		#	seminal vesicles
✓	✓	#	brain	✓		#	larynx	✓		#	skin
✓		#	caecum	✓	✓	✓	liver	✓		#	spinal cord (3 levels)[@]
✓		#	colon	✓		#	lung	✓	✓	✓	spleen
✓		#	duodenum	✓		#	lymph nodes[#]	✓			sternum w. marrow
✓	✓	#	epididymides	✓		#	mammary gland (♀)	✓		#	stomach (fore- & glandular)
✓		#	esophagus	✓		#	muscle, skeletal	✓	✓	#	testes
✓		#	eyes (with optic nerve)	✓		#	nerve, peripheral (sciatic n.)	✓	✓	#	thymus
✓			femur (with joint)	✓		#	nose/nasal cavity[‡]	✓	✓	✓	thyroid/parathyroid
			gall bladder	✓	✓	#	ovaries and oviduct**	✓		#	trachea
✓		✓	gross lesions	✓		#	pancreas	✓		#	urinary bladder
✓		#	Harderian gland	✓		#	pharynx	✓	✓	#	uterus
✓	✓	#	heart	✓		#	pituitary	✓		#	vagina
✓		#	ileum	✓		#	prostate				
S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.											
[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III											

In addition, the following special stains were carried out in kidneys of male animals:

- Mallory-Heidenain stain to investigate the presence of tubular protein
- Immunohistochemical stain against alpha-2µ-globulin in animals #01, #02, #33 and #37

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

No animal died prematurely in the study.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals. In the anogenital region a mass was palpable through the skin (< 1.5 cm) in female animal #75 of test group 3 (3600 ppm) from study day 70 until study day 76. Also on account of the transient occurrence, this single finding was assessed to be spontaneous in nature and not related to treatment.

3. Functional observation battery

Home cage observations:	no treatment-related findings
Open field observations:	no treatment-related findings
Sensimotor tests / reflexes:	no treatment-related findings
Feces:	no treatment-related findings
Rearing:	no treatment-related findings
Gripstrength forelimbs:	no treatment-related findings
Gripstrength hindlimbs:	no treatment-related findings
Landing foot splay:	no treatment-related findings

4. Motor activity

Regarding the overall motor activity as well as the single intervals, no test substance-related deviations were noted. Comparing the single intervals with the control groups, a statistically significantly increased value was measured at interval #2 for male animals at 3600 ppm and a statistically significantly decreased value was measured at interval #10 for female animals at 400 ppm. These sporadic changes were regarded as being incidental and not related to treatment.

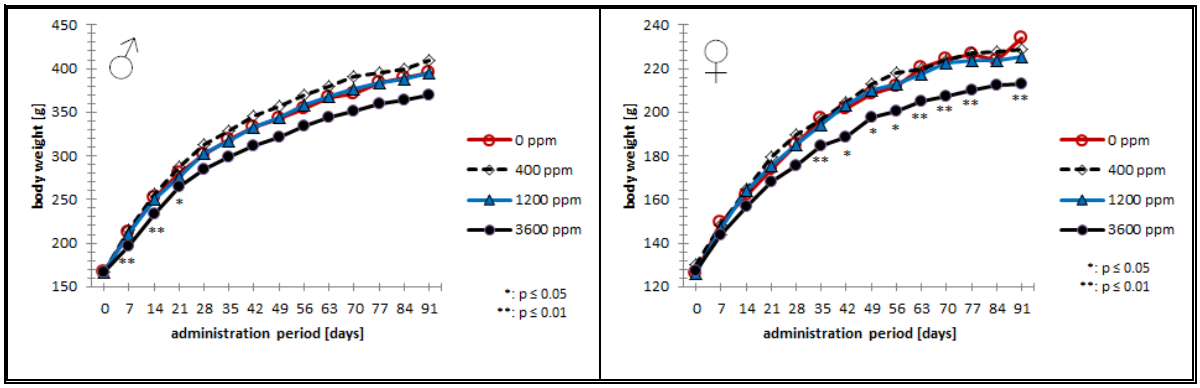
C. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight [see Figure 5.3.2-1]

Mean body weights were consistently lower in high-dose male animals (3600 ppm) throughout the treatment period; the body weight decrease attained statistical significance from study day 7 until study day 21 with a maximum of -7.4% on study day 14. At the end of the administration period, the mean body weights at 3600 ppm were 6.5% lower when compared to the controls. In female animals administered 3600 ppm, mean body weights were significantly lower from study day 35 until study day 77 as well as on study day 91 with a maximum of -8.9% on study day 91. The changes were regarded to be related to treatment and adverse.

No effects on mean body weights were observed in male and female animals of the low- and mid-dose groups (400 and 1200 ppm).

Figure 5.3.2-1: Body weight development



2. Body weight gain [see Table 5.3.2-2]

Mean body weight change values were significantly lower in high-dose male animals from study day 7 until study day 49 with a maximum by -34.5% on study day 7. At the end of the administration period, the mean body weight change value of the 3600 ppm male rats was 11% below the control group value (statistically not significant). In female animals given 3600 ppm, the mean body weight change values were significantly lower on study day 7 and from study day 28 onwards with a maximum by -28.5% on study day 7. At the end of the administration period, the mean body weight change value of high-dose females was 20% lower than the control group. Again, the changes at 3600 ppm were assessed as being related to treatment and adverse.

No effects on body weight development were observed in male and female animals of the low- and mid-dose groups (400 and 1200 ppm).

Table 5.3.2-2: Body weight development

	Males				Females			
Dose level [ppm]	**0**	**400**	**1200**	**3600**	**0**	**400**	**1200**	**3600**
Body weight [g]								
- Day 0	167.4	167.4	167.3	167.4	126.0	130.2	125.9	127.0
- Day 91	395.2	409.4	395.1	369.6	233.9	228.9	225.6	**213.0****
Δ% (compared to control[#]		3.6	0.0	-6.5		-2.2	-3.6	-8.9
Overall body weight gain (g)	227.8	242.0	227.9	202.2	108.0	98.7	99.7	**86.0****
Δ% (compared to control[#]		6.2	0.0	-11.2		-8.6	-7.7	**-20.3**

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

No test substance-related changes were observed in male and female animals.

2. Water consumption

No test substance-related, adverse changes were observed.

3. Test substance intake

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following table:

Table 5.3.2-3: Test substance intake

	Males			Females		
Dose level [ppm]	**400**	**1200**	**3600**	**400**	**1200**	**3600**
BAS 750 F (mg/kg bw/d)	27	76	256	30	91	314

E. OPHTHALMOSCOPY

No treatment-related findings were observed. All apparent findings were assessed as being incidental in nature since they occurred in individual animals only and did not show a dose-response relationship.

F. BLOOD ANALYSES

1. Hematology

No treatment-related changes of hematological parameters were observed.

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related:

Males

- ↑ Alkaline phosphatase at 3600 ppm (above HCR), adverse

Females

- ↑ Alkaline phosphatase at 3600 ppm (above HCR), adverse
- ↑ Cholesterol at 3600 ppm (above HCR), adverse
- ↓ Albumin at 3600 ppm (below HCR), adverse
- ↓ Total bilirubin (not accompanied by changes in red blood cell parameters, therefore decrease considered secondary to increased metabolism via enzyme induction), adaptive, not adverse

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

- ↑ Alkaline phosphatase at 1200 ppm (within HCR)
- ↑ Urea at 3600 ppm (in HCR)

Females

- ↑ Alkaline phosphatase at 1200 ppm (within HCR)
- ↓ Aspartate aminotransferase at 3600 ppm (within HCR; concurrent control outside HCR)
- ↑ GGT at 3600 ppm (within HCR)
- ↓ Albumin and total protein at 400 and 1200 ppm (within HCR, no dose-response)
- ↓ Total protein at 3600 ppm (within HCR)
- ↑ Urea at 3600 ppm (within HCR)
- ↑ Calcium at 400 and 3600 ppm (no dose-response)

Table 5.3.2-4: Clinical chemistry parameters (selected)

		Males				Females			
Dose level [ppm]		**0**	**400**	**1200**	**3600**	**0**	**400**	**1200**	**3600**
ALP	[µkat/l]	1.02	1.15	1.29*	**1.76****	0.51	0.63	0.67*	**0.78****
		Historical control: 0.91 – 1.45				*Historical control: 0.43 – 0.73*			
AST	[µkat/l]	1.65	1.70	1.57	1.74	2.75	2.80	1.82	1.50*
						Historical control: 1.43 – 2.35			
GGT	[nkat/l]	0	1	1	0	0	0	1	6*
						Historical control: 0 – 14			
Cholesterol	[mmol/l]	1.81	1.82	2.12	1.98	1.65	1.44	1.53	**2.21***
Albumin	[g/l]	38.43	38.25	37.50	38.24	42.88	40.36**	40.51**	38.90**
						Historical control: 39.50 – 43.65			
Total protein	[g/l]	62.75	63.28	61.64	62.47	67.74	64.68*	65.95*	63.72**
						Historical control: 62.13 – 70.12			
Urea	[mmol/l]	5.51	5.61	6.07	6.73**	6.75	6.75	6.58	7.65*
		Historical control: 4.38 – 7.42				*Historical control: 5.61 – 9.03*			
Calcium	[mmol/l]	2.50	2.53	2.52	2.52	2.56	2.46*	2.54	2.50*
Total bilirubin	[µmol/l]	1.57	1.52	1.43	0.97**	1.93	1.49	1.48	1.67

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)
Historical control data from test facility: 37 studies 90-day treatment of Wistar rats (Jul-2008 and Nov-2012)

3. Test substance concentration in blood plasma

BAS 750 F was detected in 4 out of 60 measured control samples, from which 3 values were obtained on Day 24 and comparable to the quantification limit of 5 ng/ml (♂#10, ♀#24, ♀#49). A higher BAS 750 F concentration of about 47 ng/mL was found in the 66-day sample ♀#49. No quantifiable amounts of BAS 750 F were detectable in the remaining 56 control samples.

For a given dose level, a time-dependent decrease in the plasma concentration was observed in both sexes with a greater extent in male rats. Independent of sampling time point, a three-fold increase in dietary concentration / test substance intake (i.e. from 400 to 1200 ppm or from 1200 to 3600 ppm) corresponded to a roughly five- to seven-fold increase of plasma concentration in males and to about an overall seven-fold increase in plasma concentration in females.

Table 5.3.2-5: BAS 750 F plasma concentration

	BAS 750 F plasma concentration [ng/ml]					
	Males			**Females**		
Dose level [ppm]	**400**	**1200**	**3600**	**400**	**1200**	**3600**
Day 24	110 ± 24	740 ± 300	4469 ± 1439	182 ± 106	1025 ± 476	7942 ± 3523
Day 45	119 ± 35	546 ± 244	2647 ± 680	116 ± 50	1137 ± 617	6926 ± 2168
Day 66	54 ± 20	320 ± 62	1702 ± 500	136 ± 82	722 ± 372	4749 ± 1813

G. URINALYSIS

In male rats given 3600 ppm, incidences of transitional epithelial cells and granulated and epithelial cell casts in the urine sediment were higher compared to control, whereas the urine pH value in these individuals was lower. A reduced pH value of the urine in combination with granulated/epithelial cell casts in the urine sediment is frequently seen in rats suffering from alpha-2µ-globulinurea, a species-specific finding which is not relevant for humans. Kidney sections from affected male rats were immunohistochemically stained to check for the potential presence of alpha-2µ-globulin. Upon histopathological examination, however, no treatment-related differences between the control- and high-dose groups were found (see also next section).

Table 5.3.2-6: Urinalyses

	Males				Females			
Dose level [ppm]	**0**	**400**	**1200**	**3600**	**0**	**400**	**1200**	**3600**
pH (Day 83)	6.7	6.5	6.5	6.2*	6.4	5.8	6.0	6.2
Transitional epithelial cells	1	1	1	2*	1	1	1	1
Casts	0	0	0	1*	0	0	0	0

Statistics: Wilcoxon test (1-sided), * $p \leq 0.05$; ** $p \leq 0.01$; mean severity: 1=few; 2=many

H. NECROPSY AND PATHOLOGY

1. Organ weight

When compared with control group 0, the mean absolute adrenal gland and heart weights were significantly decreased in high-dose group males. The mean absolute kidney weight of females given 1200 ppm were increased (112%); at 3600 ppm, the terminal body weight of female rats was decreased (92%) and also the adrenal glands (78%). Relative heart weights were slightly reduced in low-dose group males (90%). Relative liver weights were significantly increased in males given 1200 ppm (106%) and in both males and females given 3600 ppm (111% and 113%, respectively). Relative kidney weights were significantly increased in females at all dose levels (110%, 116% and 113% at 400, 1200 and 3600 ppm, respectively). Mean relative thyroid weights were significantly increased in females given 3600 ppm (126%). All other mean absolute and relative weight parameters did not show statistically significant differences when compared to controls.

These statistically significant changes were assessed as follows:

Terminal body weight: The significantly decreased mean body weight of females given 3600 ppm was assessed as treatment-related.

Liver: The increase in relative liver weights in males given 1200 ppm and in both sexes given 3600 ppm was assessed as treatment-related.

Thyroid glands: The increase in relative thyroid weights in high-dose group females was not accompanied by histopathological findings and was therefore not regarded as treatment-related.

Adrenal glands: Weight decreases in adrenal absolute weights were not regarded as treatment-related as there was no significant change in relative weights or a histopathological correlate.

Heart and kidneys: Heart and kidney weights were not dose-dependently altered; there were no histopathological findings in these organs. Therefore, these weight changes were not regarded as treatment-related.

Table 5.3.2-7: Organ weights

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	372.54				218.68			
	400	386.13	(+4)			216.74	(-1)		
	1200	371.69	(±0)			211.60	(-3)		
	3600	348.14	(-7)			**200.50****	**(-8)**		
Adrenal glands (mg)	0	66.7		0.018		69.1		0.032	
	400	58.9	(-12)	0.015	(-15)	70.7	(+2)	0.033	(+3)
	1200	57.0	(-15)	0.015	(-15)	66.0	(-4)	0.031	(-2)
	3600	**53.5***	**(-20)**	0.015	(-15)	**53.8****	**(-12)**	0.027	(-16)
Brain (g)	0	2.110		0.569		1.960		0.900	
	400	2.080	(-1)	0.540	(-5)	1.986	(+1)	0.919	(+2)
	1200	2.116	(±0)	0.572	(±0)	1.948	(-1)	0.921	(+2)
	3600	2.072	(-2)	0.598	(+5)	1.933	(-1)	0.966	(+7)
Epididymides (g)	0	1.088		0.294					
	400	1.082	(-1)	0.281	(-5)				
	1200	1.104	(+1)	0.299	(+2)				
	3600	1.052	(-3)	0.303	(+3)				
Heart (g)	0	1.088		0.293		0.716		0.328	
	400	1.017	(-7)	**0.264***	**(-10)**	0.692	(-3)	0.319	(-3)
	1200	1.084	(±0)	0.293	(±0)	0.659	(-8)	0.312	(-5)
	3600	**0.997***	**(-8)**	0.287	(-2)	0.679	(-5)	0.339	(+3)
Kidneys (g)	0	2.327		0.625		1.349		0.618	
	400	2.361	(+1)	0.612	(-2)	1.477	(+9)	**0.682****	**(+10)**
	1200	2.248	(-3)	0.607	(-3)	**1.509***	**(+12)**	**0.714****	**(+16)**
	3600	2.202	(-5)	0.634	(+1)	1.393	(+3)	**0.696****	**(+13)**
Liver (g)	0	8.366		2.248		5.386		2.468	
	400	8.920	(+7)	2.310	(+3)	5.100	(-5)	2.359	(-4)
	1200	8.842	(+6)	**2.376***	**(+6)**	5.369	(±0)	2.541	(+3)
	3600	8.702	(+4)	**2.502****	**(+11)**	5.570	(+3)	**2.779****	**(+13)**
Ovaries (mg)	0					90.6		0.042	
	400					91.0	(±0)	0.042	(+1)
	1200					95.6	(+6)	0.045	(+9)
	3600					89.1	(-2)	0.044	(+7)
Spleen (g)	0	0.596		0.161		0.453		0.208	
	400	0.588	(-1)	0.152	(-6)	0.421	(-7)	0.194	(-7)
	1200	0.584	(-2)	0.157	(-3)	0.387	(-15)	0.183	(-12)
	3600	0.573	(-4)	0.165	(+2)	0.434	(-4)	0.216	(+4)
Testes (g)	0	3.710		1.000					
	400	3.634	(-2)	0.945	(-6)				
	1200	3.641	(-2)	0.985	(-2)				
	3600	3.498	(-6)	1.009	(+1)				

Table 5.3.2-7: Organ weights

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Thymus (mg)	0	332.3		0.089		312.7		0.144	
	400	333.1	(±0)	0.086	(-3)	322.5	(+3)	0.149	(+4)
	1200	276.2	(-17)	0.074	(-17)	280.5	(-10)	0.133	(-8)
	3600	294.2	(-11)	0.084	(-5)	300.7	(-4)	0.150	(+4)
Thyroid (mg)	0	25.2		0.007		19.4		0.009	
	400	26.7	(+6)	0.007	(+3)	21.8	(+12)	0.010	(+13)
	1200	26.3	(+4)	0.007	(+5)	21.7	(+12)	0.010	(+16)
	3600	26.1	(+4)	0.008	(+11)	22.4	(+15)	**0.011***	**(+26)**
Uterus (g)	0					0.852		0.395	
	400					0.817	(-4)	0.380	(-4)
	1200					0.808	(-5)	0.380	(-4)
	3600					0.763	(-10)	0.383	(-3)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

2. Gross pathology

All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

Treatment-related centrilobular hepatocellular hypertrophy (grade 1) was observed in the liver of male animals given 1200 ppm and of male and female animals given 3600 ppm with incidences and grading according to the table below:

Table 5.3.2-8: Histopathology

	Males				Females			
Dose level [ppm]	**0**	**400**	**1200**	**3600**	**0**	**400**	**1200**	**3600**
No. of animals	10	10	10	10	10	10	10	10
LIVER								
examined	10	10	10	10	10	10	10	10
Necrosis, (multi)focal								1
Infiltration, lymphoid	10	10	10	10	10	10	10	10
Hypertrophy, hepatocellular			3	8				3
Grade 1			3	8				3
ADRENAL CORTEX								
examined	10	10	10	10	10	9	10	10
Vacuolation, zona fasciculata		1						
Ectopic bone						1		
HEART								
examined	10			10	10			10
Necrosis / fibrosis	3			4				
KIDNEYS								
examined	10	10	10	10	10	10	10	10
Tubules, basophilic	4	3	1	1			2	3
Mineralization, medulla					10	10	10	10
Dilation, renal pelvis		1						
Eosinophilic droplets	10			10				
THYROID GLANDS								
examined	10	10	10	10	10	10	10	10
Hypertrophy / hyperplasia, follicular cell		1		1				
Altered colloid		1		1			1	

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

The special stains performed with kidney sections of male animals did not reveal differences between control and treated males.

III. CONCLUSION

Dietary administration of BAS 750 F to Wistar rats for 90 days resulted in reduced body weight gain in females given 3600 ppm, resulting in a terminal body weight that was about 10% lower than the respective control group mean weight. The liver was identified as target organ. Serum alkaline phosphatase activities were slightly increased in both sexes at 3600 ppm; female rats of this group additionally showed marginally increased cholesterol and reduced albumin concentrations. Slight increases in relative liver weight were noted in males at 1200 ppm (+6%) and in both sexes at 3600 ppm (+11% in males and +13% in females). Histopathological examination revealed the occurrence of centrilobular hepatocellular hypertrophy of minimal severity in these groups, reflecting the slight increases of liver weight. The slight changes in liver weight and histology were regarded as an adaptive response to increased functional demand of the liver, but not as adverse per se. The NOAEL was established at 1200 ppm (76 mg/kg bw/d in males and 91 mg/kg bw/d in females), based on liver weight increases with minimal grade hypertrophy in combination with changes of clinical chemistry parameters indicating slight dysregulation of liver cell function at the LOAEL of 3600 ppm.

Report:	CA 5.3.2/2 [redacted] 2015 a 90-day oral dietary toxicity study with BAS 750 F in C57BL/6JRj mice 2014/1046542
Guidelines:	OECD 408, EPA 870.3100, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.26, JMAFF No 12 Nosan No 8147, Japanese Chemical Substances Control Law 1973 - Notification of Mar. 31 2012 by MHLW (0331 No. 7) METI (No. 5) and MOE (No. 110331009)
GLP:	yes (certified by Ministry of Health, Welfare and Sport, Utrecht, The Netherlands)

Report:	CA 5.3.2/3 Becker M.,Kamp H., 2014 a BAS 750 F - Plasma analysis for external studies 2014/1177165
Guidelines:	None
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 5.3.2/4 Becker M., 2015 a Amendment No. 1 to the report: BAS 750 F - Plasma analysis for external studies 2015/1240217
Guidelines:	None
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Note:	The amendment corrects a typing error in chapter 5.1 (discussion) of the original report

EXECUTIVE SUMMARY

BAS 750 F (batch: COD-001740, purity: 98.8%) was administered to groups of 15 male and 15 female C57BL/6JRj mice at dietary concentrations of 0, 10 (low dose), 50 (intermediate low dose), 250 (intermediate high dose) and 750 ppm (top dose) for at least 90 days. The doses corresponded to time-weighted mean intakes of 2, 11, 58 and 174 mg/kg bw/d in males and 3, 15, 67 and 211 mg/kg bw/d in females. The study was designed to select appropriate dose levels for a subsequent 18-month mouse carcinogenicity study.

Administration of BAS 750 F to C57BL/6JRj mice at the top dose level of 750 ppm caused reductions in body weight gain and clear signs of hepatotoxicity in both sexes, including changes in several clinical chemistry parameters, pronounced liver weight increases with associated hepatocellular hypertrophy, liver cell necrosis and degenerative hepatocellular (cytoplasmic) changes.

At the LOAEL of 250 ppm (58 mg/kg bw/d in males and 67 mg/kg bw/d in females), 2 male mice with single cell necrosis of minimal severity and 4 further male mice with cytoplasmic alteration of liver cells also of minimal severity were noted. In both sexes, liver weights were moderately increased and associated with liver cell hypertrophy. Cholesterol was reduced in both sexes and the albumin/globulin ratio was additionally reduced in females. Increased levels of red blood cell parameters and platelets in male mice at 250 ppm were assessed as evidence for hemoconcentration. The Maximum Tolerated Dose (MTD) was considered to be exceeded at 250 ppm in male mice and met/approached at 250 ppm in female mice.

At the dose level of 50 ppm, slight increases in hemoglobin and hematocrit were observed in male mice that were considered to be treatment-related but not adverse. Additional treatment-related findings in male mice at 50 ppm were a reduction in cholesterol, slightly increased liver weights (abs. +16, rel. +14%) with associated liver cell hypertrophy of minimal severity in 8 of 10 males. These liver changes were considered to be an adaptive response to BAS 750 F exposure and not adverse. In females at 50 ppm, treatment-related effects were confined to slightly reduced cholesterol levels. Due to the absence of adverse effects at 50 ppm after 90 days, as well as after 18 months at the same dietary concentration and dose in the much longer mouse carcinogenicity study, the dose level of 50 ppm was therefore considered to be the NOAEL in both sexes, corresponding to intakes of 11 mg/kg bw/d in males and 15 mg/kg bw/d in female mice. (DocID 2014/1046542)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The test substance was stable over the study period (expiry date 31-Mar-2014).
2. Vehicle:	Rodent diet
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6JRj
Sex:	male and female
Age:	approx. 7 weeks at start of dosing
Weight at dosing (mean):	♂: ca. 21.3 ± 1.1 g; ♀: ca. 17.3 ± 0.7 g
Source:	Janvier Labs SAS, Le Genest Saint Isle, France
Acclimatization period:	at least 5 days
Diet:	Ground Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum

Housing:	Male animals individually in Macrolon cages type MIII (height 15 cm); female animals housed 2 per cage (main groups) or 1-2 per cage (satellite groups); sterilized sawdust bedding (Litalabo, France); enrichment: paper (Enviro-dri, Wm. Lilico & Son, UK)
Environmental conditions:	
Temperature:	18 – 24°C
Humidity:	40 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 28-May-2013 to 30-Aug-2013 [in life phase: 29-May-2013 (start of treatment) to 30-Aug-2013 (necropsy)]

2. Animal assignment and treatment

The 90-day study included main test groups of 10 animals/sex and satellite groups of 5 animals/sex, which were administered 0, 10, 50, 250 or 750 ppm BAS 750 F in the diet. The satellite groups were kept under identical conditions as the main groups, and blood samples of these groups were used for plasma analysis of parent compound and metabolites. These animals were sacrificed after the last blood sampling on Study Day 63 without further investigations. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights, with all animals within ± 20% of the sex-specific mean weight.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diets were prepared once weekly.

Samples of diet preparations were analysed for homogeneity (highest and lowest test concentration) and accuracy of preparations (all concentrations determined weeks 1, 4 and 13). The 30-day stability of the test substance in the diet (highest and lowest concentration) at room temperature was investigated using a sample from the week-1 preparation. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC/UV analysis with an external standard.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change, food consumption, relative food consumption	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means, based on pooled variance
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters assumed to follow a normal distribution	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means, based on pooled variance
Blood parameters assumed not to follow a normal distribution	Pairwise comparison of each dose group with the control group using the STEEL-test (two-sided, non-parametric rank sum test) for the hypothesis of equal medians or means ➔ reticulocytes, differential white blood cell count percentage; total globulin, albumin/globulin ratio
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means, based on pooled variance
Gross necropsy and histopathology findings	FISHER-EXACT test applied to frequency data

C. METHODS

1. Observations

The animals were examined for mortality / viability at least twice daily. Observations for clinical signs of toxicity were performed once daily. The time of onset, severity grade (where applicable, otherwise presence/absence) and duration were recorded.

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly during the treatment period. Food was weighed when supplied to the animals, and remaining food was weighed 7 days later, approximately the same time each week. The weight difference was then used for the calculation of the mean food intake per animal and day. Food consumption values of ≥ 10 gram/animal/day were excluded from summary and statistics, as these values were regarded as unrealistically high for mice in this type of study (these occurred most likely due to spillage).

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and mean food consumption, according to the following equation:

$$\text{Substance intake for } Day_x = \frac{FC_x \times C}{BW_x}$$

with FC_x as the mean daily food consumption (in g/day) on Day_x, C as the dose in ppm and BW_x as body weight on Day_x of the study (in g).

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any changes in volume. Since no treatment-related change became obvious, no quantitative investigation was introduced.

5. Hematology and clinical chemistry

Clinical laboratory investigations in all main group animas were carried out at the end of the 13-week treatment period just prior to scheduled necropsy. Blood was withdrawn from fasted, isoflurane-anesthetized animals from the retro-orbital sinus. The following hematological and clinical chemistry parameters were determined:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)		Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)		Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	RBC distribution width (RDW)				
✓	Reticulocytes				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Albumin/globulin ratio (calc.)	✓	Aspartate aminotransferase (AST)
	Magnesium		Bile acids (TBA)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Cholesterol		
✓	Sodium	✓	Creatinine		
		✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

6. Urinalysis

Not performed in this study.

7. Blood sampling for plasma concentration of parent compound

Blood samples (about 200 µL) were taken from all satellite group animals (5/group/sex, non-fasted) between 8:00 and 10:00 a.m. on study days 21, 42 and 63 by puncturing the retrobulbar venous sinus under isoflurane anesthesia. After addition of EDTA-K3 and centrifugation for preparation of plasma, the plasma samples were stored at ≤ -75 °C prior to LC-MS analysis for determination of the BAS 750 F concentration.

11. Sacrifice and pathology

The fasted main group animals were necropsied following exsanguination via the thoracic aorta under deep isoflurane anesthesia. All animals were subjected to a full post mortem examination. The following organs were sampled, weighed and examined histopathologically following fixation in 10% buffered formalin, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	#	adrenals	✓		#	jejunum (w. Peyer's patches)	✓		#	rectum
✓		#	aorta	✓	✓	#	kidneys	✓		#	salivary glands*
✓		#	bone marrow[§]	✓			lachrymal glands[%]	✓		#	seminal vesicles
✓	✓	#	brain	✓		#	larynx	✓		#	skin
✓		#	caecum	✓	✓	✓	Liver (wt incl. gall bladder)	✓		#	spinal cord (3 levels)[@]
✓		#	cervix	✓		#	lung	✓	✓	#	spleen
✓		#	(clitoral gland)	✓		#	lymph nodes [ln]	✓			sternum w. marrow
✓		#	coagulating glands	✓		#	mammary gland	✓		#	stomach
✓		#	colon	✓		#	muscle, skeletal	✓	✓	#	testes***
✓		#	duodenum	✓		#	nerve, peripheral (sciatic n.)	✓	✓	#	thymus
✓	✓	#	epididymides***	✓		#	nose/nasal cavity[‡]	✓		✓	thyroid/parathyroid
✓		#	Esophagus	✓	✓	#	ovaries and oviduct**	✓			(tongue)
✓		#	eyes (with optic nerve) ***	✓		#	pancreas	✓		#	trachea
✓			femur (with joint)	✓		#	pharynx	✓			(ureter)
✓		#	gall bladder	✓		#	pituitary	✓			(urethra)
✓		✓	gross lesions	✓		#	(preputial gland)	✓		#	urinary bladder
✓		#	Harderian gland	✓		#	prostate	✓	✓	#	uterus (wt incl. cervix)
✓	✓	#	heart					✓		#	vagina
✓		#	Ileum (w. Peyer's patches)								

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose + animals died pre-schedule.

[§] from femur; [ln] axillary, mandibular and mesenteric; [@] cervical, midthoracic, lumbar; *mandibular, parotid and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III; *** fixed for at least 24 h in modified Davidson's solution before transfer to formalin fixative

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

1. Stability

Analysis of diets at the top dose level (750 ppm) after storage for 30 days at room temperature yielded a relative difference of ≤ 10%. The low-dose group (10 ppm) diet however yielded an apparent relative difference of 14% between the fresh and stored samples. Since the mean accuracy of the t=0 samples of was considered slightly high (108%) and the accuracy of the t=30 days samples was well within the acceptability criterion of 90-110%, the diets were considered to be stable under these storage conditions.

Table 5.3.2-9: Stability analysis

BAS 750 F nominal conc. [ppm]	Date of analysis	Date of analysis	Analytical concentration [ppm]		Relative difference [%]
	t = 0	t = 30 days	t = 0 [a]	t = 30 days	
10 ppm	02-Jul-2013	01-Aug-2013	10.8	9.29 [b]	-14
750 ppm	02-Jul-2013	01-Aug-2013	691	635 [c]	-8.1

[a] Mean of six samples at t=0 hours taken at 10%, 50% and 90% height of food container
[b] Mean of two samples at t=30 days taken at random position. Individual results were 9.21 and 9.36 ppm.
[c] Mean of two samples at t=30 days taken at random position. Individual results were 634 and 637 ppm.

2. Homogeneity

Homogeneity of the prepared high-dose and low-dose group diets (750 ppm and 10 ppm) were investigated after 1, 4 and 13 weeks. The obtained results were all within the acceptability range (coefficient of variation of ≤ 5%), except for the high-dose diet prepared for use in Week 1 and the reanalysed low-dose diet prepared for use in Week 13 (coefficient of variation of 6.7% and 6.4%, respectively). Since the observed variations were only slightly outside the acceptability range, the results were accepted.

Table 5.3.2-10: Homogeneity and concentration-control

		Initial Analysis			Re-Analysis (Week 13)		
BAS 750 F nominal conc. [ppm]	Week	BAS 750 F Mean analysed conc. [ppm]	[% of nominal]	Homogeneity (coeeficient of variation) [ppm]	BAS 750 F Mean analysed conc.	[% of nominal]	Homogeneity (coeeficient of variation) [%]
10	1	10.8	108	5.0			
	4	10.3	103	1.6			
	13	7.3	73	4.9	8.8	88	6.4
50	1	48.5	97	n.a.			
	4	45.8	92	n.a.			
	13	41.8	84	n.a.	46.1	92	n.a.
250	1	242.5	97	n.a.			
	4	220.5	88	n.a.			
	13	194.0	78		220	88	n.a.
750	1	690	92	6.7			
	4	652	87	2.6			
	13	595	79	2.1	666	89	3.2

3. Concentration control

No test substance was detected in the Group 1 (control group) diets at any time point. Accuracy of diets for Groups 2-5 prepared for use in Week 1 and diets for Groups 2 and 3 prepared for use in Week 4 were within the acceptability range of 90-110%. The mean accuracy of diets for Groups 4 and 5 prepared for use in Week 4 were out of the acceptability range of 90-110% (i.e. 88 or 87%). The mean accuracies of the diets prepared for use in Week 13 were all below the acceptability range of 90-110% (i.e. 73-84%). Reanalysis of these Week 13 samples resulted in mean accuracies between 88 and 92%. Since these deviations from the acceptability range were small and still within the range of 85-115%, the results were considered acceptable.

B. OBSERVATIONS

1. Mortality

No animal from the main groups died prematurely in the study. However, four satellite females given 750 ppm died on Day 21 during isoflurane anesthesia prior to blood sampling. The cause of death could not be ascertained.

2. Clinical signs of toxicity

There were no clinical signs of toxicity noted during the observation period.

Alopecia, incidentally observed among female groups including control, occurred within the normal range of biological variation to be expected for mice of this age and strain.

C. BODY WEIGHT AND BODY WEIGHT GAIN (Table 5.3.2-11 and Figure 5.3.2-2)

No statistically significant differences were obtained for absolute body weights of male test groups. For body weight gain, lower values were throughout treatment at 750 ppm, and statistical significance was obtained from Week 5 onwards. Overall, for male mice at 750 ppm, decreased mean body weight gain (-32%) was observed at Day 91, as compared to controls. Consistently decreased mean body weight gain was observed for the high-dose males at Day 29 (i.e. -50%) and at Day 57 (i.e. -26%). For male mice at 250 ppm, overall decreased mean body weight gain (-9%) was observed at Day 91, as compared to controls.

In female mice administered the top dose level of 750 ppm, statistically significantly lower absolute body weights were observed in Weeks 2 to 5, and Week 11. Body weight gain was decreased over the first week of treatment but no statistical significance was reached. No treatment-related changes in absolute mean body weights and body weight gain were noted over the study period in females up to 250 ppm.

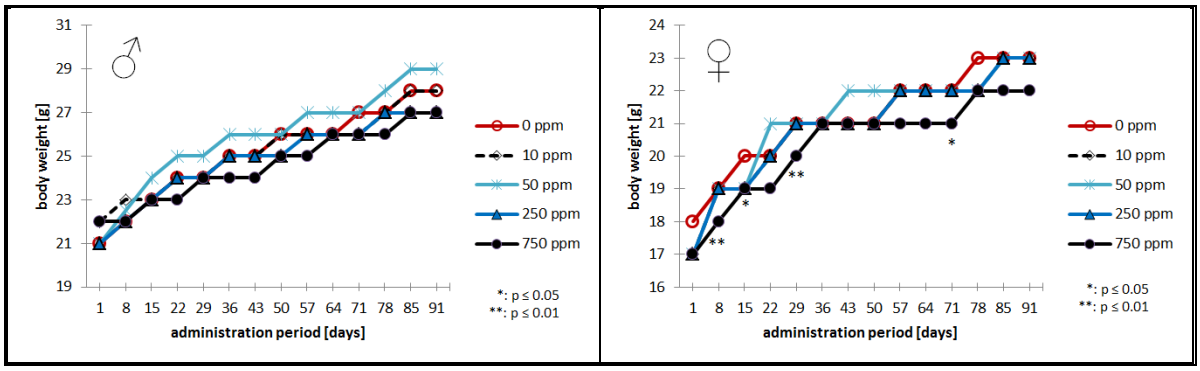
Final mean body weight of male and female mice given 750 ppm was 95% and 96% of the control weight value, respectively (see Table 5.3.2-11).

Table 5.3.2-11: Body weight and body weight gain

	Males					Females				
Dose level [ppm]	**0**	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
Body weight [g]										
- Day 1	21	22	21	21	22	18	17	17	17	17
- Day 91	28	28	29	27	27	23	23	23	23	22
Δ% (compared to control)[#]		±0	+4	-4	-5		±0	±0	±0	-4
Overall body weight gain (%, compared to Day 1)	34	28	33	31	**23****	31	29	35	32	31
Δ% (compared to control)[#]		-18	-3	-9	**-32**		-6	+12	+3	±0

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided); values are rounded

Figure 5.3.2-2: Body weight development



D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

In males up to 750 ppm, no clear changes in absolute food consumption (expressed in g/animal/day) or relative food consumption (expressed in g/kg body weight/day) were noted.

In females treated at 250 or 750 ppm there was a tendency towards lower values for absolute and relative food consumption in the second half of the treatment phase. No clear relationship with the dose was established. Further, an initial decrease in females at 750 ppm in Week 1 was seen for absolute and relative food consumption.

Table 5.3.2-12: Food consumption

	Males					Females				
Dose level [ppm]	**0**	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
Food consumption (g/day)										
- Days 1-8	8±2.5	6±1.6	6±1.8	**6±0.4***	**5±0.8***	9±3.8	7±1.8	6±0.7	6±1.6	**5±0.9***
- Mean of means over study	6	6	6	6	6	9	8	7	6	6

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett-test (two-sided); values are rounded

2. Water consumption

Water intake was not measured, however subjective appraisal of water intake maintained throughout the study did not raise any suspicion of a test substance-related change.

3. Test substance intake

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following table:

Table 5.3.2-13: Test substance intake

	Males				Females			
Dose level [ppm]	**10**	**50**	**250**	**750**	**10**	**50**	**250**	**750**
BAS 750 F (mg/kg bw/d)	2	11	58	174	3	15	67	211

E. BLOOD ANALYSES

1. Hematology

The following changes of hematological parameters were considered to be related to treatment and adverse:

Males

- ↑ Platelet counts at 250 ppm and 750 ppm
- ↑ Red blood cell counts* at 250 ppm
- ↑ Hemoglobin level* at 250 ppm
- ↑ Hematocrit* at 250 ppm
- ↑ Mean corpuscular hemoglobin (MCH)* at 250 ppm

* Red blood cell (RBC) counts hemoglobin, hematocrit and mean corpuscular hemoglobin (MCH) values could not be assessed for 8 male mice at 750 ppm due to (a) clotting of EDTA blood samples (#121, #125, #127, #133, #139), (b) insufficient sample volume (#129) or (c) technical errors (#12, #135). Therefore only two values of the above mentioned parameters were measured in the 750 ppm male group.

The changes in the red blood cell parameters in combination with increased platelet counts at 250 ppm were considered by the study authors to be indicative of hemoconcentration.

There were no signs of hemoconcentration in either the 28-day or much longer 18-month carcinogenicity study at any dose.

Normally, the hemoconcentration is caused by decreased water consumption and/or stress. Water consumption and urine volume were not quantified in this study, and adrenal cortex hypertrophy which might indicate a stress-related response, was not observed in the affected animals. Alternatively, polycythemia may lead to hemoconcentration. However, in this study and also in the 18-month carcinogenicity study, there was no histopathological evidence to support the diagnosis of polycythemia, such as histopathological changes in the bone marrow or in the red pulpa of the spleen. Therefore, polycythemia as potential cause for hemoconcentration can be excluded.

The study authors considered effects at 50 ppm (4% increase of hemoglobin and 5% increase of hematocrit compared to controls) to be potentially adverse in view of a possible association to hemoconcentration. However, 1) since the blood cell counts (red and white blood cells and platelet counts) were not significantly changed at 50 ppm; 2) in the absence of a dose-related increase in any hematological parameter in females; 3) in the absence of polycythemia as an explanation for the observation; and 4) given other more plausible explanations for the observation (decreased water consumption, stress response), the slight increase of Hb and Ht values compared to control levels at this dose level are considered to be treatment-related in view of a possible dose-response relationship, but not sufficiently severe or robust to be considered adverse. Although only 2 males were assessed at 750 ppm, there was no indication of an effect on Hb, Ht or RBCs in either animal.

Females

↑ Platelet counts at 750 ppm

↓ Relative eosinophil counts at 750 ppm

Other changes in hematological parameters in male and female mice did not show a dose-related trend, and therefore these alterations were considered to be unrelated to treatment.

Table 5.3.2-14: Hematology

	Males					Females				
Dose level [ppm]	**0**	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
RBC [10^{12}/l]	9.78 [6]	9.95 [6]	10.12 [8]	**10.23*** [9]	10.07 [2]	9.68 [9]	**10.13*** [10]	10.05 [10]	**10.48**** [10]	9.72 [6]
Hemoglobin [mmol/l]	9.1 [6]	9.3 [6]	**9.5**** [8]	**9.8**** [9]	9.2 [2]	9.3 [9]	9.4 [10]	9.3 [10]	**9.7*** [10]	9.0 [6]
Hematocrit [l/l]	0.455 [6]	0.468 [7]	**0.480**** [8]	**0.484**** [9]	0.471 [2]	0.457 [9]	**0.475*** [10]	0.468 [10]	**0.484**** [10]	0.458 [6]
RDW [%]	13.4 [5]	13.5 [7]	**12.9*** [8]	13.1 [9]	13.4 [2]	13.8 [9]	13.5 [10]	13.5 [10]	**12.8**** [10]	**13.2**** [6]
Reticulocytes [% RBC]	1.7 [5]	**2.7**$^{++}$ [7]	**2.4**$^{+}$ [8]	2.1 [9]	2.1 [2]	2.3 [9]	**2.6**$^{+}$ [10]	**2.5**$^{+}$ [10]	2.2 [10]	2.2 [6]
MCH [fmol]	0.93 [6]	0.93 [6]	0.94 [8]	**0.96**** [9]	0.92 [2]	0.96 [9]	**0.93*** [10]	**0.92**** [10]	**0.93*** [10]	**0.93*** [6]
MCHC [mmol/l]	19.95 [6]	19.76 [6]	19.77 [8]	20.32 [9]	19.60 [2]	20.28 [9]	**19.74*** [10]	19.85 [10]	20.00 [10]	**19.64**** [6]
MCV [fl]	46.6 [6]	47.0 [7]	47.4 [8]	47.3 [9]	46.7 [2]	47.2 [9]	46.9 [10]	46.6 [10]	**46.2*** [10]	47.1 [6]
Platelets [10^{9}/l]	1260 [7]	1234 [7]	1313 [8]	**1511**** [10]	**1579**** [4]	1115 [10]	**1249**** [10]	1205 [10]	1188 [10]	**1257**** [6]
WBC [10^{9}/l]	4.1 [7]	5.7 [7]	5.5 [8]	**8.3**** [10]	3.7 [4]	3.5 [10]	4.9 [10]	**6.1**** [10]	**8.7**** [10]	5.2 [6]
Eosinophils [% WBC]	1.4 [7]	1.5 [7]	1.7 [8]	2.3 [10]	2.3 [5]	3.1 [10]	2.4 [10]	2.2 [10]	2.3 [10]	**1.5**$^{+}$ [7]
Neutrophils [% WBC]	8.8 [7]	**6.3**$^{+}$ [7]	8.4 [8]	7.6 [10]	8.6 [5]	7.7 [10]	7.8 [10]	8.6 [10]	8.4 [10]	8.6 [7]
Monocytes [% WBC]	1.4 [7]	1.3 [7]	1.4 [8]	1.4 [10]	1.1 [5]	1.3 [10]	1.7 [10]	**2.1**$^{++}$ [10]	1.2 [10]	1.8 [7]

$^{+/++}$ Steel-test significant at 5% (+) or 1% (++) level
*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level
[n] = number of samples that could be evaluated

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related:

Males

↑ Alkaline phosphatase (ALP) at 750 ppm

↓ Albumin / globulin ratio at 750 ppm

↓ Cholesterol at ≥ 10 ppm, considered adverse at ≥ 250 ppm

Females

↓ Albumin / globulin ratio at 750 ppm

↓ Cholesterol at ≥ 10 ppm, considered adverse at ≥ 250 ppm

The group mean value for albumin/globulin ratio in females at 250 ppm was statistically significantly different from controls, but not regarded to represent a change of toxicological relevance as values were identical to controls.

In males at 750 ppm, increased ALP levels, reduced albumin/globulin ratio and reduced cholesterol indicated possible liver toxicity. However, the increase in ALP at 750 ppm was minor (25%) while changes in ALP in males were already quite variable (slightly decreased at 10-250 ppm without dose response, slightly increased at 750 ppm); and the change in Alb/Glob ratio was marginal (1.2 vs 1.3 in controls). Similarly, in females, the change of two parameters indicative of impaired liver function (marginal reduction of albumin/globulin ratio [1.2 vs 1.4 in controls] in combination with reduced cholesterol) at 750 ppm was assessed by the study authors as clearly adverse. At lower dose levels (up to 250 ppm), significant clinical chemistry changes were confined to a reduction of cholesterol in both male and female mice.

Other statistically significant changes of clinical-chemistry parameters did not show a dose-related trend, and therefore these alterations were assessed to be incidental, i.e. not considered treatment-related.

Table 5.3.2-15: Clinical chemistry parameters (with statistically significant change)

		Males					Females				
Dose level [ppm]		**0**	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
AST	[U/l]	139.6	66.2	108.2	55.0	144.8	119.6	64.9*	67.2*	60.2*	117.6
ALP	[µkat/l]	80	71*	69**	77	**100****	117	111	110	113	104
Cholesterol	[mmol/l]	2.36	2.09**	1.63**	**1.33****	**1.03****	2.03	1.76**	1.63**	**1.01****	**1.01****
Total protein	[g/l]	52.0	49.0**	48.5**	46.8**	49.3**	49.7	48.5	47.1**	45.1**	47.6*
Total globulin	[g/l]	22.5	21.2	21.1	20.7	22.3	20.5	19.6	19.5^{+}	19.1^{+}	21.3
Albumin	[g/l]	29.6	27.8**	27.5**	26.1**	27.0**	29.2	28.9	27.6**	26.0**	26.3**
Alb / Glob ratio		1.3	1.3	1.3	1.3	**1.2**$^{+}$	1.4	1.5	1.4	**1.4**$^{+}$	**1.2**$^{++}$
Total bilirubin	[µmol/l]	2.4	2.4	2.1	1.2**	1.8**	2.6	2.0**	1.7**	1.3**	1.6**
Glucose	[mmol/l]	13.72	17.17**	18.84**	17.95**	13.48	13.75	14.28	15.12	16.81*	14.58
Urea	[mmol/l]	10.5	11.7	10.4	11.0	10.6	10.1	10.1	11.7*	9.9	10.2
Triglycerides	[mmol/l]	1.05	1.01	0.76	0.80	1.05	0.75	1.02*	0.98	0.56	0.70
Potassium	[mmol/l]	4.47	4.53	4.46	4.31	4.74	4.57	3.86**	4.00**	4.04**	4.33
Calcium	[mmol/l]	2.19	2.20	2.19	2.20	2.15	2.18	2.19	2.14	2.11**	2.13
Phosphate	[mmol/l]	1.63	1.82	1.89	1.73	1.87	2.17	1.65**	1.69**	1.70**	2.01

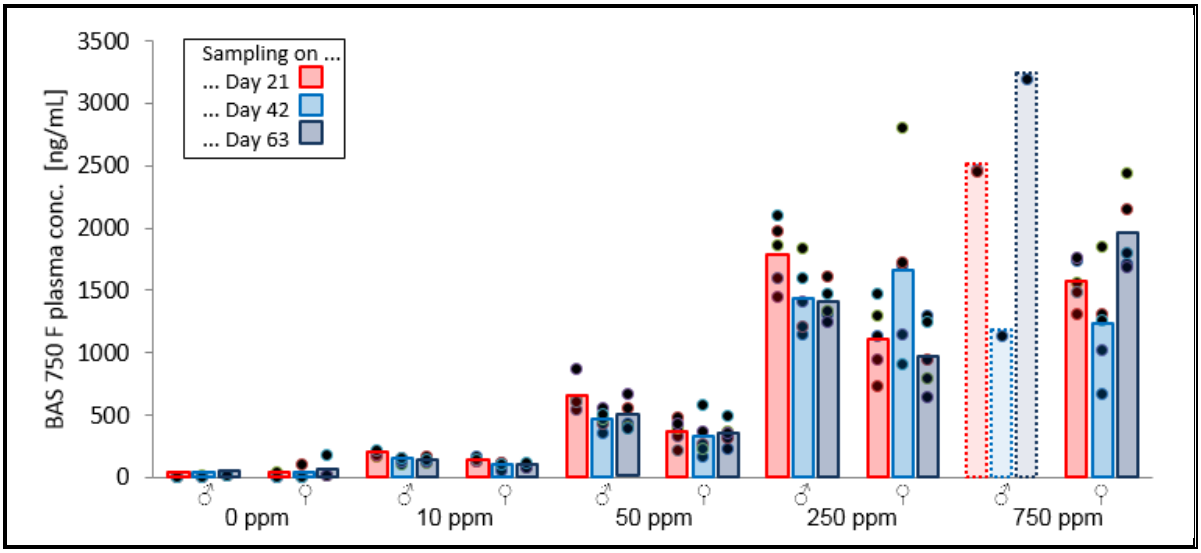
$^{+}$/$^{++}$ Steel-test significant at 5% (+) or 1% (++) level
*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

3. Test substance concentration in blood plasma

Results of the BAS 750 F plasma concentration measurements are presented in Figure 5.3.2-3 and in Table 5.3.2-16. BAS 750 F was detected in 8 of 15 measured male control samples and from 13 of 15 measured female control samples. In male mice, the 1, 2, and 5 detects were found for sampling days 21, 42 and 63, respectively; the concentrations ranged 1.94–12.80 ng/mL and were thus comparable to the quantification limit of 5 ng/mL. In female control mice, a comparably low BAS 750 F concentration range was found for 10 of the control samples with detects (0.64–10.52 ng/mL). The study director considered the presence of BAS 750 F in control samples resulted from a systematic carry-over problem that could not be fully circumvented due to the sticky behavior of the chemical compound. The carry-over issue was considered to be of no practical relevance in view of the high mean plasma concentrations obtained in test groups. Three samples with higher BAS 750 F concentrations were noted in female control group mice, i.e. with female #163 (sampling Day 21: 40.2 ng/mL), with female #162 (sampling Day 42: 93.55 ng/mL) and with female #165 (sampling Day 63: 176.60 ng/mL); the underlying cause of these outlier values could not be identified.

For the highest dose satellite group (750 ppm) 4 of 5 male animals died on Day 21 during the sampling procedure. Therefore, on day 21 only two animals could be sampled and on sampling days 42 and 63, blood from only one animal was available for BAS 750 F measurement. Consequently, in the Figure 5.3.2-3 below, means are not depicted for male satellite group mice given 750 ppm BAS 750 F.

Figure 5.3.2-3: BAS 750 F plasma concentration in satellite group mice



For a given dose level, there were no clear differences in the mean plasma concentration at the three sampling time points - the mean Day 42 value in 250 ppm females could be exaggerated due to a potential outlier value. Generally, plasma concentrations were marginally higher in males than in females (roughly about 1.5-fold) at a given dietary concentration, but very similar if correcting for test substance intake. Independent of sampling time point, a five-fold increase in dietary concentration / test substance intake (i.e. from 10 to 50 ppm or from 50 to 250 ppm) corresponded to a roughly three- to four-fold increase of plasma concentration in both sexes. The 3-fold increase in diet concentration from 250 to 750 ppm corresponded to an about 1.5-fold increase in female plasma concentration, which might suggest saturated absorption at the top dose level.

Table 5.3.2-16: BAS 750 F plasma concentration

		Males		Females	
BAS 750 F Diet concentration [ppm]	**Sampling on Study Day**	**Samples evaluated [N]**	**Mean ± SD [ng/mL]**	**Samples evaluated [N]**	**Mean ± SD [ng/mL]**
0	21	5	0.3879	5	8.838 ± 17.6
	42	5	2.977 ± 5.1	5	19.45 ± 41.4
	63	5	10.87 ± 1.5	5	43.15 ± 74.6
10	21	5	185.4 ± 19.4	5	136.0 ± 16.5
	42	5	134.0 ± 24.7	5	88.98 ± 27.2
	63	5	134.8 ± 19.9	5	85.25 ± 12.5
50	21	5	642.8 ± 125.7	5	366.8 ± 98.8
	42	5	458.4 ± 71.9	5	316.1 ± 162.2
	63	5	487.7 ± 120.7	5	346.5 ± 91.9
250	21	5	1791 ± 274.0	5	1108 ± 290.4
	42	5	1433 ± 283.3	5	1652 ± 735.2
	63	5	1389 ± 145.1	5	980.6 ± 282.4
750	21	2	2454 ± 15.1	5	1565 ± 186.9
	42	1	815.8	5	1215 ± 431.1
	63	1	3183	5	1951 ± 328.4

F. NECROPSY AND PATHOLOGY

1. Organ weight

The following statistically significant alterations of organ weight parameters were considered treatment-related and organ-specific (not secondary to body weight change):

Males

↑ Liver weight and liver/body weight ratio at 50, 250 and 750 ppm

Females

↑ Liver weight and liver/body weight ratio at 250 and 750 ppm

Table 5.3.2-17: Organ weights (selected)

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	26.9				22.6			
	10	26.5	(-1)			22.6	(±0)		
	50	27.2	(+1)			23.2	(+3)		
	250	26.5	(-1)			22.2	(-2)		
	750	25.9	(-4)			21.9	(-3)		
Adrenal glands (mg)	0	4.1		0.0154		100		0.0444	
	10	5.1	(+24)	0.0194	(+26)	98	(-2)	0.0435	(-2)
	50	4.9	(+20)	0.0181	(+18)	110	(+10)	0.0473	(+7)
	250	5.5	(+34)	0.0209	(+36)	98	(-2)	0.0441	(-1)
	750	**6.0***	**(+46)**	**0.0232****	**(+51)**	107	(+7)	0.0489	(+10)
Brain (mg)	0	431		1.604		447		1.977	
	10	432	(±0)	1.633	(+2)	447	(±0)	1.980	(±0)
	50	443	(+3)	1.633	(+2)	442	(-1)	1.917	(-3)
	250	435	(+1)	1.642	(+2)	442	(-1)	1.996	(+1)
	750	434	(+1)	1.677	(+5)	**431***	**(-4)**	1.973	(±0)
Kidneys (mg)	0	358		1.333		275		1.217	
	10	344	(-4)	1.299	(-3)	272	(-1)	1.203	(-1)
	50	361	(+1)	1.327	(±0)	257	(-7)	1.120	(-8)
	250	**320***	**(-11)**	**1.208***	**(-9)**	257	(-7)	1.157	(-5)
	750	**317****	**(-11)**	1.224	(-8)	259	(-6)	1.180	(-3)
Liver (g)	0	1.19		4.45		1.10		4.87	
	10	1.24	(+4)	4.69	(+5)	1.09	(-1)	4.81	(-1)
	50	**1.38****	**(+16)**	**5.09****	**(+14)**	1.16	(+5)	5.03	(+3)
	250	**1.62****	**(+36)**	**6.12****	**(+38)**	**1.36****	**(+24)**	**6.12****	**(+26)**
	750	**2.16****	**(+82)**	**8.34****	**(+87)**	**1.78****	**(+62)**	**8.12****	**(+67)**
Spleen (mg)	0	61		0.228		81		0.359	
	10	62	(+2)	0.235	(+3)	78	(-4)	0.345	(-4)
	50	65	(+7)	0.239	(+5)	77	(-5)	0.334	(-7)
	250	63	(+3)	0.237	(+4)	**66****	(-19)	**0.296****	(-18)
	750	55	(-10)	0.211	(-7)	**62****	(-23)	**0.282****	(-21)

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, two sided)

The following statistically significant changes of organ weight parameters were assessed to be not treatment related or of no toxicological relevance:

Males

- ↑ Adrenal weight and adrenal/body weight ratio at 750 ppm: no corresponding histopathological findings
- ↓ Kidney weight at 250 ppm and 750 ppm: no dose-related trend was apparent when corrected for body weight; no corresponding histopathological findings

Females

- ↓ Brain weight at 750 ppm (abs.); no corresponding significant change in brain/body weight ratio, no histopathological changes
- ↓ Spleen weight and spleen/body weight ratio at 250 and 750 ppm: no corresponding histopathological findings

2. Gross pathology

Macroscopic observations at necropsy did not reveal any alterations that were considered to have arisen as a result of treatment.

The incidences of necropsy findings among control and treated animals were considered to be within the background range of findings that may be expected among mice of this age and strain, and did not show a dose-related trend. These necropsy findings were therefore considered to be of no toxicological relevance.

3. Histopathology

The **liver** was identified as the only target organ. Hepatocellular hypertrophy was observed in males at and above 50 ppm in a centrilobular pattern and in a diffuse pattern in females at and above 250 ppm. The hypertrophy correlated in both sexes with the observed weight increases.

At 250 ppm in males and at 750 ppm in both sexes, the hypertrophy was associated with hepatocellular necrosis and/or cytoplasmic alteration. The cytoplasmic alteration was seen in hypertrophied hepatocytes and was characterized by numerous hyaline inclusions and concentric whorls of cell organelles, and interpreted as an early degenerative change. The centrilobular hepatocellular hypertrophy of minimal-grade severity observed in 50 ppm group male mice was regarded as an adaptive response.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 5.3.2-18: Histopathology

	Males					Females				
Dose level [ppm]	**0**	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
No. of animals	10	10	10	10	10	10	10	10	10	10
LIVER										
examined	10	10	10	10	10	10	10	10	10	10
Infiltration, lymphocytic	9	9	7	10	8	9	8	10	8	9
Grade 1	7	8	6	8	7	7	7	8	5	4
Grade 2	2	1	1	2	1	2	1	2	2	5
Grade 3									1	
Mean grade/tissue affected	*1.2*	*1.1*	*1.1*	*1.2*	*1.1*	*1.2*	*1.1*	*1.2*	*1.5*	*1.6*
Hypertrophy, centrilobular			8	10	10					
Grade 1			8							
Grade 2				4						
Grade 3				6	8					
Grade 4					2					
Mean grade/tissue affected			*1.0*	*2.6*	*3.2*					
Hypertrophy, diffuse									10	10
Grade 1									9	1
Grade 2									1	9
Mean grade/tissue affected									*1.1*	*1.9*
Cytoplasmic alteration				4	5					2
Grade 1				4	3					1
Grade 2					2					1
Mean grade/tissue affected				*1.0*	*1.4*					*1.5*
Necrosis, single cell				2	8					
Grade 1				2	7					
Grade 2					1					
Mean grade/tissue affected				*1.0*	*1.1*					
Necrosis, (multi)focal										6
Grade 1										2
Grade 2										4
Mean grade/tissue affected										*1.7*
Fatty change									*2*	

Severity grades: 1 = minimal; 2 = slight; 3 = moderate; 4 = marked

III. CONCLUSION

Administration of BAS 750 F to C57BL/6JRj mice at the top dose level of 750 ppm caused reductions in body weight gain and clear signs of hepatotoxicity in both sexes, including changes in several clinical chemistry parameters, pronounced liver weight increases with associated hepatocellular hypertrophy, liver cell necrosis and degenerative hepatocellular (cytoplasmic) changes.

At the LOAEL of 250 ppm (58 mg/kg bw/d in males and 67 mg/kg bw/d in females), degenerative liver changes of minimal severity were observed in 6 of 10 male mice. In both sexes, liver weights were moderately increased and associated with liver cell hypertrophy. Cholesterol was statistically significantly reduced in both sexes. Increased levels of red blood cell parameters and platelets in male mice at 250 ppm were assessed as evidence for hemoconcentration, although there was no evidence of polycythemia and no evidence of this in the 2 males assessed at 750 ppm or in females. The equivocal observation may have been caused by decreased water consumption or stress. The Maximum Tolerated Dose (MTD) was considered to be exceeded at 250 ppm in male mice and met/approached at 250 ppm in female mice.

At the dose level of 50 ppm, slight increases in hemoglobin and hematocrit were observed in male mice that were considered to be treatment-related but not adverse. Additional treatment-related findings in male mice at 50 ppm were a reduction in cholesterol, and slightly increased liver weights (abs. +16, rel. +14%) with associated liver cell hypertrophy of minimal severity in 8 of 10 males. These liver changes were considered to be an adaptive response to BAS 750 F exposure and not adverse. In females at 50 ppm, treatment-related effects were confined to slightly reduced cholesterol levels only. The dose level of 50 ppm was therefore considered to be the NOAEL in both sexes, corresponding to intakes of 11 mg/kg bw/d in males and 15 mg/kg bw/d in female mice.

Report:	CA 5.3.2/5 [redacted] 2015 a BAS 750 F - Repeated-dose 90-day oral toxicity study in beagle dogs - Oral administration (capsule) 2015/1000530
Guidelines:	OECD 409 (1998), EPA 870.3150, JMAFF No 12 Nosan No 8147, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.27
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch: COD-001880; Purity: 98.6%) was administered to groups of 5 male and 5 female Beagle dogs at dose levels of 0, 15, 90 and 180 mg/kg bw/d by capsule over a period of 3 months. The scope of investigations was in compliance with OECD Guideline 409. Treatment at the high dose level of 180 mg/kg bw/d caused vomiting, reduced and retarded food consumption in one male and two females; the mean food intake was significantly decreased in females (max. -17%) on study day 7. Reduced body weight was seen in both sexes (with a maximum of -6.4% in males and -5.3% in females) together with statistically significantly decreased body weight gains in both sexes (-49.4% in males, -59.6% in females from day 0-91).

The liver was identified as the major target organ as indicated by changes in a number of clinical chemistry parameters at 180 mg/kg bw/d. Clinical chemistry changes consisted of decreased serum protein values after 6-week treatment in males and after 3-month treatment in females, transiently decreased creatinine values in females after 6-week treatment and increased alkaline phosphatase (ALP) activity in both sexes after 3-month exposure. Relative liver weights were significantly increased in males dogs (+20), which in combination with the clinical chemistry changes were assessed to be adverse. There were no treatment-related macro- or histopathological findings in the liver and other organs and tissues at any dose level.

Based on the observed effects on body weight, food intake, clinical chemistry changes in both sexes and increased liver weight in male dogs at 180 mg/kg bw/d, the NOAEL in this study was identified at 90 mg/kg in male and female dogs.

(DocID 2015/1000530)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F
Description:	solid / white
Batch #:	COD-001880
Purity / content:	98.6%
Stability of test compound:	The test substance was stable over the study period (expiry date 30-Nov-2015).
2. Vehicle:	Hard gelatine capsules, size 11 (volume: 7 mL), Torpac Inc., Fairfield, NJ, USA
3. Test animals:	
Species:	Dog
Strain:	purebred Beagle
Sex:	male and female
Age (Day 0):	♂: 7.2 – 8.5 months; ♀: 7.6 – 8.5 months
Weight at dosing (Day 0):	range: ♂: 10.5 – 13.5 kg; ♀: 8.1 – 11.6 kg means: ♂: 11.8 – 12.1 kg; ♀: 9.8 – 9.9 kg
Source:	BASF; Ludwigshafen/Rhein; Germany Fed.Rep.
Acclimatization period:	at least 7 days
Diet:	Dog maintenance KLIBA laboratory diet, Provimi Kliba SA, Kaiseraugst, Switzerland A daily food ration of about 400 g (males) and 350 g (females) were offered to each animal for a maximum of 2 hours. For animal welfare reasons, when retarded food consumption was observed, the feeding period was extended up to the morning of the subsequent study day.
Water:	Drinking water ad libitum from automatic watering device; in metabolism cage (during urine collection): 500 ml drinking water (from bottle).
Housing:	Single housing in kennels of ~ 2.7 m² indoor and ~ 2.7 m² outdoor; the animals had free access to the outdoor kennel 24h/day.
Environmental conditions:	
Temperature:	ambient (in winter additional heating of the air to achieve approx. 22 °C)
Humidity:	not measured
Air changes:	Forced ventilation, approx. 8 air changes/hour
Photo period:	Natural day/night rhythm with additional artificial light as required during working hours

B. STUDY DESIGN

1. Dates of work: 19-Sep-2014 to 30-Sep-2015 [administration period: 09-Dec-2014 up to 16-Mar-2015

2. Animal assignment and treatment

At the beginning of the acclimatization period, the animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

BAS 750 F was orally administered in capsules once daily in the morning, immediately before the feed ration (portions of 400 g for males and 350 g for females) was offered to the animals. Control group animals received the same number of gelatin capsules as the animals of the high dose group (however empty).

Groups of 5 male and 5 females dogs of test groups 0, 1, 2 and 3 were given BAS 750 F at dose levels of 0, 15 (low dose), 90 (mid dose) or 180 mg/kg bw/d (high dose), respectively.

3. Test substance preparation and analysis

On each day immediately prior to administration, gelatin capsules were filled with appropriate amounts of test substance, which were determined on the basis of each individual animal's weekly body weight. The weights of representative capsules containing the test substance were checked against empty capsules at the start of the study, to ensure dose correctness. The results were documented in the raw data. Homogeneity and concentration control analyses of the test substance in the gelatin capsules were not considered necessary and therefore not carried out.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change and food consumption	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Blood parameters with uni-directional changes	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians
Urinalysis parameters (except pH, urine volume, specific gravity, color and turbidity	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians
Urine pH, volume, specific gravity, color and turbidity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians *Urine color and turbidity are not evaluated statistically.*
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test for the hypothesis of equal medians.

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Each animal was examined once a day for any evident signs of toxicity before and after treatment and, if signs occurred, several times daily.

Detailed clinical observations (open field observations) were performed in all animals prior to the start of the administration period and weekly thereafter. The findings were ranked according to the degree of severity. The chosen housing conditions with kennels of appropriate size including areas inside and outside the building allowed the animals to move freely. Therefore, deviations in motor activity and altered behavior could be determined if evident. The scope of examinations and the scoring of the findings included but was not limited to the following parameters listed:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Mucosal membranes
6. Salivation
7. Respiration
8. Activity/arousal level
9. Tremors
10. Convulsions
11. Abnormal movements
12. Impairment of gait
13. Lacrimation
14. Visible swellings masses
15. Feces (appearance/consistency)
16. Urine (volume color)
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals, on study day -7, at the beginning of the administration period (day 0) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption

The extent of food intake was monitored several times daily. Food consumption of the animals was quantified at weekly intervals, starting one week before the start of test substance administration (day -7). For this purpose, the extent of food intake was measured within a two-hour period immediately following the capsule administration.

4. Ophthalmoscopy

Before the beginning of the administration period, all dogs used in the study were examined for changes of their eyes using a KOWA-fundus camera after administration of a mydriatic. At the end of the administration period the eyes of all animals of the control and highest dose group was examined. The eyes of the animals of the other dose groups were examined only in case of a striking discrepancy between the highest dose group and the control group.

5. Hematology and clinical chemistry

For hematology and clinical chemistry investigation, blood was taken in the morning from the *vena cephalica antebrachii* from fasted animals before start of treatment on study day -11 and on study days 41 and 85. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to ensure reliable test results.

The results of clinical pathology examinations were expressed in International System (SI) units. The following examinations were carried out in all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Quick's test)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)	✓	Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

6. Urinalysis

Urinalysis investigations were carried with fasted animals before the start of test substance administration on study day -13 in males and day -14 in females, and on study days 44/45 and 86/87 in males/females, respectively. For this purposes, food was withdrawn in the afternoon and the animals were transferred to individual metabolism cages for overnight collection of urine. During this time, the dogs had access to about 500 ml of water, but to no food. Urine samples were evaluated in a randomized sequence.

Urinalysis					
	Quantitative parameters		*Semi-quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilinogen
		✓	Glucose	✓	Sediment (microscop. examination)
		✓	Ketones		

7. Plasma concentration analysis

On the morning of day 83 before and 6 hours after capsule administration, approx. 1.2 mL blood samples were taken from the *vena cephalica antebrachii of* all (non-fasted) animals. After addition of EDTA-K3 and centrifugation for preparation of plasma, the plasma samples were stored at -80 °C prior to LC-MS analysis for determination of the BAS 750 F concentration.

8. Sacrifice and pathology

Test substance administration continued through the day prior to necropsy, which was carried out between study days 92-98. The animals were anesthetized and sacrificed by exsanguination from the cervical and brachial vessels. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	✓	adrenals	✓		✓	jejunum (w. Peyer's patches)	✓		✓	rectum
✓		✓	aorta	✓	✓	✓	kidneys	✓		✓	salivary glands*
✓		✓	bone marrow[§]	✓			lachrymal glands[%]				seminal vesicles
✓	✓	✓	brain	✓		✓	larynx	✓		✓	skin
✓		✓	caecum	✓	✓	✓	liver	✓		✓	spinal cord (3 levels)[@]
✓		✓	colon	✓		✓	lung	✓	✓	✓	spleen
✓		✓	duodenum	✓		✓	lymph nodes[#]	✓		✓	sternum w. marrow
✓	✓	✓	epididymides	✓		✓	mammary gland (♀ + ♂)	✓		✓	stomach
✓		✓	esophagus	✓		✓	muscle, skeletal	✓	✓	✓	testes
✓		✓	eyes (with optic nerve)	✓		✓	nerve, peripheral (sciatic n.)	✓	✓	✓	thymus
✓			femur (with joint)	✓		✓	nose/nasal cavity[‡]	✓	✓	✓	thyroid/parathyroid
✓			gall bladder	✓	✓	✓	ovaries and oviduct**	✓		✓	trachea
✓		✓	gross lesions	✓		✓	pancreas	✓		✓	urinary bladder
			Harderian gland	✓		✓	pharynx	✓	✓	✓	uterus
✓	✓	✓	heart	✓	✓	✓	pituitary	✓		✓	vagina
✓		✓	ileum	✓	✓	✓	prostate				

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.

[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

Test substance analyses (stability, homogeneity and concentration control analyses in the test preparation) were not required, because the test substance was not mixed with any vehicle but administered unchanged via gelatin capsules.

1. Test substance concentration in blood plasma

Two blood samples were taken from each dog on day 83 of the study, at the time points 0 hours (t_0), and 6 hours (t_6) post dosing (which was chosen as time point corresponding to the approx. T_{max} based on results of the 28-day range finding study. The plasma from the t=0 sampling time point were taken immediately before the administration of the daily capsule and therefore the corresponding plasma concentrations reflect the residual test substance burden from the previous administration period (day 0-82). Results are presented in Table 5.3.2-19 below.

BAS 750 F was not detected in plasma from control groups. In the test groups, BAS 750 F was detectable in all individual samples and at both time-points of investigation. A dose-dependent increase of plasma concentration was noted in both sexes. At the assumed tmax value (t_6), mean plasma concentrations in males increased from 1101 ng/mL at the low dose to 13604 ng/ml at the high dose; in females, similar mean concentrations of 890 / 14217 ng/mL were obtained at the low / high dose, respectively. However, the mean plasma concentration at the mid dose was about 1.7-fold higher in males than in females.

By comparison of the t_6 with the t_0 data (sampled just before capsule administration), the BAS 750 F concentrations at t_0 were about 16-19% of the t_6 values at the low dose of both sexes and at the mid-dose level of the female dogs. At the mid-dose level in males, a concentration ratio of 32% was obtained, and at the high dose level in males and females, even higher concentration ratios of 54% and 40% were obtained, which appeared to indicate that the excretion at the mid-dose in males and at the high-dose in females is slower than at the low dose.

Table 5.3.2-19: BAS 750 F plasma concentration in dogs (day 83)

	BAS 750 F plasma concentration [ng/ml]					
Dose level [mg/kg bw/d]	**15**		**90**		**180**	
MALES						
0 h	# 6	130.59	# 11	1857.60	# 16	6525.76
	# 7	210.05	# 12	3457.12	# 17	5014.91
	# 8	339.50	# 13	2709.12	# 18	8342.46
	# 9	224.10	# 14	2338.04	# 19	3752.30
	# 10	107.63	# 15	2843.36	# 20	4094.18
	Mean ± SD	**202.4 ± 91.46**	Mean ± SD	**2641 ± 595.2**	Mean ± SD	**5546 ± 1897**
6 h	# 6	1007.62	# 11	7906.69	# 16	13696.30
	# 7	1116.94	# 12	9328.58	# 17	15573.05
	# 8	1250.35	# 13	8596.87	# 18	15540.98
	# 9	1022.19	# 14	7282.47	# 19	12069.10
	# 10	1108.16	# 15	8111.95	# 20	11138.39
	Mean ± SD	**1101 ± 96.88**	Mean ± SD	**8245 ± 767.6**	Mean ± SD	**13604 ± 2005**
FEMALES						
0 h	# 36	145.16	# 41	1373.48	# 46	2109.00
	# 37	144.84	# 42	719.85	# 47	3539.30
	# 38	74.68	# 43	543.26	# 48	12448.26
	# 39	140.07	# 44	1411.30	# 49	16728.67
	# 40	221.28	# 45	877.21	# 50	2410.57
	Mean ± SD	**145.2 ± 51.93**	Mean ± SD	**985.0 ± 390.4**	Mean ± SD	**7447 ± 6714**
6 h	# 36	933.77	# 41	3427.43	# 46	11796.25
	# 37	911.80	# 42	3772.94	# 47	9104.20
	# 38	559.04	# 43	4411.99	# 48	16193.20
	# 39	1024.93	# 44	7342.90	# 49	23373.01
	# 40	1022.20	# 45	5683.48	# 50	10618.35
	Mean ± SD	**890.3 ± 192.1**	Mean ± SD	**4928 ± 1601**	Mean ± SD	**14217 ± 5760**

B. OBSERVATIONS

1. Mortality

No animal died prematurely in the study.

2. Clinical signs of toxicity

In the highest test group (180 mg/kg bw/d), one male animal (No. 140021) showed reduced and retarded food consumption on some isolated days (study days 3, 6, 8 and 10). This animal showed also vomitus on study day 2.

Four female animals of the highest test group (180 mg/kg bw/d) showed reduced and retarded food consumption on some isolated days during the whole study period. Two of these four females showed also vomitus on study days 1 and 2. At 90 mg/kg bw/d, three female animals showed reduced and retarded food consumption on some isolated days in the second half of the study. Male animals of test group 2 showed no clinical findings. At 15 mg/kg bw/d, one female animal showed reduced and retarded food consumption from day 4 onwards over nearly the whole administration period. No findings were observable in male animals of test group 1. In the control group, one female animal also showed reduced and retarded food consumption from day 14 onwards over nearly the whole administration period.

The isolated occurrence of reduced and retarded food consumption was considered to be not-treatment related since one control animal was also affected. However, the findings reduced and retarded food consumption together with vomitus of some male and female animals administered 180 mg/kg bw/d were regarded as treatment-related.

C. FOOD CONSUMPTION

Other than in one male animal (No. 140021) in the highest dose group that also experienced vomitus, food consumption was unaffected by treatment in males.

In females in the highest test group (180 mg/kg bw/d), food consumption was reduced over the whole study period but only with statistical significance on study days 0 and 7 (up to -17% on study day 7).

Food consumption was reduced in female animals given 90 mg/kg bw/d from day 49 onwards (up to -23% on study day 63) but the reduction was not dose-related and also not statistically significant compared to control animals.

The reduced food consumption of females from the top-dose group was considered to be treatment related.

D. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight

In males and females of test group 3 (180 mg/kg bw/d), body weight was reduced over the whole study period but without statistical significance compared to control (up to -6.4% in male and -5.3% in female animals on study day 91). Body weight was also slightly reduced in female animals of test group 2 (90 mg/kg bw/d) over the whole study period (up to -4.2% on study day 91).

Table 5.3.2-20: Mean body weight of dogs administered BAS 750 F for 91 days

	Males				Females			
Dose level [mg/kg bw/d]	0	15	90	180	0	15	90	180
Body weight [kg]								
- Day 0	11.8	11.9	12.1	11.8	9.8	9.9	9.8	9.9
- Day 91	13.4	13.6	13.8	12.6	10.9	10.7	10.4	10.3
Δ% (compared to control)		1.0	2.5	-6.4		-1.1	-4.2	-5.3
Body weight gain [kg]	1.6	1.6	1.6	**0.8***	1.0	0.8	0.6	0.4
Δ% (compared to control)		3.8	2.5	**-49.4**		-19.2	-42.3	-59.6

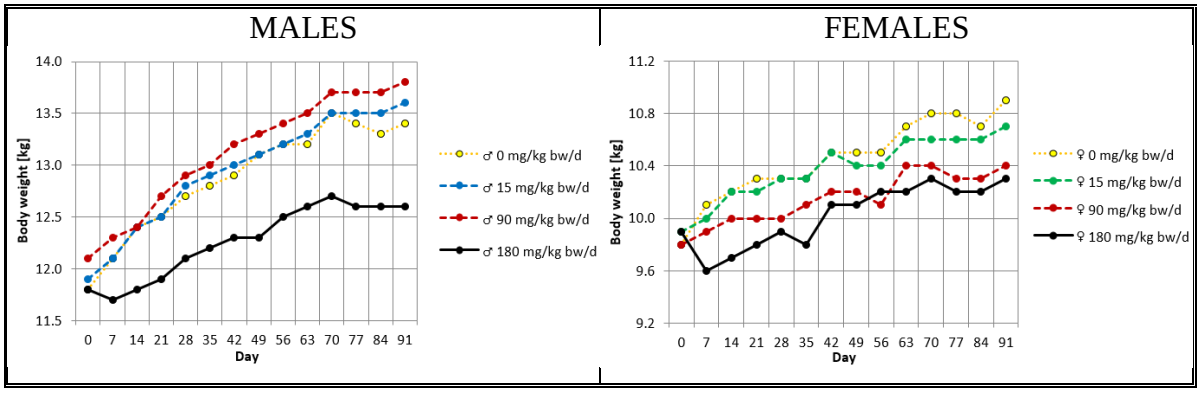
* $p \leq 0.05$ (Dunnett's test, two sided);

2. Body weight change

Mean body weight gain data is summarized in Table 5.3.2-20, the mean body weight development is presented in Figure 5.3.2-4. At 180 mg/kg bw/d in male dogs, body weight change was reduced statistically significantly with a maximum decrease of 49.4% over the whole treatment period (study day 0 to 91). In female animals, body weight change was also reduced at 180 mg/kg bw/d with a maximum decrease of 59.6% over the whole treatment period. The decrease was only statistically significant in the first five weeks of the study (with a maximum decrease of 207% in the first week) and from study day 0 to 77.

The reduced body weight development and change observed in male and female dogs of the high-dose group were considered to be treatment-related and adverse.

Figure 5.3.2-4: Body weight development



E. OPHTHALMOSCOPY

No test substance-related effects were observed.

F. BLOOD ANALYSES

1. Hematology

No treatment-related changes of hematological parameters were observed after 6-wk or 13-wk treatment of male or female dogs up to the highest dose level tested.

It was noted that in females of test groups 2 and 3, mean platelet concentrations were increased after 6- and 13-week treatment. Statistical significance was reached probably due to low mean platelet concentrations in control group females, which were outside of the HCR of the test facility [see Table 5.3.2-21]. After a six-week administration period, activated partial thromboplastin time (PTT) was reduced in females of test group 2 (90 mg/kg bw/d) and relative large unstained cell (LUC) counts were decreased in females of test group 1 (15 mg/kg bw/d). Both parameters were not dose-dependently changed and therefore considered to be incidental.

Table 5.3.2-21: Hematology parameters (selected)

	Week	Males				Females			
Dose level [mg/kg bw/d]		**0**	**15**	**90**	**180**	**0**	**15**	**90**	**180**
Platelets [giga/L]	6	354	325	348	390	250	314	**343****	**367****
	13	336	323	346	318	272	302	**350****	**374****
Historical control (15 studies, sampling 2004-2015) [giga/L]	*6*					*mean: 352; min-max: 289-406$^{\$}$*			
	13					*mean: 331; min-max: 294-386$^{\$}$*			
LUC [%]	6	0.3	0.4	0.5	0.3	0.6	**0.3***	0.5	0.4
	13	0.3	0.3	0.5	0.3	0.5	0.4	0.5	0.3
PTT [s]	6	11.7	11.6	11.1	11.4	12.3	12.0	**11.4****	11.8
	13	12.2	11.8	11.6	11.6	12.6	11.9	11.7	12.1

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)
$^{\$}$ minimum and maximum values obtained in studies with sampling performed between 2011-2012

2. Clinical chemistry

The following changes of clinical-chemistry parameters were considered treatment-related:

Males

- ↑ **Alkaline phosphatase** increased after 3-mo treatment at 180 mg/kg bw/d in both sexes and at 90 mg/kg bw/d in males. At 90 mg/kg bw/d, the mean was only slightly (1.8-fold) increased over the concurrent mean control. Moreover, the mean control group value was below the HCR. The increased ALP value at 180 mg/kg bw/d in male dogs is considered to be adverse. At 90 mg/kg bw/d, the slight ALP increase in male dogs was the only relevantly changed parameter, therefore, this difference to controls was not considered an adverse finding.
- ↓ **Total protein** in group 3 (180 mg/kg bw/d) after 6-wk treatment, which might be the result of reduced albumin (non-significant reduction of about 10%). The finding is considered to be treatment-related. Although the extent of the change is minor (not significantly affecting albumin or globulin concentrations) and not statistically significant after 13 months, the transient finding was assessed to be adverse in combination with other findings indicating a slight impairment of liver function (see discussion).

Females

- ↑ **Alkaline phosphatase** increased at 180 mg/kg bw/d after 3-mo treatment (adverse).
- ↓ **Total protein** at 180 mg/kg bw/d after 6-wk and 13-wk treatment (adverse).
- ↓ **Creatinine** at 180 mg/kg bw/d in females after 6–wk treatment (adverse)
- ↓ **Total bilirubin** at 180 mg/kg bw/d. The slight decrease was probably secondary to increased enzyme induction and resulting enhanced phase II metabolism and excretion of glucuronidated bilirubin, since there was no indication of a hypoplastic anemia. The change was considered to reflect an adaptation response to increased metabolism demand.

Table 5.3.2-22: Clinical chemistry parameters (selected)

	Week	Males				Females			
Dose level [mg/kg bw/d]		**0**	**15**	**90**	**180**	**0**	**15**	**90**	**180**
Alkaline phosphatase [µkat/L]	6	1.54	1.48	2.51	2.59	1.47	1.38	1.99	1.97
	13	1.13	1.14	**2.08***	**3.27***	1.18	1.08	1.73	**2.20***
Hist. control (11 studies, sampling 2004-2015) [µkat/L]	*13*	*mean: 1.44; min-max: 1.35-1.50[$]*							
Creatinine [mmol/L]	6	54.8	59.6	58.1	53.5	59.9	60.2	55.5	**52.4***
	13	54.9	61.1	**61.5***	56.0	61.7	60.9	57.5	52.9
Total protein [g/L]	6	55.18	52.93	54.96	**51.78****	55.66	**51.97****	54.15	**52.50***
	13	55.37	53.35	53.80	51.34	54.84	51.66	53.36	**50.95***
Total bilirubin [µmol/L]	6	1.36	1.49	1.53	1.23	1.93	1.60	1.61	**1.04****
	13	0.62	0.79	0.63	0.34	1.22	0.75	0.82	**0.33****

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)
[$] minimum and maximum values obtained in studies with sampling performed in 2013

The following changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Females

- ↓ **Total protein** in group 1 (15 mg/kg bw/d) after 6-wk treatment (no dose-response related change).

G. URINALYSIS

No treatment-related changes among urinalysis parameters were observed.

H. NECROPSY AND PATHOLOGY

1. Organ weight

The following alterations of organ weight parameters were considered treatment-related and organ-specific (not secondary to body weight change), see Table 5.3.2-23:

Males

- ↑ Liver weight and liver/body weight ratio at 90 and 180 mg/kg bw/d (adverse at 180 mg/kg bw/d)

The following significant changes of organ weight parameters were assessed to be not treatment related or of no toxicological relevance:

Males

- ↑ Relative testes weight at 180 mg/kg bw/d
- ↓ Prostate weight at 90 and 180 mg/kg bw/d (not statistically significant)

Females

- ↑ Relative kidney weight at 180 mg/kg bw/d
- ↑ Relative spleen weight at 90 mg/kg bw/d
- ↓ Uterus and ovary weight at 180 mg/kg bw/d (not statistically significant)

Table 5.3.2-23: Organ weights (selected)

Sex		Males				Females			
Organ weight	Dose [mg/kg bw/d]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	13520				10940			
	15	13640	(+1)			10800	(-1)		
	90	13880	(+3)			10500	(-4)		
	180	12720	(-6)			10280	(-6)		
Liver (g)	0	377.45		2.799		340.95		3.112	
	15	372.38	(-1)	2.739	(-2)	304.89	(-11)	2.825	(-9)
	90	**477.21****	**(+26)**	3.436	(+23)	332.13	(-3)	3.186	(+2)
	180	429.09	(+14)	**3.371****	**(+20)**	378.60	(+11)	3.675	(+18)
Testes / Ovaries [g]	0	17.74		0.131		1.192		0.011	
	15	19.58	(+10)	0.144	(+9)	1.228	(+3)	0.011	(+2)
	90	22.89	(+29)	0.163	(+24)	0.934	(-22)	0.009	(-18)
	180	22.70	(+28)	**0.178****	**(+35)**	0.888	(-26)	0.008	(-22)
Epididymides /	0	3.94		0.029		8.784		0.077	
Uterus [g]	15	4.08	(+4)	0.030	(+3)	13.344	(+52)	0.118	(+53)
	90	3.95	(±0)	0.028	(-3)	7.348	(-16)	0.069	(-10)
	180	3.96	(+1)	0.031	(+7)	4.926	(-44)	0.046	(-40)
Kidneys [g]	0	67.73		0.501		46.47		0.424	
	15	59.79	(-12)	0.437	(-13)	43.03	(-7)	0.396	(-7)
	90	59.64	(-12)	0.431	(-14)	42.14	(-9)	0.401	(-5)
	180	56.95	(-16)	0.447	(-11)	49.17	(+6)	**0.479***	**(+13)**

Table 5.3.2-23: Organ weights (selected)

Sex		Males				Females			
Organ weight	Dose [mg/kg bw/d]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Prostate [g]	0	7.28		0.053					
	15	7.33	(+1)	0.053	(-1)				
	90	4.12	(-43)	0.029	(-46)				
	180	3.71	(-49)	0.029	(-45)				
Spleen [g]	0	29.73		0.220		26.93		0.246	
	15	27.44	(-8)	0.201	(-9)	25.30	(-6)	0.234	(-5)
	90	26.17	(-12)	0.186	(-15)	18.86	(-30)	**0.179****	**(-27)**
	180	28.92	(-3)	0.226	(+3)	21.99	(-18)	0.215	(-12)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis H and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

Details on the organ weight assessment are given below.

Terminal body weights were slightly decreased in males and females of the high dose group (94% of control), but the change did not attain statistical significance. In view of the body weight development curves, the slightly decreased terminal body weights in group 3 animals are considered to be treatment-related.

Liver: The increase in absolute liver weight of test group 2 (90 mg/kg bw/d) males and relative liver weight of test group 3 (180 mg/kg bw/d) males was significant. A treatment-related effect cannot be excluded. In the absence of adverse effects on any other liver parameter, the increased liver weight in males of test group 2 was considered adaptive and non-adverse.

Testes: The significant increase in relative testes weight of males of test group 3 (180 mg/kg bw/d) did not have a histopathologic correlate and the absolute testes weights were within historical control values. It was therefore regarded to be incidental.

Kidney: In females the increase in relative kidney weights in test group 3 (180 mg/kg bw/d) was thought to be caused by the decreased terminal body weight in this test group and there was no histopathologic correlate that could explain the weight increase.

Spleen: The increase of relative spleen weight in females of test group 2 (90 mg/kg bw/d) did not show a dose-response relationship and no histopathologic correlate and was therefore regarded to be not related to treatment.

Prostate: In the male test groups given 90 and 180 mg/kg bw/d, the mean prostate weights were significantly (not statistically) lower than the concurrent control group mean. The weight decrease corresponded to a reduced organ size noted upon gross pathology and histopathology examination in one male dog of the mid-dose and in two dogs of the high-dose level. The change is not regarded to be related to treatment (see discussion below).

Uterus: At the high-dose level of 180 mg/kg bw/d, mean rel. uterus weights were significantly (not statistically) lower than the control mean (-40%). At the mid-dose level, mean uterus weights were decreased by 10%, which was considered to be unremarkable, while at the low dose, the mean uterus weight was increased over controls (+53%). The changes are not regarded to be related to treatment (see discussion below).

Ovaries: Mean rel. ovary weights were decreased by about 20% at 90 and 180 mg/kg bw/d (not statistically significant). At the low dose level of 15 mg/kg bw/d, ovary weight values were comparable to those of the control group. The changes are not regarded to be related to treatment (see discussion below).

2. Gross pathology

The decrease in organ size of the prostate in one male in test group 2 (90 mg/kg bw/d) and in two males of test group 3 (180 mg/kg bw/day) corresponded to the decreased weight in these groups. All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

The liver was identified as only target organ upon histopathological examination. Eosinophilic change of minimal severity was diagnosed in the livers of all dogs from the high-dose group (males and females), at the mid-dose level in all male dogs and in 2 of 5 females, and at the low-dose in 2 of 5 male dogs. The term eosinophilic change was used to describe the cytoplasmic aspect of centrilobular hepatocytes observed in treatment group male and female animals, which differed from the more vacuolated aspect of control animal hepatocytes that are characteristic for the cytoplasmic storage of glycogen.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 5.3.2-24: Histopathology

	Males				Females			
Test group	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Dose level [mg/kg bw/d]	**0**	**15**	**90**	**180**	**0**	**15**	**90**	**180**
No. of animals	5	5	5	5	5	5	5	5
Centrilobular hepatocytes Eosinophilic change of cytoplasm, grade 1	0	2	5	5	0	0	2	5

III. DISCUSSION

Reduced body weight development together with clinical observations comprising reduced and retarded food consumption with single episodes of vomiting in some male and female animals of group 3 (180 mg/kg bw/d) were regarded as treatment-related and adverse.

Regarding clinical pathology, at the dose level of 180 mg/kg bw/d, clinical pathology changes were noted in both sexes, i.e. reduced total protein levels in males (after six weeks) and in females (after six weeks and 3 months), as well as increased alkaline phosphatase activity after 3 months in both sexes. The reason may be some effect on liver cells. On the other hand, an ALP activity increase may be also due to an increased bone growth after a retarded growth at the beginning of the study. The latter hypothesis would fit to the observed lower creatinine values in high-dose group females after six weeks compared to controls which reflected a lower muscle activity. At 90 mg/kg bw/d, the only treatment-related clinical pathology change was a slightly increased alkaline phosphatase activity in males.

Regarding pathology, the liver weight of male animals at 90 and 180 mg/kg bw/d was increased. In combination with changes in several clinical pathology parameters this was regarded to be adverse for the high-dose group (180 mg/kg bw/d) only. For the liver weight increase at 90 mg/kg bw/d there was no dose-response relationship present. Although a treatment-related effect cannot be excluded, the increased liver weight in males at 90 mg/kg bw/d was not regarded to be adverse due to the absence of relevant findings in clinical pathology (limited to a slight change of a single parameter, see above). In almost all test groups, there was a so-called eosinophilic change of the cytoplasmic aspect of hepatocytes of the centrilobular area without any size difference of the hepatocytes. The eosinophilic change itself was regarded as non-adverse due to missing additional findings such as inflammation or degeneration.

Weight deviations observed in genital organs of males and females can be explained by age-related variability in the extent of sexual maturity within the control and test group animals investigated in this study. This is especially true for the observed reduced prostate sizes noted in one male dog at 90 mg/kg bw/d and two male dogs of the high-dose group (180 mg/kg bw/d), and increased mean testes weights in the high-dose group.

Sexual maturity in male Beagle dogs has been reported to occur at an age of about 7-8 months [Goedken MJ et al. (2008), Toxicol. Pathol. 36: 465-471; James RW and Heywood R (1979), Toxicology 12: 234-247]. However, inter-individual variations with regard to the age of reaching maturity are known to occur and are a frequent observation of the test facility. The variability in prostate and testes weights in this study are considered to reflect the variability in the status of sexual maturity since there were no histopathological findings in the prostate, testes or associated organs e.g. epididymides that would have indicated a treatment-related or toxicologically relevant process in any of the individual animals from the control or treatment groups.

Female Beagle dogs reach their first estrus between 8 and 14 month of age. (Rehm S et al. (2007), Birth Defects Res. (Part B) 80: 233-245). In the current study, the absolute and relative uterus and ovaries weight in females of all test groups showed varying weights. Histopathological examination showed that only single animals of control or treated females had already reached the first estrus (presence of functional or old degraded corpora lutea). Furthermore, mammary glands were immature in most animals (characterized by undifferentiated ducts in the dermis of the nipples). In spite of this, a great range of variability in sexual organ development was noted when examining ovaries, vagina, uterus and mammary gland of each female animal. Therefore, the weight changes in uterus and ovaries in females of all test groups was attributed to sexual immaturity, which was ascribed to the age-related variations in reaching the first estrus and sexual maturity.

IV. CONCLUSION

The oral administration of BAS 750 F by capsules to male and female Beagle dogs for 3 months caused test substance-related, adverse signs of toxicity at a dose level of 180 mg/kg bw/d (reductions in food intake body weight and body weight gain, increased alkaline phosphatase, decreased serum protein concentration in both sexes, transiently decreased creatinine in females and increased liver weight in males). Therefore, under the conditions of the present study the no observed adverse effect level (NOAEL) was 90 mg/kg bw/d in male and in female Beagle dogs.

CA 5.3.3 Other routes

Report:	CA 5.3.3/1 [redacted] 2015 b BAS 750 F - Repeated dose 28-day dermal toxicity study in Wistar rats 2014/1170751
Guidelines:	OECD 410, EPA 870.3200, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch: COD-001880; Purity: 98.6%) was applied by dermal application to groups of 10 male and 10 female Wistar rats at dose levels of 0 mg/kg mg/kg bw/d (drinking water containing 0.5% carboxymethylcellulose served as vehicle), 100, 300 and 1000 mg/kg bw/d for 6 hours per day on 5 days a week during a period of 4 weeks (males: 21 applications, females: 22 applications). The scope of examinations were in compliance with OECD Guideline 410 requirements. No treatment-related effects were identified in the study. Based on the results of the study, the NOAEL in both sexes was identified at 1000 mg/kg bw/d, the highest dose tested. (DocID 2014/1170751)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F
Description:	solid / white
Batch #:	COD-001880
Purity / content:	98.6%
Stability of test compound:	The test substance was stable over the study period (expiry date 30-Nov-2015).
2. Vehicle:	0.5% aqueous carboxymethyl cellulose in drinking water
3. Test animals:	
Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Sex:	male and female
Age:	57 ± 1 days at delivery; 63 ± 1 days at start of dosing
Weight at dosing (mean):	♂: ca. 252.3 ± 6.2 g; ♀: ca. 174.9 ± 5.6 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimatization period:	6 days

Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in polycarbonate type III cages (Tecniplast, Hohenpeissenberg, Germany, floor area about 800 cm²) with dust free wooden bedding. Wooden gnawing blocks (NGM E -022 from Abedd Lab and Vet Service GmbH, Vienna, Austria) were provided as environmental enrichtment. Motor activity measurements were conducted in polycarbonate cages from Tecniplast, Germany (floor area about 800 cm²) with small amounts of absorbent material.
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 24-Jun-2014 to 02-Dec-2014 [in life phase: 30-Jun-2014 (start of treatment) to 30-Jul-2014 (necropsy)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 10 male and 10 female rats at dermal dose levels of 0 (vehicle control), 100 (low dose), 300 (mid dose), and 1000 mg/kg bw/d (high dose) for four weeks. The animals were assigned to the treatment groups by means of a computer generated randomization list based on body weights.

The fur of the animals allocated for the study was clipped one day before the first application of the test substance, thereafter when necessary but at least once a week. The test substance was administered uniformly to the clipped dorsal skin (dorsal and dorsolateral parts of the trunk; at least 10% of the body surface) using 3 ml syringes, for about 4 weeks on a 5 days per week basis (males: 21 applications, females: 22 applications). The administration volume was 4 ml/kg body weight, based upon the latest individual body weight determination. After application the skin was covered for 6 hours with a semi-occlusive dressing, consisting of 4 layers of porous gauze dressing and an elastic dressing. After removal of the dressing, the skin was washed with lukewarm water. Control animals were treated with vehicle. All rats were sacrificed after a fasting period (withdrawal of food) of at least 16 hours.

3. Test substance preparation and analysis

The test substance was applied as a suspension in 0.5% aqueous carboxymethylcellulose (0.5% CMC). To prepare the suspension, the appropriate amount of test substance was weighed out depending on the desired concentration. Then the vehicle was filled up to the desired volume and subsequently mixed with Ultraturrax. During application, the test substance preparations were kept homogeneous using a magnetic stirrer. The test substance preparations were prepared at least once a week and stored in a refrigerator during this time.

The stability of the test substance in 1% CMC was demonstrated with a similar batch prior to the start of the study, and found to be stable over a period of seven days. Although the drinking water that was used for the test substance preparations during the study contained only 0.5% CMC, the stability in the water vehicle was assessed to be given.

Before the beginning of the administration period, three samples were taken from all concentrations for homogeneity and concentration analyses.

Table 5.3.3-1: Analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [g/100 mL]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± RSD
2.5	30-Jun-2014	09-Jul-2014	3	2.281	91.2	83.3 ± 14.6
			4	2.231	89.2	
			5	1.733	69.3	
7.5	30-Jun-2014	09-Jul-2014	6	6.541	87.2	
25	30-Jun-2014	09-Jul-2014	7	22.983	91.9	93.3 ± 2.1
			8	23.091	92.4	
			9	23.890	95.6	

Based on the obtained analytical results, the quality criteria for the specification were not reached for the low and mid concentration (90% - 110% of the nominal concentrations, RSD ≤ 5%). Therefore retain probes of samples 03 – 06 were analysed:

Table 5.3.3-2: Re-analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [g/100 mL]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± RSD
2.5	30-Jun-2014	15-Jul-2014	3R	2.415	96.6	95.8 ± 0.7
			4R	2.384	95.4	
			5R	2.385	95.4	
7.5	30-Jun-2014	15-Jul-2014	6R	6.790	90.5	

The mean values (samples 03R – 05R and samples 07 – 09) and the single value (sample 06R) of BAS 750 F in 0.5% CMC were found to be in the range of 90.5–95.8% of the nominal concentrations. Relative standard deviations of the homogeneity samples 3R-5R and 7-9 in the range of 0.7 to 2.1% indicated the homogenous distribution of BAS 750 F in the test substance preparations.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
feces, rearing, grip strength forelimbs and hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily. The findings on the treated skin were recorded immediately before application once on each working day.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly over a period of 1 day and calculated as mean food consumption in grams per animal and day.

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any changes in volume.

5. Ophthalmoscopy

Prior to the start of the administration period on day -4 the eyes of all animals, and on day 25 the eyes of the control and high dose animals were examined for any changes using an ophthalmoscope after administration of a mydriatic agent.

6. Functional observation battery (FOB)

A functional observational battery was performed in all animals at the end of the administration period starting at about 10.00 a.m. For this purpose, the animals were placed individually in polycarbonate cages during the time of the examination. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The findings were ranked according to the degree of severity, if applicable (for details see Part III of the report).

Home cage observations:

During the home cage observation, special attention was paid to posture, tremors, convulsions, abnormal movements and impairment of gait.

Open field observations:

For open field observation, the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm high) and observed for at least 2 minutes. The following parameters were assessed:

1. Behavior on removal from the cage
2. Fur
3. Skin
4. Salivation
5. Nasal discharge
6. Lacrimation
7. Eyes / pupil size
8. Posture
9. Palpebral closure
10. Respiration
11. Tremors
12. Convulsions
13. Abnormal movements / stereotypes
14. Gait abnormalities
15. Activity / arousal level
16. Feces excreted within 2 min (number of fecal pellets / appearance / consistency)
17. Urine excreted within 2 min (amount / color)
18. Number of rearings within 2 minutes

Sensimotor tests / reflexes:

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. Approach response (reaction to an object being moved towards the face)
2. Touch response (touch sensitivity)
3. Visual placing response (vision)
4. Pupillary reflex
5. Pinna reflex
6. Auditory startle response
7. Righting response (coordination of movements)
8. Behavior during handling
9. Vocalisation
10. Tail pinch (pain perception)
11. Forelimb grip strength
12. Hindlimb grip strength
13. Landing foot-splay test
14. Other findings

7. Motor activity measurement

Motor activity was measured on the same day as the FOB was performed, from 14:00 h onwards. The examinations were performed using the TSE Labmaster System (TSE Systems GmbH, Bad Homburg, Germany). For this purpose, the animals were placed in new clean polycarbonate cages with a small amount of bedding for the duration of the measurement. Eighteen beams were allocated per cage. The number of beam interrupts was counted over 12 intervals for 5 minutes per interval. The sequence in which the animals were placed in the cages was selected at random. On account of the time needed to place the animals in the cages, the starting time was "staggered" for each animal. The measurement period began when the first beam was interrupted and finished exactly 1 hour later. No food or water was offered to the animals during these measurements and the measurement room was darkened after the transfer of the last animal.

8. Hematology and clinical chemistry

Blood was withdrawn in the morning of study day 29 from fasted, isoflurane-anesthetized animals from the retro-orbital plexus. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
✓	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis

Urinalysis was not performed in this study.

10. Sacrifice and pathology

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	#	adrenals	✓		#	larynx	✓		#	rectum
✓		#	aorta	✓	✓	✓	liver	✓		#	salivary glands*
✓		#	bone marrow[§]	✓		#	lung	✓		#	seminal vesicles
✓	✓	#	brain	✓		#	lymph nodes[#]	✓		#	Skin treated / untreated
✓		#	cecum	✓		#	mammary gland	✓		#	spinal cord (3 levels)[@]
✓		#	colon	✓		#	muscle, skeletal	✓	✓	#	spleen
✓		#	duodenum	✓		#	nerve, peripheral (sciatic n.)	✓		#	stomach (fore- & glandular)
✓	✓	#	epididymides	✓		#	nose/nasal cavity[‡]	✓	✓	#	testes
✓		#	esophagus	✓	✓	#	ovaries	✓	✓	#	thymus
✓		#	eyes (with optic nerve)	✓		#	pancreas	✓	✓	#	thyroid/parathyroid**
✓		✓	gross lesions	✓		#	pharynx	✓		#	trachea
✓	✓	#	heart	✓		#	pituitary	✓		#	urinary bladder
✓	✓	#	kidneys	✓		#	prostate	✓	✓	#	uterus

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.

[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, [‡] histopathology at level III; ** additional histopathology for low and mid-dose for males

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See ‘I. Material and Methods, B.3’ above

B. OBSERVATIONS

1. Mortality

No animal died prematurely in the present study.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals.

3. Treated skin

No test substance-related effects were observed.

4. Functional observation battery

Home cage observations:	no treatment-related findings
Open field observations:	no treatment-related findings
Sensory motor tests / reflexes:	no treatment-related findings
Feces:	no treatment-related findings
Rearing:	no treatment-related findings
Grip strength forelimbs:	no treatment-related findings
Grip strength hindlimbs:	no treatment-related findings
Landing foot splay:	no treatment-related findings

4. Motor activity

No test-substance-related changes were observed.

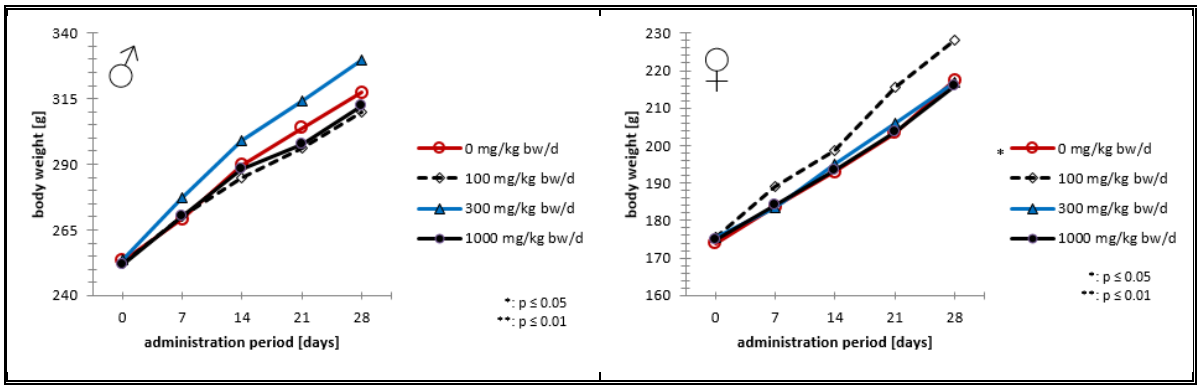
Comparing the single intervals of test substance-treated groups with the control group, isolated changes within the single interval No. 6 was observed for male animals of test groups 2 and 3 (300 and 1000 mg/kg bw/d), i.e. significantly increased in test group 2 (300 mg/kg bw/d) and significantly decreased in test group 3 (1000 mg/kg bw/d). However, as there were no significant deviations with regard to other single intervals as well as to the overall motor activity (summation of all intervals) in male animals of test groups 1-3 (100, 300 and 1000 mg/kg bw/d) these findings were assessed as being not relevant and underscore the inherent variability of motor activity measurements.

C. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight [see Figure 5.3.1-1]

No test substance-related effects on body weight were observed.

Figure 5.3.3-1: Body weight development



2. Body weight gain [see Table 5.3.3-3]

No test substance-related effects on body weight change were observed. A statistically significant difference from controls was obtained for body weight gain of male animals from the mid-dose group during the first treatment week (Days 0-7), which was considered to be an incidental finding.

Table 5.3.3-3: Body weight development

Dose level [mg/kg bw/d]	Males				Females			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Body weight [g]								
- Day 0	253.2	252.1	253.4	251.6	173.9	175.4	175.6	174.7
- Day 28	317.4	309.8	329.9	312.1	217.5	228.1	216.9	216.0
Δ% (compared to control[)]		-2.4	+4.0	-1.7		+4.9	-0.3	-0.7
Overall body weight gain (g)	64.2	57.7	76.5	60.5	43.6	52.7	41.3	41.4
Δ% (compared to control[#]		-10.2	+19.1	-5.8		20.7	-5.3	-5.2
Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)								

D. FOOD CONSUMPTION AND WATER CONSUMPTION

1. Food consumption

No test substance-related effects were observed.

2. Water consumption

No test substance-related effects were observed.

E. BLOOD ANALYSES

1. Hematology

No statistically significant or treatment-related changes of hematological parameters were observed.

2. Clinical chemistry

No statistically significant or treatment-related changes of hematological parameters were observed.

F. NECROPSY AND PATHOLOGY

1. Organ weight

Statistically significant changes of organ weights comprised reduced absolute thyroid weights in mid-dose group males (+21%), decreased relative heart weights in mid-dose group males (-7%) and increased relative liver weights in high-dose group females (+5%).

The significant thyroid weight increase occurred without a dose-dependent relationship and was therefore regarded as incidental and not related to treatment. The decreased heart weight occurred without a dose-dependent relationship and was therefore regarded as incidental. The liver weight increase had no histopathological correlate and was of low magnitude and weak statistical significance. Furthermore, the increased relative liver weight (2.728%) was within the HCR values (2.5–2.842%). Therefore, this change was considered to be incidental and not treatment-related. All other mean absolute and relative weight parameters did not show significant differences when compared to the control group 0.

Table 5.3.3-4: Organ weight findings (statistically significant)

Sex		Males				Females			
Organ weight	Dose [mg/kg bw/d]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	289.45				194.79			
	100	282.52	(-2)			205.08	(+5)		
	300	301.58	(+4)			194.19	(±0)		
	1000	283.30	(-2)			194.58	(±0)		
Heart [g]	0	0.940		0.325		0.773		0.397	
	100	0.918	(-2)	0.325	(±0)	0.783	(+5)	0.382	(-4)
	300	0.916	(-3)	**0.304***	**(-7)**	0.718	(+1)	0.369	(-7)
	1000	0.918	(-2)	0.324	(-1)	0.720	(+5)	0.370	(-7)
Thyroid [mg]	0	17.9		0.006		18.4		0.009	
	100	17.9	(±0)	0.006	(+2)	18.4	(±0)	0.009	(-5)
	300	**21.7***	**(+21)**	0.007	(+16)	17.3	(-6)	0.009	(-6)
	1000	18.3	(+2)	0.006	(+5)	17.7	(-4)	0.009	(-4)
Liver [g]	0	7.129		2.462		5.066		2.601	
	100	7.047	(-1)	2.494	(+1)	5.320	(+5)	2.592	(±0)
	300	7.543	(+6)	2.504	(+2)	5.097	(+1)	2.622	(+1)
	1000	7.022	(-2)	2.477	(+1)	5.305	(+5)	**2.728***	**(+5)**
Historical control range: (10 studies started 2005–2011)						Mean: 5.089 g Min 4.600 g Max: 5.626 g		Mean: 2.641% Min 2.500% Max: 2.842%	

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

2. Gross pathology

All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

Two out of 10 males of test group 3 (1000 mg/kg bw/d) and one out of 10 male control animals showed minimal hypertrophy/hyperplasia of the follicular cells in the thyroid glands. This finding was regarded as incidental and not related to treatment.

All other changes occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 5.3.3-5: Histopathology findings (thyroid)

	Males				Females			
Dose level [mg/kg bw/d]	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
No. of animals	10	10	10	10	10	10	10	10
THYROID								
examined	10	10	10	10	10			10
Hypertrophy/hyperplasia foll	1			2				
Altered colloid	1		1	2				
Cyst, branchiogenic	2		1	1				
Ectopia, thymic tissue				1	2			1
Size, reduced	1							

III. CONCLUSION

The dermal application of BAS 750 F over a period of 4 weeks revealed no signs of local and systemic toxicity in male and female Wistar rats up to a dose level of 1000 mg/kg bw/d. Therefore, under the conditions of the present study, the no observed adverse effect level (NOAEL) was 1000 mg/kg bw/d (limit dose).

CA 5.4 Genotoxicity Testing

BAS 750 F (Reg.No. 5834378) was tested for potential genotoxic properties in a battery of in-vitro and in-vivo assays as is summarized in Table 5.4-1.

Exposure of *Salmonella typhimurium* and *Escherichia coli* tester strains to BAS 750 F up and including the limit concentration of 5000 µg/plate did not produce an increased number of reversions, neither with nor without metabolic activation. Two in-vitro assays for gene mutations (thymidine kinase locus) in mouse lymphoma cells with and without S9-mix were negative, when tested up to precipitating concentrations of BAS 750 F. Likewise, BAS 750 F did not induce any evidence of clastogenic or aneugenic effects in a micronucleus test in V79 cells and in human lymphocytes with and without S9-mix.

The genotoxicity of BAS 750 F was tested under in-vivo conditions in a bone marrow micronucleus assay in NMRI mice. The test did not reveal an increased frequency of micronucleated polychromatic erythrocytes following single oral doses of up to 1500 mg/kg bw. The genotoxicity data set for BAS 750 F is complete and was negative without exceptions. Thus, BAS 750 F is concluded to be free of a genotoxic potential.

A photomutagenicity study with BAS 750 F was not performed. According to Comm. Reg. (EU) No 283/2013, photomutagenicity testing is not required for substances with a UV/VIS molar extinction/absorption coefficient less than 1000 L x mol^{-1} x cm^{-1}. In the UV/VIS wavelength range of 295-700 nm, the molar absorption coefficient of BAS 750 F is clearly below the value 1000 L x mol^{-1} x cm^{-1}; a higher extent of light absorption of BAS 750 F occurs only in the UVC range (ca. 194-290 nm), which is not relevant. Moreover, BAS 750 F did not show any evidence of phototoxicity when tested in an *in-vitro* 3T3 NRU phototoxicity assay (see section MCA 5.2.7, Cetto & Landsiedel, DocID 2015/1117503). According to a tiered approach proposed by the Committee on Mutagenicity of Chemicals in Food consumer Products and the Environment (COM/13/S1), there would be no need for photomutagenicity testing in case of a negative *in-vitro* 3T3 NRU phototoxicity test. In conclusion, photomutagenicity testing of BAS 750 F is not triggered and therefore not required.

General note on photomutagenicity testing requirement:
Currently, no validated or reliable test methods are available for photomutagenicity testing. Comm Reg. (EU) 283/2013 does not provide any guidance on test methods. The recently updated ICH Guideline on Photosafety Evaluation of Pharmaceuticals S10 (2013), explicitly discourages photogenotoxicity testing: *"Testing for photogenotoxicity is not recommended as a part of the standard photosafety testing program. In the past, some regional guidelines (e.g., CPMP/SWP/398/01) have recommended that photogenotoxicity testing be conducted, preferentially using a photoclastogenicity assay (chromosomal aberration or micronucleus test) in mammalian cells in vitro. However, experience with these models since the CPMP/SWP guideline was issued has indicated that these tests are substantially oversensitive and even incidences of pseudo-photoclastogenicity have been reported (Ref. 8). Furthermore, the interpretation of photogenotoxicity data regarding its meaning for clinically relevant enhancement of UV-mediated skin cancer is unclear."*

Table 5.4-1: Genotoxicity studies

Study type	Test system	Dose / concentr. range (batch / purity)	Result	Reference (BASF DocID)
In-vitro reverse mutation assay in bacteria (Ames test)	*S. typhimurium* strains TA 1535, TA 1537, TA 98, TA 100; *E. coli* strain WP2 uvrA; plate incorporation and pre-incubation assay; with/without S9-mix	1 - 5000 µg/plate (COD-001880 / 98.6%)	negative	CA 5.4.1/1 Woitkowiak, 2014a (2014/1128030)
		3.3 - 5000 µg/plate (001651-181 / 97.9%)	negative	CA 5.4.1/3 Woitkowiak, 2015a (2015/1116956)
In-vitro forward mutation assay in mammalian cells (mouse lymphoma assay)	Mouse lymphoma L5178Y cells; with/without S9-mix	3.75 - 60 µg/mL; (COD-001740 / 98.8%)	negative	CA 5.4.1/4 Wollny, 2013a (2015/1112683)
		3.1 - 62.5 µg/mL (001651-181 / 97.9%)	negative	CA 5.4.1/5 Wollny, 2015b (2015/1101908)
In-vitro cytogenicity assay in mammalian cells (micronucleus test)	V79 cells; with/without S9-mix	0.39 – 50 µg/mL (COD-001740 / 98.8%)	negative	CA 5.4.1/6 Schulz & Landsiedel, 2014a (2013/1375108)
	human lymphocytes; with/without S9-mix	2.0 - 8.2 µg/mL (001651-181 / 97.9%)	negative	CA 5.4.1/7 Sokolowski A., 2015a (2015/1101907)
In-vivo micronucleus test	Male NMRI mice; single oral (gavage) application; vehicle: DMSO/corn oil	0-375-750-1500 mg/kg bw (COD-001740 / 98.8%)	negative	CA 5.4.2/1 Schulz et al., 2014a (2014/1043159)

CA 5.4.1 In vitro studies

Report:	CA 5.4.1/1 Woitkowiak C., 2014 a BAS 750 F - Salmonella typhimurium / Escherichia coli reverse mutation assay 2014/1128030
Guidelines:	OECD 471 (1997), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142, EPA 870.5100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 5.4.1/2 Becker M.,Kamp H., 2013 b BAS 750 F - Stability analysis in Dimethyl sulfoxide 2015/1040886
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

S. typhimurium and *E. coli* were exposed to BAS 750 F (Batch: COD-001880, Purity: 98.6%) using dimethyl sulfoxide (DMSO) as a solvent in the presence and absence of metabolic activation in standard plate and pre-incubation tests. Triplicate plates were used per dose and per test condition. Vehicle and positive controls were included in each experiment. In the standard plate and pre-incubation test BAS 750 F was tested at concentrations of 1 to 5000 µg/plate dependent on the strain used. A bacteriotoxic effect (reduced his^- or trp^- background growth, decrease in the number of his^+ revertants) was observed in the standard plate test depending on the strain and test conditions from about 333 µg/plate onward; in the pre-incubation assay bacteriotoxicity was observed from about 33 µg/plate onward. Test substance precipitation was found from about 1000 µg/plate onward with and without S9-mix. A biologically relevant increase in the number of revertant colonies was not noticed in any of the strains tested in the presence or absence of metabolic activation in any of the experiments. The number of revertant colonies in the negative controls was within the range of the historical negative control data for each tester strain in the presence or absence of S9-mix. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

According to the results of the study, BAS 750 F was not mutagenic in the *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay under the experimental conditions of the study. (DocID 2014/1128030)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description: Solid, white

Lot/Batch #: COD-001880

Purity: 98.6% (tolerance ±1.0%)

Stability of test compound: The test substance was stable over the study period under the storage conditions. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically using a comparable batch.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60.0 µg/plate

In order to demonstrate the efficacy of the S9-mix in this assay, the S9 batch was further characterized with benzo(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

3. Activation:

S9 was produced from the livers of induced male Wistar rats. The rats received phenobarbital (80 mg/kg bw; i.p.) and β-naphthoflavone (80 mg/kg bw; orally) each on three consecutive days. 24 h after the last administration the animals were sacrificed and the livers were prepared. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.

The S9-mix was prepared immediately before use and had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10 %

4. Test organisms:

S. typhimurium strains TA98, TA100, TA1535, TA1537; *E. coli* strain: WP2 uvrA

The *Salmonella* strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (uvrB); ampicillin resistance (R factor plasmid).

E. coli WP2 uvrA is checked for UV sensitivity.

Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

Standard Plate incorporation assay (SPT)

- all strains, with/without S9: 0, 33, 100, 333, 1000, 2500, 5000 µg/plate
- TA 1537, without S9: 0, 1.0, 3.3, 10, 33, 100, 333 µg/plate
  (due to strong bacteriotoxicity observed in the first experiment with TA 1537 in the absence of S9-mix, an additional experiment was carried out)

Pre-incubation assay (PIT)

- *S. typhimurium* strains: 0, 3.3, 10, 33, 100, 333, 1000 µg/plate
- *E. coli* WP2 uvrA: 0, 33, 100, 333, 1000, 2500, 5000 µg/plate
  (both with and without S9-mix).

B. TEST PERFORMANCE:

1. Dates of experimental work: 04-Mar-2014 to 21-Mar-2014

2. Plate incorporation assay

To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto Vogel-Bonner agar plates (minimal glucose agar plates). In the experiments with *E. coli* the warm soft agar contains 0.5 mM tryptophan instead of histidine + biotin. After incubation for 48-72 h at 37°C, his+ or trp+ revertants were counted. Three test plates per concentration or per control incubation were carried out.

3. Pre-incubation assay

100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9-mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C the bacterial colonies were counted. Three test plates per concentration or per control incubation were carried out.

4. Statistics

No special statistical tests were performed.

5. Evaluation criteria

Solubility:

Precipitation of the test material was recorded and indicated in the tables. Even in the case of relatively insoluble test compounds, 5 mg/plate was generally selected as the maximum dose at least in the 1st experiment and analyzed (in cases of nontoxic compounds) to detect possible mutagenic impurities, as long as precipitation did not interfere with the colony scoring. Furthermore, doses > 5 mg/plate might also be tested in repeat experiments for further clarification or substantiation.

Toxicity:

Toxicity detected by a

- Decrease in the number of revertants (factor ≤ 0.6)
- Clearing or diminution of the background lawn (reduced his- or trp- background growth)

was recorded for all test groups both with and without S9-mix in all experiments and indicated in the tables.

Mutagenicity:

The test substance is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and *E. coli* WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9-mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance (comparable batch) at room temperature in the vehicle DMSO was verified analytically (Project No. 01Y0741/11Y125, see DocID 2015/1040886).

B. TOXICITY AND SOLUBILITY

In the standard plate test, a bacteriotoxic effect (reduced his- or trp- background growth, decrease in the number of his+ revertants) was observed depending on the strain and test conditions from about 333 µg/plate onward.

In the pre-incubation assay, bacteriotoxicity was observed depending on the strain and test conditions from about 33 µg/plate onward.

Test substance precipitation was found from about 1000 µg/plate onward with and without S9-mix.

C. MUTATION ASSAYS

Neither in the plate incorporation test (see Table 5.4.1-1) nor in the pre-incubation experiment (see Table 5.4.1-2) with and without metabolic activation a biologically relevant increase in number of revertants was observed in any strain tested.

Table 5.4.1-1: Ames test (SPT) with BAS 750 F- Mean number of revertants

Experiment 1: Plate incorporation assay[#]										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	35	16	57	47	9	12	7	6	60	60
Test substance										
33 µg/plate	24	21	54	30	11	12	7	6	60	45
100 µg/plate	28	19	51	41	12	10	8	5	64	56
333 µg/plate	19	14	37	27	11	8	5 B	0.3 B	57	47
1000 µg/plate	7 BP	6 BP	12 BP	7 BP	7 P	9 BP	4 BP	0 BP	61 P	54 P
2500 µg/plate	0.3 BP	1 BP	0 BP	0 BP	2 P	2 BP	0 BP	0 BP	58 P	54 P
5000 µg/plate	0 BP	0 BP	0 BP	0 BP	0 P	0 BP	0 BP	0 BP	55 P	48 BP
Pos. control[§]	2497	374	2867	3732	264	5343	258	1022	275	864
Experiment 2[+]: Plate incorporation assay[#]										
Strain							TA 1537			
Metabol. activation								-S9		
Neg. control (DMSO)								9		
Test substance										
1.0 µg/plate								7		
3.3 µg/plate								8		
10 µg/plate								8		
33 µg/plate								8		
100 µg/plate								7		
333 µg/plate								2 B		
Pos. control[§]								2127		

[+]: Data from repeated experiments were included in the table for TA 1537
[#]: Numbers may differ from original data due to rounding
[§] = Compound and concentrations see Material and Methods (I.A.2.) above
B = reduced background growth, P = precipitation

The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system.

Table 5.4.1-2: Ames test (PIT) with BAS 750 F - Mean number of revertants

Experiment 3/4[+]: Pre-incubation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	23	17	61	28	9	7	10	5	80	94
Test substance										
3.3 µg/plate	29	11	46	36	10	7	8	6	NA	NA
10 µg/plate	25	14	56	28	10	9	8	7	NA	NA
33 µg/plate	20	13	49	35	9	8	5	6	96	89
100 µg/plate	28	12	54	28	9	5	4	2 [B]	91	84
333 µg/plate	22	7 [B]	35	16 [B]	6	6 [B]	4 [B]	0 [B]	79	60
1000 µg/plate	16 [BP]	4 [BP]	9 [BP]	0 [BP]	7 [BP]	4 [BP]	0 [BP]	0 [BP]	80 [P]	55
2500 µg/plate	NA	NA	NA	NA	NA	NA	NA	NA	62 [P]	73 [P]
5000 µg/plate	NA	NA	NA	NA	NA	NA	NA	NA	74 [BP]	77 [BP]
Pos. control[§]	1164	397	1527	1936	215	1560	117	1633	208	367

[+]: Data from experiment 3 with S. typhimurium strains and from experiment 4 with E.coli strain WP2 uvrA
[#]: Numbers may differ from original data due to rounding; NA = test concentration not assayed
[§] = Compound and concentrations see Material and Methods (I.A.2) above
B = reduced background growth, P = precipitation

III. CONCLUSION

According to the results of the present study, the test substance BAS 750 F is not mutagenic in the bacterial reverse mutation assay (Ames test) in the absence and the presence of metabolic activation.

Report:	CA 5.4.1/3 Woitkowiak C., 2015 a BAS 750 F - Salmonella typhimurium / Escherichia coli - Reverse mutation assay 2015/1116956
Guidelines:	OECD 471, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142, EPA 870.5100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

S. typhimurium and *E. coli* strains were exposed to BAS 750 F (Batch: 01651-181, Purity: 97.9%) using dimethyl sulfoxide (DMSO) as a solvent in the presence and absence of metabolic activation (hepatic S9-mix of phenobarbital/ß-naphthoflavone induced rats) in standard plate (SPT) and pre-incubation tests (PIT). Triplicate plates were used per dose and per test condition. Vehicle and positive controls were included in each experiment. Tested concentrations ranged from 3.3 to 5000 µg/plate and from 1.0 to 1000 µg/plate for SPT and PIT, respectively.

A bacteriotoxic effect (reduced his^- or trp^- background growth, decrease in the number of his^+ revertants) was observed in SPT and PIT from about 100 µg/plate onward. Test substance precipitation was found from about 1000 and 333 µg/plate onward in SPT and PIT, respectively, with and without addition of S9-mix. A biologically relevant increase in the number of revertant colonies was not noticed in any of the strains tested in the presence or absence of metabolic activation in any of the experiments. The number of revertant colonies in the negative controls was within the range of the historical negative control data for each tester strain in the presence or absence of S9-mix. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

According to the results of the study, BAS 750 F was not mutagenic in the *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay under the experimental conditions of the study. (DocID 2015/1116956)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description: Solid, white

Lot/Batch #: 01651-181

Purity: 97.9% (tolerance ±1.0%)

Stability of test compound: The test substance was stable over the study period under the storage conditions (Expiration date: 01-Jun-2018). The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically using a comparable batch.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control, without bacteria) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60.0 µg/plate

In order to demonstrate the efficacy of the S9-mix in this assay, the S9 batch was further characterized with benzo(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

3. Activation:

S9 was produced from the livers of at least 5 induced male Wistar (Crl:WI(Han)] rats. The rats received phenobarbital (80 mg/kg bw; i.p.) and β-naphthoflavone (80 mg/kg bw; orally) each on three consecutive days. 24 hours after the last administration the animals were sacrificed and the liver S9 fraction were prepared at stored at ≤ -70°C. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.

The S9-mix was prepared immediately before use and had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10 %

4. Test organisms:

S. typhimurium strains TA98, TA100, TA1535, TA1537; *E. coli* strain: WP2 uvrA

The *Salmonella* strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (Δ uvrB); ampicillin resistance (R factor plasmid).

E. coli WP2 uvrA is checked for UV sensitivity.

Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

Standard Plate incorporation assay (SPT)

- all strains, with/without S9: 0, 33, 100, 333, 1000, 2500, 5000 µg/plate
- TA 1537, with/without S9: 0, 3.3, 10, 33, 100, 333, 1000 µg/plate (due to strong bacteriotoxicity observed in the first experiment)

Pre-incubation assay (PIT)

- all strains, with/without S9: 0, 3.3, 10, 33, 100, 333, 1000 µg/plate
- TA 1537, with/without S9: 0, 1.0, 3.3, 10, 33, 100, 333 µg/plate (due to strong bacteriotoxicity observed in the first experiment)

B. TEST PERFORMANCE:

1. Dates of experimental work: 06-May-2015 to 29-May-2015

2. Plate incorporation assay

To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto minimal glucose agar plates. In the experiments with *E. coli* the warm soft agar contains 0.5 mM tryptophan instead of histidine + 0.5 mM biotin. After incubation for 48-72 h at 37°C, his+ or trp+ revertants were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perceptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System. Three test plates per concentration or per control incubation were carried out.

3. Pre-incubation assay

100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9-mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C the bacterial colonies were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perceptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System. Three test plates per concentration or per control incubation were carried out.

4. Statistics:

No special statistical tests were performed.

5. Evaluation criteria:

Solubility:

Precipitation of the test material was recorded and indicated in the tables. As long as precipitation did not interfere with the colony scoring, 5 mg/plate was generally selected and analyzed (in cases of nontoxic compounds) as the maximum dose at least in the 1st experiment even in the case of relatively insoluble test compounds to detect possible mutagenic impurities. Furthermore, doses > 5 mg/plate might also be tested in repeat experiments for further clarification or substantiation.

Toxicity:

Toxicity detected by a

- Decrease in the number of revertants (factor ≤ 0.6)
- Clearing or diminution of the background lawn (reduced his- or trp- background growth)

was recorded for all test groups both with and without S9-mix in all experiments and indicated in the tables.

Mutagenicity:

The test substance is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and *E.coli* WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9-mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance (comparable batch, COD-001740) at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (Project No. 01Y0741/11Y125, see DocID 2015/1040886).

B. TOXICITY AND SOLUBILITY

A bacteriotoxic effect, evident by reduced his^- or trp^- background growth and/or decrease in the number of his^+ or trp^+ revertants was observed depending on the strain and test conditions from about 100 µg/plate onward, both in the SPT and PIT.

Test substance precipitation was noticed with and without S9-mix from about 1000 and 333 µg/plate onward in the SPT and PIT, respectively.

C. MUTATION ASSAYS

Neither in the SPT (see Table 5.4.1-3) nor in the PIT experiments (see Table 5.4.1-4) with and without metabolic activation a biologically relevant increase in number of revertants was observed in any strain tested. The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system.

Table 5.4.1-3: Ames test (SPT) with BAS 750 F - Mean number of revertants

Experiment 1: Plate incorporation assay[#]										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	17	19	95	104	17	17	12	8	21	21
Test substance										
33 µg/plate	20	16	96	91	15	16	8	6	21	17
100 µg/plate	18	13	100	89	11	13	10	6	20	17
333 µg/plate	16	12	85	98	14	14	12	6	22	21
1000 µg/plate	16 BP	6 BP	36 BP	54 BP	10 P	12 P	11 BP	0 BP	16 P	15 P
2500 µg/plate	8 BP	4 BP	16 BP	26 BP	8 BP	8 BP	0 BP	0 BP	13 BP	13 BP
5000 µg/plate	0 BP	0 BP	0 BP	0 BP	5 BP	7 BP	0 BP	0 BP	14 BP	12 BP
Pos. control[§]	2351	443	2819	3192	323	5311	241	1404	149	1495
Experiment 2[+]: Plate incorporation assay[#]										
Strain							TA 1537			
Metabol. activation							+S9	-S9		
Neg. control (DMSO)							9	7		
Test substance										
3.3 µg/plate							9	6		
10 µg/plate							8	10		
33 µg/plate							7	7		
100 µg/plate							4	6		
333 µg/plate							6	4 B		
1000 µg/plate							6 BP	0 BP		
Pos. control[§]							182	1081		

[+]: Data from repeated experiments were included in the table for TA 1537
[#]: Numbers may differ from original data due to rounding
[§] = Compound and concentrations see Material and Methods (I.A.2.) above
B = reduced background growth, P = precipitation

Table 5.4.1-4: Ames test (PIT) with BAS 750 F - Mean number of revertants

Experiment 3: Pre-incubation assay [#]										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	25	18	92	80	12	10	15	7	22	20
Test substance										
3.3 µg/plate	22	21	96	97	8	11	9	7	19	23
10 µg/plate	26	12	94	89	7	12	10	8	22	18
33 µg/plate	20	15	89	92	10	14	13	6	26	16
100 µg/plate	26	12	101	74	8	10	14	3	24	17
333 µg/plate	29 BP	10 BP	91 BP	9 BP	11 BP	6 BP	0 BP	0 BP	21 BP	23 BP
1000 µg/plate	12 BP	0 BP	75 BP	0 BP	5 BP	0 BP	0 BP	0 BP	10 BP	12 BP
Pos. control[§]	1682	451	2327	2308	222	2470	167	1006	68	498
Experiment 4[+]: Pre-incubation assay [#]										
Strain							TA 1537			
Metabol. activation							+S9	-S9		
Neg. control (DMSO)							7	5		
Test substance										
1.0 µg/plate							8	7		
3.3 µg/plate							10	9		
10 µg/plate							9	6		
33 µg/plate							9	7		
100 µg/plate							6 B	6 B		
333 µg/plate							0 BP	0 BP		
Pos. control[§]							144	553		

[+]: Data from repeated experiments were included in the table for TA 1537
[#]: Numbers may differ from original data due to rounding
[§] = Compound and concentrations see Material and Methods (I.A.2.) above
B = reduced background growth, P = precipitation

III. CONCLUSION

According to the results of the present study, the test substance BAS 750 F is not mutagenic in the bacterial reverse mutation assay (Ames test) in the absence and the presence of metabolic activation, under conditions applied.

Report:	CA 5.4.1/4 Wollny H.-E., 2015 a BAS 750 F: In vitro cell mutation assay at the thymidine kinase locus (TK+/-) in mouse lymphoma L5178Y cells 2015/1112683
Guidelines:	OECD 476 (1997), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.17, EPA 870.5300, EPA 712-C-98-221
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

EXECUTIVE SUMMARY

BAS 750 F (Batch: COD-001740, purity: 98.8%) was tested *in vitro* for its ability to induce forward mutations in mammalian cells by assessing the mutation of the TK locus in Mouse Lymphoma L5178Y cells. Two independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. Based on the results of a preliminary cytotoxicity assay concentrations up to 60 µg/mL were used in the main experiment. The treatment intervals for both experiments in the presence and absence of metabolic activation were generally 4 hours, except in experiment II (in the absence of metabolic activation) where a treatment interval of 24 h was applied. Methylmethanesulfonate (MMS) and cyclophosphamide (CPA) served as positive controls in the experiments without and with metabolic activation, respectively. After the incubation period treatment media were replaced by culture medium in both experiments and the cells were incubated for 48 h for expression of mutant cells. This was followed by incubation of cells in selection medium containing TRIFLUOROTHYMIDINE (TFT) for about 10 - 15 days.

No substantial and reproducible dose dependent increase in mutant colony numbers was observed in both main experiments. No relevant shift of the ratio of small versus large colonies was observed up to the maximal concentration of the test substance. Appropriate reference mutagens (MMS and CPA) were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

Based on the results of the study it is concluded that BAS 750 F is considered to be non-mutagenic in this mouse lymphoma assay.

(DocID 2015/1112683)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description: Solid, white

Lot/Batch #: COD-001740

Purity: 98.8%

Stability of test compound: The test substance was stable over the study period under the storage conditions. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Solvent control: 0.5% (v/v) DMSO in culture medium

Positive control -S9: Methylmethanesulfonate (MMS) 19.5 µg/mL (exp. I) and 13.0 µg/mL (exp. II) (purity: ≥99%; dissolved in nutrient medium)

Positive control +S9: Cyclophosphamide (CPA) 3 and 4.5 µg/mL (purity: ≥98%; dissolved in 0.9% saline)

3. Activation: S9 was produced from the livers of rats pretreated with β-naphthoflavone/phenobarbital. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction (protein concentration 38.4 mg/mL) was thawed at room temperature and an appropriate amount of S9-fraction was mixed with S9-cofactor solution to result in a final protein concentration of 15 mg/mL in the S9-mix (the corresponding protein concentration in the cultures was set to 0.75 mg/mL).

The S9-mix had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9-supernatant (protein concentration)	15 mg/mL

In order to demonstrate the efficacy of the S9-mix in this assay, the S9 batch was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene.

4. Test organism: L5178Y mouse lymphoma cells were used. They have a high proliferation rate (doubling time about 10-12 h), a high cloning efficiency (>50%) and a stable karyotype with a near diploid (40 ± 2) chromosome number. Stocks of the L5178Y cell line were maintained at -196°C in liquid nitrogen. Each batch used for mutagenicity testing was checked for mycoplasma contamination, karyotype stability, and spontaneous mutant frequency. Prior to treatment, spontaneous TK-deficient mutants were eliminated from the stock cultures by growing the cells for one day in pretreatment medium 1 and two days in pretreatment medium 2 (see below).

5. Culture media

Complete Culture medium: RPMI 1640 medium with 15% horse serum (3% horse serum during 4-h treatment), 1% of 100 U/100 µg/mL penicillin/streptomycin, 220 µg/mL sodium pyruvate, and 0.5 - 0.75% amphotericin used as antifungal agent.

Pretreatment medium 1: ("HAT" medium): RPMI medium containing hypoxanthine (5 mM), aminopterin (0.02 mM), thymidine (1.6 mM), and glycin (5 mM).

Pretreatment medium 2: RPMI 1640 medium containing hypoxanthine (0.1 mM) and thymidine (1.6 mM).

Selection medium: Complete culture medium supplemented with 5 µg/mL trifluorothymidine (TFT).

6. Locus examined: thymidine kinase (TK)

7. Test concentrations:

Preliminary toxicity assay: Eight concentrations ranging from 31.5 to 4026 µg/mL (corresponding to a max. concentration of ca. 10 mM)

Mutation assay: The dose range of the main experiments was set according to data generated in the pre-experiment. In both main experiments the individual concentrations were generally spaced by a factor of 2.0 in the lower range. A narrower spacing was used at the highest concentrations to cover the expected cytotoxic range more closely.

- Experiment I + II: 0, 3.75, 7.5, 15, 30, 45, 60 µg/mL
(with / without metabolic activation)

B. TEST PERFORMANCE:

1. Dates of experimental work: 10-July-2013 to 19-Aug-2013

2. Preliminary cytotoxicity assay

A pre-test was performed in order to determine the concentration range of the mutagenicity experiments. Both, pH value and osmolarity were determined at the maximal concentration of the test substance (4026 µg/mL corresponding to about 10 mM) and in the solvent control without metabolic activation.

$1x10^7$ cells ($3x10^6$ cells at the beginning of 24-hour treatment) were exposed to each concentration of the test substance for 4 and 24 hours without and 4 hours with metabolic activation. The serum concentration was 3% during 4-hour treatment and 15% during 24-h treatment. Following treatment the cells were washed twice by centrifugation (425 x g, 10 min) and re-suspended in "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium for a 2-day growth period. The cell density was determined immediately after treatment and at each day of the growth period and adjusted to $3x10^5$ cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated at the end of the growth period.

3. Mutation Assay:

Cell treatment and expression period:

The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment period of 24 hours in the absence of metabolic activation and with a treatment period of 4 hours in the presence of metabolic activation.

For each test group, about $1x10^7$ ($3x10^6$ during 24 h exposure) cells per flask (80 cm^2 flasks) suspended in 10 mL RPMI medium with 3% horse serum (15% horse serum during 24-h exposure) were exposed to various concentrations of the test substance either in the presence or absence of metabolic activation. Positive and solvent controls were performed in parallel. After 4 hours (24 hours in the second experiment without S9-mix) the test substance was removed by centrifugation (425 x g, 10 min) and the cells were washed twice with "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium and incubated for an expression and growth period of 48 hours. The cell density was determined each day and adjusted to $3×10^5$ cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number according to the method of Clive and Spector.

Seeding for selection and cloning efficiency

After the expression period the cultures were selected. Cells from each experimental group were seeded into 2 microtiter plates so that each well contained approximately 4×10^3 cells in selective medium with TFT. The viability (cloning efficiency) was determined by seeding about 2 cells per well into microtiter plates (same medium without TFT). The plates were incubated at 37°± 1.5°C in 4.5% CO_2/95.5% water saturated air for 10-15 days. Then the plates were evaluated. The relative total growth (RTG) is calculated by the RSG multiplied by the viability.

Colony counting and determination of colony size distribution

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test substance. Criteria to determine colony size were the absolute size of the colony (more than 1/3 of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the optical density of the large ones).

Calculations and processing of the data:

The data listed in the tables of results are calculated and processed as described in Table 5.4.1-5.

Table 5.4.1-5: Overview on calculation and processing of data

Pre-test	
Total suspension growth (4-h treatment):	(cell number at 24 h / cell number at 4 h) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
Total suspension growth (24-h treatment):	(cell number at 24 h / cell number of seeded cells per mL (100000)) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
Relative suspension growth:	total suspension growth × 100 / total suspension growth of corresponding control
Main test	
Total suspension growth (4-h treatment):	(cell number at 24 h / cell number at 4 h) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
Total suspension growth (24-h treatment):	(cell number at 24 h / cell number of seeded cells per mL (100000)) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h) × (cell number at 72 h / if cell number at 48 h > 300000 then 300000, if cell number at 48 h < 300000 then cell number at 48 h)
Relative suspension growth:	total suspension growth × 100 / total suspension growth of corresponding control
Relative total growth:	relative suspension growth × relative cloning efficiency / 100
Cloning efficiency (viability):	-ln(mean number of empty wells per plate / 96) / cells seeded per well
Relative cloning efficiency:	cloning efficiency × 100 / cloning efficiency of corresponding control
Cells survived:	cloning efficiency × cell number seeded in TFT medium
Mutant colonies / 10^6 cells:	(small mutant colonies + large mutant colonies)
Small mutant colonies / 10^6 cells:	(-ln(mean number of wells not containing small colonies / 96) / cells survived) × 10^6
Large mutant colonies / 10^6 cells:	(-ln(mean number of wells not containing large colonies / 96) / cells survived) × 10^6
Threshold	number of mutant colonies per 10^6 cells of each solvent control plus 126
cloning efficiency (viability):	Cloning efficiency determined after the expression period to measure viability of the cells without selective agent

4. Statistics:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT®11 (SYSTAT Software, Inc.) statistics software. The number of mutant colonies obtained for the groups treated with the test substance was compared to the solvent control groups. A trend was judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological relevance and statistical significance were considered together.

5. Acceptability criteria:

A mutation assay is considered acceptable if it meets the following criteria (the current recommendations of the International Workgroup on Genotoxicity Test Procedures (IWGT) are considered):

- All plates, from either the cloning efficiency or the TFT resistance-testing portion of the experiment are analyzable.
- The absolute cloning efficiency at the time of mutant selection (CE) of the solvent controls is 65-120%.
- The total suspension growth of the solvent control calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number is 8-32. Following 24 h treatment the total suspension growth is 32-180.
- The range of the solvent control mutant frequency (MF) is in the range of 50-170 x 10^{-6} cells.
- The positive controls (MMS and CPA) should yield an absolute increase in total MF that is an increase above spontaneous background MF (an induced MF [IMF]) of at least 300 x 10^{-6} cells. At least 40% of the induced mutation frequency (IMF) should be reflected in the small colony MF. Alternatively, the positive controls should induce at least 150 small colonies.
- The upper limit of cytotoxicity observed in the positive control culture should be the same as for the experimental cultures (i.e. the relative total growth -RTG- should be greater than 10% of the concurrent selective control group).
- The highest concentration of the test substance should be 10 mM or 5000 µg/mL, unless limited by toxicity or solubility of the test substance. If toxicity occurred, the highest concentration should lower the relative total growth to approximately 10 to 20% of survival. If precipitation is noted, the highest analyzed concentration should be the lowest concentration where precipitation is observed by the naked eye.

6. Evaluation criteria:

A test substance is classified as mutagenic if at least one of the following criteria is met:

- The induced mutation frequency reproducibly exceeds a threshold of 126 colonies per 10^6 cells above the corresponding solvent control.
- A relevant increase of the mutation frequency should be dose-dependent and correspondingly statistically significant.
- A mutagenic response is considered to be reproducible if it occurs in both parallel cultures.

The test substance is considered non-mutagenic if at least one of the following criteria is met:

- The mutation frequency is below a threshold of 126 colonies per 10^6 cells above the concurrent solvent control value.
- No evidence of reproducibility of an increase in mutant frequencies is obtained.
- No statistical significant dose-related increase in mutant frequencies using an appropriate statistical trend.
- If the threshold is reproducibly exceeded but the increase of the mutation frequency is not dose dependent and a biological relevance of the effect can be excluded.

However, in the evaluation of the test results the historical variability of the mutation rates in the solvent controls of this study were taken into consideration.

Results of test groups are generally rejected if the relative total growth is less than 10% of the vehicle control unless the exception criteria specified by the IWGT recommendations are fulfilled.

Whenever a test substance is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated.

A test substance is classified as non-mutagenic if the induced mutation frequency does not reproducibly exceed a threshold of 126 colonies per 10^6 cells above the corresponding solvent control.

A test substance not meeting the conditions for a classification as mutagenic or non-mutagenic will be considered equivocal in this assay and may be considered for further investigation.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

Cytotoxic effects leading to RSG values below 50% were observed following 4 hours treatment at 62.9 µg/mL and above with and without metabolic activation. After 24 hours treatment cytotoxic effects as described above were noted at 31.5 µg/mL and above. The test medium was checked for precipitation at the end of each treatment period (4 or 24 hours) before the test substance was removed. Precipitation was observed by the unaided eye at 62.9 µg/mL and above after 4 and 24 hours treatment with and without metabolic activation.

There was no relevant shift of the osmolarity and pH value even at the maximum concentration of the test substance.

B. MUTAGENICITY ASSAYS

To overcome problems with possible deviations in toxicity the main experiments were started with more than four concentrations. Following the expression phase of 48 hours the cultures at the highest concentration in experiment I with and without metabolic activation and in experiment II with metabolic activation were not continued due to exceedingly severe cytotoxic effects. In experiment II the cultures at the lowest concentration without metabolic activation were not continued as a minimum of only four analyzable concentrations is required by the guidelines.

Relevant cytotoxic effects indicated by a relative total growth of less than 50% of survival in both parallel cultures were observed in the first experiment at 45.0 µg/mL with and without metabolic activation. In the second experiment cytotoxic effects as described above were noted at 30 µg/mL and above with and without metabolic activation.

Table 5.4.1-6: Results of Mouse Lymphoma Assay - Experiment I

Without metabolic activation, 4-hour exposure period		Rel. total growth	# mutant colonies / 10^6 cells	Threshold	Rel. total growth	# mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Neg. ctrl. (DMSO)		100	98	224	100	75	201
BAS 750 F	3.75	83.3	94	224	85.3	62	201
	7.5	99.1	87	224	82.3	91	201
	15	99.7	102	224	91.1	50	201
	30	53.0	43	224	38.7	47	201
	45	2.3	84	224	3.8	93	201
	60		#			#	
Pos. ctrl. (MMS)	19.5	15.8	269	224	24.9	323	201
With metabolic activation, 4-hour exposure period		Rel. total growth	# mutant colonies / 10^6 cells	Threshold	Rel. total growth	# mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2*		
Neg. ctrl. (DMSO)		100	82	208	100	94	220
BAS 750 F	3.75	58.0	124	208	62.6	128	220
	7.5	59.1	134	208	79.9	100	220
	15	76.5	126	208	79.6	93	220
	30	64.8	79	208	55.9	163	220
	45	33.9	90	208	21.6	248	220
	60		#			#	
Pos. ctrl. (CPA)	3	47.7	267	208	59.7	223	220
	4.5	23.0	387	208	28.5	368	220

#: culture was not continued due to exceedingly severe cytotoxic effects*

In this study the range of the solvent controls was from 75 up to 116 mutant colonies per 10^6 cells; the range of the groups treated with the test substance was from 43 up to 248 mutant colonies per 10^6 cells. MMS (19.5 µg/mL in experiment I and 13.0 µg/mL in experiment II) and CPA (3.0 and 4.5 µg/mL) were used as positive controls and showed a distinct increase in induced total mutant colonies and an increase of the relative quantity of small versus large induced colonies.

Although the MMS control of the first culture of the first experiment induced a substantial increase of total colonies (269 total colonies versus 98 of the corresponding solvent control) the acceptance criteria of at least 150 induced small colonies was not quite met as the spontaneous rate of small colonies of the solvent control was comparatively high (200 small colonies versus 71 of the corresponding solvent control). The data are acceptable however, since the total number of induced colonies remained within the historical range of MMS controls and the acceptance criteria were easily met by the parallel culture (263 small colonies versus 49 of the solvent control).

No substantial and reproducible dose dependent increase of the mutation frequency was observed with and without metabolic activation. An isolated increase exceeding the threshold of 126 above the corresponding solvent control was noted at 45.0 µg/mL in the second culture of the first experiment with metabolic activation; the statistical analysis indicated a significant dose-dependent trend of the mutation frequency, based on a probability value of < 0.05. This increase however, was not reproduced in the parallel culture at identical experimental conditions or in the second experiment with metabolic activation. As the statistically significant trend is based on the isolated increase described above, the trend was judged as biologically irrelevant. No significant trend was noted in the parallel culture or in the second experiment with metabolic activation.

Table 5.4.1-7: Results of Mouse Lymphoma Assay - Experiment II

Without metabolic activation, 24-hour exposure period		Rel. total growth	# mutant colonies / 10^6 cells	Threshold	Rel. total growth	# mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Neg. ctrl. (DMSO)		100	100	226	100	116	242
BAS 750 F	3.75	##			##		
	7.5	65.8	77	226	54.0	113	242
	15	31.4	126	226	54.0	123	242
	30	25.8	145	226	24.4	110	242
	45	20.1	154	226	16.6	100	242
	60	14.7	123	226	9.8	146	242
Pos. ctrl. (MMS)	19.5	15.0	614	226	23.3	477	242
With metabolic activation, 4-hour exposure period		Rel. total growth	# mutant colonies / 10^6 cells	Threshold	Rel. total growth	# mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Neg. ctrl. (DMSO)		100	77	203	100	76	202
BAS 750 F	3.75	65.8	75	203	76.9	74	202
	7.5	71.2	117	203	132.4	62	202
	15	71.2	106	203	39.4	115	202
	30	31.9	134	203	48.9	94	202
	45	22.7	108	203	22.2	72	202
	60	#			#		
Pos. ctrl. (CPA)	3	25.5	457	203	38.6	263	202
	4.5	10.6	642	203	18.5	468	202

#: culture was not continued due to exceedingly severe cytotoxic effects

##: culture was not continued as a minimum of only four concentrations is required by the guidelines

III. CONCLUSION

Under the experimental conditions reported the test substance BAS 750 F did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.

Therefore, BAS 750 F is considered to be non-mutagenic in this mouse lymphoma assay.

Report:	CA 5.4.1/5 Wollny H.-E., 2015 b BAS 750 F: In vitro cell mutation assay at the thymidine kinase locus (TK+/-) in mouse lymphoma L5178Y cells 2015/1101908
Guidelines:	OECD 476 (1997), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.17, EPA 870.5300
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

EXECUTIVE SUMMARY

BAS 750 F (Batch: 01651-181, purity: 97.9%) was tested *in vitro* for its ability to induce forward mutations in mammalian cells by assessing the mutation of the TK locus in Mouse Lymphoma L5178Y cells. Three independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. Based on the results of a preliminary cytotoxicity assay concentrations up to 62.5 µg/mL were used in the main experiments. The treatment intervals for all experiments in the presence and absence of metabolic activation were generally 4 hours, except in experiment II (in the absence of metabolic activation) where a treatment interval of 24 h was applied. Methylmethanesulfonate (MMS) and Cyclophosphamide (CPA) served as positive controls in the experiments without and with metabolic activation, respectively. After the incubation period treatment media were replaced by culture medium and the cells were incubated for 48 h for expression of mutant cells. This was followed by incubation of cells in selection medium containing TFT for about 10 - 15 days.

No substantial and reproducible dose dependent increase in mutant colony numbers was observed in both main experiments. Appropriate reference mutagens (MMS and CPA) were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

Based on the results of the study, BAS 750 F is considered to be non-mutagenic in this mouse lymphoma assay, under the test conditions applied.

(DocID 2015/1101908)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description: Solid, white

Lot/Batch #: 01651-181

Purity: 97.9%

Stability of test compound: The test substance was stable over the study period under the storage conditions (Expiration date: 01-Jun-2018). The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified using a comparable batch.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Solvent control: 0.5% (v/v) DMSO in culture medium

Positive control -S9: Methylmethanesulfonate (MMS) 19.5 µg/mL (Exp. I) and 13.0 µg/mL (exp. II) (purity: ≥ 99%; dissolved in nutrient medium)

Positive control +S9: Cyclophosphamide (CPA) 3.0 and 4.5 µg/mL (purity: ≥ 97%; dissolved in 0.9% saline)

3. Activation: S9 was produced from the livers of rats pretreated with β-naphthoflavone/phenobarbital. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction (protein concentration 35.0 mg/mL (Exp. I and II) and 32.3 mg/mL (Exp. III)) was thawed at room temperature and an appropriate amount of S9-fraction was mixed with S9-cofactor solution to result in a final protein concentration of 15 mg/mL in the S9-mix (the corresponding protein concentration in the cultures was set to 0.75 mg/mL).

The S9-mix had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9-supernatant (protein concentration)	15 mg/mL

In order to demonstrate the efficacy of the S9-mix in this assay, the S9 batch was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene.

4. Test organism: L5178Y mouse lymphoma cells were used. They have a high proliferation rate (doubling time about 10-12 h), a high cloning efficiency (> 50%) and a stable karyotype with a near diploid (40 ± 2) chromosome number. Stocks of the L5178Y cell line were maintained at -196°C in liquid nitrogen. Each batch used for mutagenicity testing was checked for mycoplasma contamination, karyotype stability, and spontaneous mutant frequency. Prior to treatment, spontaneous TK-deficient mutants were eliminated from the stock cultures by growing the cells for one day in pretreatment medium 1 and two days in pretreatment medium 2 (see below).

5. Culture media

Complete Culture medium: RPMI 1640 medium with 15% horse serum (3% horse serum during 4-h treatment), 1% of 100 U/100 µg/mL penicillin/streptomycin, 220 µg/mL sodium pyruvate, and 0.5 – 0.75% amphotericin used as antifungal agent.

Saline G Solution: Consists of 8 g/L NaCl, 0.4 g/L KCl, 1.1 g/L Glucose, 0.192 g/L Na_2HPO_4 x $2H_2O$, 0.15 g/L KH_2PO_4, adjusted to a pH of 7.2

Pretreatment medium 1: ("HAT" medium): RPMI medium containing hypoxanthine (5 mM), aminopterin (0.02 mM), thymidine (1.6 mM), and glycin (5 mM).

Pretreatment medium 2: RPMI 1640 medium containing hypoxanthine (0.1 mM) and thymidine (1.6 mM).

Selection medium: Complete culture medium supplemented with 5 µg/mL trifluorothymidine (TFT).

6. Locus examined: thymidine kinase (TK)

7. Test concentrations:

Preliminary toxicity assay:	see CA 5.4.1/4 (DocID 2015/1112683)
Mutation assay:	The dose range of the main experiments was set either according to data generated in the pre-experiment or previous main experiments. In the main experiments the individual concentrations were generally spaced by a factor of 2.0 in the lower range. A narrower spacing was used at the highest concentrations to cover the expected cytotoxic range more closely.
- Experiment I (4 h):	0, 3.1, 6.3, 12.5, 25.0, 37.5 µg/mL (- S9)
	0, 6.3, 12.5, 25.0, 50.0 µg/mL (+ S9)
- Experiment II (24 h):	12.5, 25.0, 37.5, 50.0, 62.5 µg/mL (- S9)
(4 h):	0, 6.3, 12.5, 25.0, 50.0 µg/mL (+ S9)
- Experiment III (4 h):	0, 6.3, 12.5, 25.0, 50.0 µg/mL (+ S9)

B. TEST PERFORMANCE:

1. Dates of experimental work: 18-May-2015 to 10-Aug-2015

2. Preliminary cytotoxicity assay

Please refer to CA 5.4.1/4 (DocID 2015/1112683)

3. Mutation Assay:

Cell treatment and expression period:

The assay was performed in three independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment period of 24 hours in the absence of metabolic activation and with a treatment period of 4 hours in the presence of metabolic activation. The third experiment was performed with a treatment period of 4 hours in the presence of metabolic activation.

For each test group, about $1x10^7$ ($3x10^6$ during 24 h exposure) cells per flask (80 cm^2 flasks) suspended in 10 mL RPMI medium with 3% horse serum (15% horse serum during 24-h exposure) were exposed to various concentrations of the test substance either in the presence or absence of metabolic activation. Positive and solvent controls were performed in parallel. After 4 hours (24 hours in the second experiment without S9-mix) the test substance was removed by centrifugation (425 x g, 10 min) and the cells were washed twice with "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium and incubated for an expression and growth period of 48 hours. The cell density was determined each day and adjusted to $3×10^5$ cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number according to the method of Clive and Spector.

Seeding for selection and cloning efficiency

After the expression period the cultures were selected. Cells from each experimental group were seeded into 2 microtiter plates so that each well contained approximately 4×10^3 cells in selective medium with TFT. The viability (cloning efficiency) was determined by seeding about 2 cells per well into microtiter plates (same medium without TFT). The plates were incubated at 37°± 1.5°C in 4.5% CO_2/95.5% water saturated air for 10-15 days. Then the plates were evaluated. The relative total growth (RTG) is calculated by the RSG multiplied by the viability.

Colony counting and determination of colony size distribution

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test substance. Criteria to determine colony size were the absolute size of the colony (more than 1/3 of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the optical density of the large ones).

Calculations and processing of the data:

The data listed in the tables of results are calculated and processed as described in Table 5.4.1-8.

Table 5.4.1-8: Overview on calculation and processing of data

Main test	
Total suspension growth (4-h treatment):	(cell number at 24 h / cell number at 4 h) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
Total suspension growth (24-h treatment):	(cell number at 24 h / cell number of seeded cells per mL (100000)) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h) × (cell number at 72 h / if cell number at 48 h > 300000 then 300000, if cell number at 48 h < 300000 then cell number at 48 h)
Relative suspension growth:	total suspension growth × 100 / total suspension growth of corresponding control
Relative total growth:	relative suspension growth × relative cloning efficiency / 100
Cloning efficiency (viability):	-ln(mean number of empty wells per plate / 96) / cells seeded per well
Relative cloning efficiency:	cloning efficiency × 100 / cloning efficiency of corresponding control
Cells survived:	cloning efficiency × cell number seeded in TFT medium
Mutant colonies / 10^6 cells:	(small mutant colonies + large mutant colonies)
Small mutant colonies / 10^6 cells:	(-ln(mean number of wells not containing small colonies / 96) / cells survived) × 10^6
Large mutant colonies / 10^6 cells:	(-ln(mean number of wells not containing large colonies / 96) / cells survived) × 10^6
Threshold	number of mutant colonies per 10^6 cells of each solvent control plus 126
cloning efficiency (viability):	Cloning efficiency determined after the expression period to measure viability of the cells without selective agent

4. Statistics:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT®11 (SYSTAT Software, Inc.) statistics software. The number of mutant colonies obtained for the groups treated with the test substance were compared to the solvent control groups. A trend was judged as significant whenever the p-value (probability value) was below 0.05. However, both, biological relevance and statistical significance were considered together.

5. Acceptability criteria:

A mutation assay is considered acceptable if it meets the following criteria (the current recommendations of the International Workgroup on Genotoxicity Test Procedures (IWGT) are considered):

- All plates, from either the cloning efficiency or the TFT resistance-testing portion of the experiment are analyzable.
- The absolute cloning efficiency at the time of mutant selection (CE) of the solvent controls is 65-120%.
- The total suspension growth of the solvent control calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number is 8-32. Following 24 h treatment the total suspension growth is 32-180.
- The range of the solvent control mutant frequency (MF) is in the range of 50-170 x 10^{-6} cells.
- The positive controls (MMS and CPA) should yield an absolute increase in total MF that is an increase above spontaneous background MF (an induced MF [IMF]) of at least 300 x 10^{-6} cells. At least 40% of the induced mutation frequency (IMF) should be reflected in the small colony MF. Alternatively, the positive controls should induce at least 150 small colonies.
- The upper limit of cytotoxicity observed in the positive control culture should be the same as for the experimental cultures (i.e. the relative total growth -RTG- should be greater than 10% of the concurrent selective control group).
- The highest concentration of the test substance should be 10 mM or 5000 µg/mL, unless limited by toxicity or solubility of the test substance. If toxicity occurred, the highest concentration should lower the relative total growth to approximately 10 to 20% of survival. If precipitation is noted, the highest analyzed concentration should be the lowest concentration where precipitation is observed by the naked eye.

6. Evaluation criteria:

A test substance is classified as mutagenic if at least one of the following criteria is met:

- The induced mutation frequency reproducibly exceeds a threshold of 126 colonies per 10^6 cells above the corresponding solvent control.
- A relevant increase of the mutation frequency should be dose-dependent and correspondingly statistically significant.
- A mutagenic response is considered to be reproducible if it occurs in both parallel cultures.

The test substance is considered non-mutagenic if at least one of the following criteria is met:

- The mutation frequency is below a threshold of 126 colonies per 10^6 cells above the concurrent solvent control value.
- No evidence of reproducibility of an increase in mutant frequencies is obtained.
- No statistical significant dose-related increase in mutant frequencies using an appropriate statistical trend.
- If the threshold is reproducibly exceeded but the increase of the mutation frequency is not dose dependent and a biological relevance of the effect can be excluded.

However, in the evaluation of the test results the historical variability of the mutation rates in the solvent controls of this study were taken into consideration.

Results of test groups are generally rejected if the relative total growth is less than 10% of the vehicle control unless the exception criteria specified by the IWGT recommendations are fulfilled.

Whenever a test substance is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated.

A test substance is classified as non-mutagenic if the induced mutation frequency does not reproducibly exceed a threshold of 126 colonies per 10^6 cells above the corresponding solvent control.

A test substance not meeting the conditions for a classification as mutagenic or non-mutagenic will be considered equivocal in this assay and may be considered for further investigation.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

Please refer to CA 5.4.1/4 (DocID 2015/1112683)

B. MUTAGENICITY ASSAYS

The experimental part of the first experiment without metabolic activation was prematurely terminated due to exceedingly severe cytotoxicity. This experimental part was repeated with an extended concentration range and the data are reported as first experiment without metabolic activation. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation. The confirmatory third experiment was performed solely in the presence of metabolic activation with a treatment time of 4 hours.

Cytotoxic effects, indicated by a relative total growth (RTG) below 50% in both parallel cultures occurred in the second experiment at 12.5 µL/mL and above in the absence of metabolic activation (24 hours treatment) and at 50.0 µL/mL in the presence of metabolic activation. The data generated at 62.5 µg/mL without metabolic activation (both cultures) were not considered valid as the relative total growth remained far below the lower acceptable limit of 10%. In the additional third experiment relevant cytoxic effects of less than 50% were noted at 50.0 µg/mL. The recommended cytotoxic range of approximately 10 - 20% RTG was covered with and without metabolic activation.

Table 5.4.1-9: Results of Mouse Lymphoma Assay - Experiment I

Without metabolic activation, 4-hour exposure period		Rel. total growth	mutant colonies / 10^6 cells	Threshold	Rel. total growth	mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Solvent ctrl. (DMSO)		100	113	239	100	63	189
BAS 750 F	3.1	127.8	115	239	102.8	97	189
	6.3	225.8	108	239	46.1	126	189
	12.5	178.9	93	239	100.5	95	189
	25.0	184.9	76	239	171.1	97	189
	37.5	66.3	45	239	50.1	72	189
Pos. ctrl. (MMS)	19.5	52.4	900	239	20.5	751	189
With metabolic activation, 4-hour exposure period		Rel. total growth	mutant colonies / 10^6 cells	Threshold	Rel. total growth	mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Solvent ctrl. (DMSO)		100	79	205	100	70	196
BAS 750 F	6.3	67.3	151	205	118.5	175	196
	12.5	52.9	149	205	77.8	**271**	196
	25.0	96.2	57	205	88.4	**304**	196
	50.0	30.4	**238**	205	62.8	149	196
Pos. ctrl. (CPA)	3.0	43.5	728	205	57.3	445	196
	4.5	12.9	1877	205	35.5	495	196

The threshold of 126 above the mutation frequency of the solvent control was solely exceeded in experiment I in the presence of metabolic activation at 50.0 µg/mL in culture I (238 compared to 205 colonies per 10^6 cells), and at 12.5 and 25.0 µg/mL in culture II (271 and 304 compared to 196 colonies per 10^6 cells). However, the increases were not reproduced in the parallel cultures under identical conditions and were therefore, not considered as true mutagenic effects. Furthermore, the additional third experiment performed to verify the isolated increased effects described above did not show any increase above the threshold and thus, confirmed the interpretation of the data as non-mutagenic.

Table 5.4.1-10: Results of Mouse Lymphoma Assay - Experiment II

Without metabolic activation, 24-hour exposure period		Rel. total growth	mutant colonies / 10^6 cells	Threshold	Rel. total growth	mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Solvent ctrl. (DMSO)		100	153	279	100	67	193
BAS 750 F	12.5	40.4	123	279	35.3	54	193
	25.0	34.3	129	279	27.5	90	193
	37.5	23.6	168	279	14.0	54	193
	50.0	9.1	181	279	8.0	56	193
	62.5	3.8	97	279	2.3	60	193
Pos. ctrl. (MMS)	13.0	13.7	1904	279	14.6	482	
With metabolic activation, 4-hour exposure period		Rel. total growth	mutant colonies / 10^6 cells	Threshold	Rel. total growth	mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Solvent ctrl. (DMSO)		100	105	231	100	140	266
BAS 750 F	6.3	101.5	81	231	103.7	131	266
	12.5	87.1	117	231	78.9	167	266
	25.0	56.1	140	231	66.0	168	266
	37.5	52.6	70	231	39.7	205	266
	50.0	19.2	91	231	31.4	101	266
Pos. ctrl. (CPA)	3.0	17.1	1044	231	31.7	668	266
	4.5	10.7	945	231	11.8	952	266

In this study the range of the solvent controls was from 63 - 153 mutant colonies per 10^6 cells; the range of the groups treated with the test item was 45 - 304 mutant colonies per 10^6 cells.

Both positive control substances CPA and MMS showed a distinct increase in induced total mutant colonies, demonstrating the sensitivity of the test system by the ability to detect known mutagens that do or do not require metabolic activation, respectively.

Table 5.4.1-11: Results of Mouse Lymphoma Assay - Experiment III

With metabolic activation, 4-hour exposure period		Rel. total growth	mutant colonies / 10^6 cells	Threshold	Rel. total growth	mutant colonies / 10^6 cells	Threshold
Test item	Conc. [µg/mL]	Culture 1			Culture 2		
Solvent ctrl. (DMSO)		100	64	190	100	149	275
BAS 750 F	6.3	114.3	76	190	100	190	275
	12.5	94.0	79	190	83.8	179	275
	25.0	154.7	46	190	67.5	198	275
	50.0	28.0	53	190	6.9	102	275
Pos. ctrl. (CPA)	3.0	90.8	217	190	51.0	287	275
	4.5	36.8	341	190	38.9	406	275

Overall, no substantial and reproducible increase of the mutation frequency was noted in the main experiments with and without metabolic activation.

III. CONCLUSION

Under the experimental conditions, the test substance BAS 750 F did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.

Therefore, BAS 750 F is considered to be non-mutagenic in this mouse lymphoma assay.

Report:	CA 5.4.1/6 Schulz M.,Landsiedel R., 2014 a BAS 750 F - In vitro micronucleus assay in V79 cells (Cytokinesis Block Method) 2013/1375108
Guidelines:	OECD 487 (2010), Commission Regulation EU No. 640/2012 of 06 July 2012 - B.49: In vitro Mammalian Cell Micronucleus Test
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch: COD-001740; Purity: 98.8%) was tested in vitro for its potential to induce micronuclei in V79 Chinese Hamster lung fibroblasts (clastogenic or aneugenic activity). Two independent experiments were carried out with and without the addition of liver S9-mix from induced rats (exogenous metabolic activation). The vehicle DMSO served as negative control, EMS served as positive control in the absence of metabolic activation and cyclophosphamide as positive control in the presence of metabolic activation. Cells were incubated with BAS 750 F at concentrations in the range of 0.39 to 50 µg/mL. Two independent experiments were performed where the cells were incubated for four (with and without S9-mix) or 24 hours (without S9-mix). Following exposure to the test or control substances, the cell cultures were incubated with Cytochalasin B, subsequently fixed and DNA and cytoplasm stained. Cytotoxicity parameters and number of micronucleated cells were determined in at least 1000 binucleated cells/culture, i.e. 2000 cells for each test group.

Cytotoxicity was indicated by a dose-dependent decrease of proliferative activity of treated cells; relative increase in cell count (RICC), proliferation index (CBPI) or replicative index (RI) were clearly reduced at least at the highest applied test substance concentration in all experimental parts of this study. Effects on cell attachment/morphology and precipitation in culture medium were observed in both experiments dose-dependently.

The test substance BAS 750 F did not lead to a biologically relevant increase in the number of micronucleated cells either without S9-mix or after the addition of a metabolizing system in two experiments performed independently of each other. The increase in the frequencies of micronuclei induced by the positive control substances EMS and CPP clearly demonstrated the sensitivity of the test system and of the metabolic activity of the S9-mix employed.

In conclusion, BAS 750 F is considered not to have a chromosome-damaging (clastogenic) effect nor to induce numerical chromosomal aberrations (aneugenic activity) under in vitro conditions in V79 cells in the absence and the presence of metabolic activation when tested up to cytotoxic concentrations.

(DocID 2013/1375108)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description: Solid, white

Lot/Batch #: COD-001740

Purity: 98.8%

Stability of test compound: The test substance was stable over the study period under the storage conditions. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified.

Vehicle used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Vehicle/solvent control: DMSO

Positive control: Without metabolic activation:

Ethylmethanesulfonate (EMS, 300, 400, and 500 µg/mL; dissolved in Minimal Essential Medium with Earle's salts (MEM) without fetal calf serum (FCS))

With metabolic activation:

Cyclophosphamide (CPP, 1 and 2.5 µg/mL; dissolved in MEM without FCS)

3. Activation: S9 was produced from the livers of rats pretreated with β-naphthoflavone/phenobarbital. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction was thawed at room temperature and 1 volume of S9-fraction was mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so called S9-mix, was kept on ice until used.

The concentrations of the co-factors in the S9-mix were as follows:

Component	***Concentration***
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

4. Test organism: V79 Chinese hamster lung fibroblasts

5. Culture medium / conditions:

Culture media:	Minimal essential medium with Earle's salts (MEM) containing a L-glutamine source supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) penicillin / streptomycin (10 000 IU / 10 000 µg/mL) and 1% (v/v) amphotericin B (250 µg/mL). During exposure to the test substance in the presence of S9-mix, MEM was used without FCS supplementation.
Cell culture:	Deep-frozen cell stocks were thawed at 37°C in a water bath, and volumes of 0.5 mL were transferred into 25 cm² plastic flasks containing about 5 mL MEM supplemented with FCS. Cells were grown with 5% (v/v) CO_2 at 37°C and ≥ 90% humidity and subcultured twice weekly. Cell monolayers were suspended in culture medium after detachment with 0.25% (w/v) trypsin solution.
Cell cycle and harvest time:	The cell cycle of the untreated V79 cells lasts for about 12-14 hours under the selected culture conditions. Thus, a harvest time of 24 hours is about 2 times the normal cell cycle length. V79 cells are an asynchronous cell population, i.e. at the time of test substance treatment there are different cell stages. Since the effect on these cell stages may vary for different test substances, more than one harvest time after treatment may be appropriate. Furthermore, substance-induced mitotic delay may considerably delay the first post-treatment mitosis. Therefore, delayed harvest times (e.g. 44 hours) and prolonged exposure periods (e.g. 24 hours treatment) were considered.

6. Test concentrations:

Micronucleus assay

- Experiment I	1.56, 3.13, 6.25, 12.5, 25, 50 µg/mL with / without metabolic activation, 4-hour exposure
- Experiment II	1.56, 3.13, 6.25, 12.5, 25, 50 µg/mL with metabolic activation, 4-hour exposure
	0.39, 0.78, 1.56, 3.13, 6.25, 12.5 µg/mL without metabolic activation, 24-hour exposure

B. TEST PERFORMANCE

1. Dates of experimental work: 01-July-2013 to 31-Jan-2014

2. Dose selection

Following the requirements of the current guidelines a test substance should be tested up to a maximum concentration of 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest.

In case of toxicity, the top concentration should produce 55 ± 5% cytotoxicity (based on determinations of the relative increase in cell count [RICC] and/or proliferation index [CBPI] and/or replicative index [RI]) compared to the respective vehicle control. For relatively insoluble test substances at least one concentration should be scored showing no precipitation in culture medium at the end of exposure period.

Preliminary cytotoxicity assay

A cytotoxicity pretest was performed following the method described for the main experiment. 4000 µg/mL (approx. 10 mM) BAS 750 F was used as top concentration. The cells were prepared at a harvest time of 24 hours (about 2 cell cycles) after 4 and 24 hours exposure time without S9-mix and after 4 hours exposure time with S9-mix. As indication of test substance toxicity the relative increase in cell count (RICC) and cell attachment (morphology) were determined for dose selection. Additionally, the pH-value, osmolarity and solubility were determined.

In the pretest the pH value was not influenced by the addition of the test substance preparation to the culture medium at the concentrations tested. In addition, no test substance precipitation in the vehicle DMSO was observed in the stock solution (Test group: 4 000 µg/mL). However, in culture medium test substance precipitation occurred at the end of exposure (macroscopically) at and above concentrations of 15.63 µg/mL. In tests with a 4-hour treatment period (in the absence or presence of S9-mix), cytotoxicity was observed at 31.25 µg/mL and above, indicated by reduced RICC of about or below 40-50%. In the pretest with 24-hour exposure in the absence of S9-mix, RICC was clearly reduced after treatment with BAS 750 F concentrations of 7.81 µg/mL and above. Based on these results the concentrations stated above were used for the main experiments.

3. Micronucleus test

Seeding and treatment of the cultures

A single cell suspension with the required cell count (3-5 x10^5 cells per culture, depending on the schedule) was prepared in MEM incl. 10% (v/v) FCS. 5 mL cell suspension was transferred into 25 cm² cell culture flasks using a dispenser. After attachment (20-24 h) the medium was removed and replaced by the treatment medium.

The cultures were treated according to the following scheme:

	Without S9-mix		With S9-mix	
	Experiment I	Experiment II	Experiment I	Experiment II
Exposure time	4 h	24 h	4 h	4 h
Recovery time	20 h	-	20 h	40 h
Harvest time	24 h	24 h	24 h	44 h

At the end of the exposure period, the treatment medium was removed and the cultures were rinsed twice with 5 mL HBSS (Hanks Balanced Salt Solution). Subsequently, 5 mL MEM (incl. 10% [v/v] FCS) supplemented with Cytochalasin B (CytB, final concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO) was added and incubated at 37°C, 5% (v/v) CO_2 and ≥ 90% relative humidity for the respective recovery time.

In the case of 24-hour continuous exposure, CytB was added to the treatment medium at start of treatment, and cell preparation was started directly at the end of exposure. In cells exposed for four hours without S9-mix and harvested for cell preparation after 44 hours, CytB was supplemented 24 hours before preparation of the cultures.

Cell harvest, preparation of slides and staining

Just before preparation the culture medium was completely removed. Single cell suspensions were prepared from each test group by trypsination. Then, the cell numbers per flask of each single cell suspension were determined using a cell counter. Subsequently, 5x10^4 cells per slide were centrifuged at 1400 rpm for 7 minutes onto labelled slides using a Cytospin centrifuge. After drying, the slides were fixed in 90% (v/v) methanol for 10 minutes.

Before scoring, the slides were stained with a mixture of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; stock: 5 mg/mL) and propidium iodide (PI, stock: 5 mg/mL) in Fluoroshield™ at a concentration of 0.25 µg/mL each. By the use of the combination of both fluorescence dyes it is possible to differentiate between DNA (DAPI; excitation: 350 nm, emission: 460 nm) and cytoplasm (PI; excitation: 488 nm, emission: 590 nm).

4. Cytotoxicity evaluation

Relative increase in cell count (RICC)

Before preparing the slides, cell numbers were determined from cultures. The relative increase in cell counts was calculated based on the following formula:

$$RICC = \frac{\text{Increase in the number of cells in treated cultures (final – start)}}{\text{Increase in the number of cells in control cultures (final – start)}} \times 100$$

Cytokinesis-block proliferation index (CBPI)

To describe a cytotoxic effect the CBPI ("Cytokinesis-block proliferation index") was determined in at least 1000 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (i.e. all cells are mononucleated) is equivalent to 100 % cytostasis.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mono-nucleated cells
BINC	Bi-nucleated cells
MUNC	Multi-nucleated cells

$$\text{Cytostasis (\%)} = 100 - 100 \times \frac{(CBPI_T - 1)}{CBPI_C - 1}$$

T	Test substance
C	Solvent control

Replicative index (RI)

The Replicative Index (RI) is an additional parameter for proliferation that indicates the relative number of cells in treated cultures compared to the respective control cultures:

$$RI = \frac{[(BINC) + (MUNC \times 2)] / (\text{total number of cells})_T}{[(BINC) + (MUNC \times 2)] / (\text{total number of cells})_C}$$

BINC	Bi-nucleated cells
MUNC	Multi-nucleated cells

Thus, an RI of 53% means that in comparison to the respective control cultures only 53% of the cells divided in the treated test group (= 47% cytotoxicity/cytostasis).

Cell morphology

At the end of the treatment period, the test cultures of all test groups will be examined microscopically with regard to cell morphology, which is a further indication for cytotoxicity.

5. Micronucleus evaluation

The dose selection for scoring was based on slide/cell quality, number of analyzable cells, and nuclear fragmentation. Evaluation of the slides was performed by fluorescence microscopy. At least 1000 binucleated cells per culture (i.e. 2000 binucleated cells per test group) were evaluated for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. The analysis of the micronuclei was carried out based on the following criteria:

- The diameter of the micronucleus is less than 1/3 of the main nucleus.
- The micronucleus and main nucleus retain the same color.
- The micronucleus is not linked to the main nucleus and is located within the cytoplasm of the cell.
- Only cells clearly surrounded by a membrane were scored.

6. Acceptability criteria

The in vitro micronucleus assay is considered acceptable if the following criteria are met:

- The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.
- The rate of micronuclei in the solvent controls falls within the range of the test laboratory's recent negative control data.
- The rate of micronuclei in the positive controls both with and without S9-mix induced a distinct increase in the number of micronucleated cells.

7. Assessment criteria

A test substance is considered "negative" in the in-vitro micronucleus test if the following criteria are met:

- the number of micronucleated cells in the test groups is not distinctly increased above the concurrent vehicle control
- the number of micronucleated cells in the test groups is within the test laboratory's recent negative control data range

A test substance is considered "positive" in the in-vitro micronucleus test if the following criteria are met:

- a significant, dose-related and reproducible increase in the number of cells containing micronuclei was observed
- The number of micronucleated cells exceeded both the value of the concurrent vehicle control and the range of the laboratory's recent negative control data

4. Statistics:

The statistical evaluation of the data was carried out using the MUVIKE program system (BASF SE). The proportion of cells containing micronuclei was calculated for each group. A comparison of each dose group with the concurrent vehicle control group was carried out using Fisher's exact test for the hypothesis of equal proportions. This test is Bonferroni-Holm corrected versus the dose groups separately for each time and was performed one-sided.

If the results of this test were statistically significant compared with the respective vehicle control, labels (* $p \leq 0.05$, ** $p \leq 0.01$) have been be printed in the tables.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS AND TREATMENT CONDITIONS

All formulations were prepared freshly before treatment. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (see CA 5.4.1/2; DocID 2015/1040886).

Osmolarity and pH values were not influenced by test substance treatment. No precipitation of the test substance in the vehicle DMSO was observed. Test substance precipitation in culture medium was observed at the end of exposure period (macroscopical assessment) in the 1st experiment from 25 µg/mL onward in the absence and presence of S9-mix and in the 2nd experiment at 50 µg/mL in the presence of S9-mix.

B. CYTOTOXICITY

Growth inhibition indicated by reduced cell counts of below 50% was observed in both main experiments in the absence and the presence of S9-mix at least at the highest applied test substance concentrations: after 4-h exposure in the absence of metabolic activation, the cell counts were clearly reduced from 25 µg/mL onward (RICC: -3.3%) in the 1st experiment; after 24 hours exposure in the absence of metabolic activation, the cell counts were below 50% of controls from 3.13 µg/mL onward (RICC: -41.1%) in the 2nd experiment. In the presence of metabolic activation, strong cytotoxicity was observed after 4 hours exposure in the 1st and 2nd experiment at 50 µg/mL (RICC: -48.0% or -10.0%, respectively).

The proliferative activity of the cells was dose-dependently reduced as indicated by the CBPI cytostasis values and by the replicative index (RI). Under all experimental conditions the highest tested concentrations could not be evaluated for cytogenetic damage due to strong cytotoxicity.

Cell attachment / cell morphology was adversely influenced (grade > 2) in the 1st experiment in the absence of S9-mix from 25 µg/mL onward and in the presence of S9-mix at 50 µg/mL. In addition, in the 2nd experiment adverse effects were found without S9-mix from 3.13 µg/mL onward and with S9-mix from 25 µg/mL onward.

B. MICRONUCLEUS ASSAY

In this study, no biologically relevant increase in the number of micronucleated cells was observed either without S9-mix or after the addition of a metabolizing system. In both experiments in the absence and presence of metabolic activation after 4 and 24 hours treatment with the test substance, the obtained percentages of micronucleated cells (0.2 - 0.8%) were close to the concurrent vehicle control values (0.4 - 0.9% micronucleated cells) and were clearly within the historical negative control data range (0.1 - 1.8% micronucleated cells).

Table 5.4.1-12: Results of in-vitro micronucleus test in V79 cells – without S9-mix

Without metabolic activation (S9-mix): 4-hour exposure period, harvest at 24 hours						
Experiment I			Cytotoxicity			Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	RICC [%]	CBPI [%]	RI [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO)		n.d.	100	0.0	100	0.7
BAS 750 F	1.56	-	126.1	n.d.	n.d.	n.d.
	3.13	-	97.2	2.5	97.5	0.4
	6.25	-	122.5	4.5	95.5	0.5
	12.5	-	76.3	13.4	86.5	0.3
	25.0	+	-3.3	43.5	56.5	0.2
	50.0	+	-20.8	n.d.	n.d.	n.d.
Pos. ctrl. (EMS)	300.0	n.d.	104.7	3.0	97.0	0.6
	400.0	n.d.	86.9	-1.5	101.5	**2.4***
Without metabolic activation (S9-mix): 24-hour exposure period, harvest at 24 hours						
Experiment II			Cytotoxicity			Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	RICC [%]	CBPI [%]	RI [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO)		n.d.	100.0	0.0	100.0	0.4
BAS 750 F	0.39	-	99.8	12.6	87.4	0.4
	0.78	-	97.3	37.2	62.8	0.2
	1.56	-	124.6	42.4	57.6	0.4
	3.13	-	-41.1	n.d	n.d	n.d
	6.25	-	-63.3	n.s.	n.s.	n.s.
	12.5	-	-66.6	n.s.	n.s.	n.s.
Pos. ctrl. (EMS)	300.0	n.d.	151.8	-1.0	101.0	**2.4***

* statistically significantly increased over corresponding control values

n.d. = not determined; n.s. = not scorable due to strong cytotoxicity

The positive control substances EMS (without S9-mix; 400 µg/mL [Exp. 1] and 300 µg/mL [Exp. 2]) and CPP (with S9-mix; 1.0 µg/mL) induced statistically significant increased micronucleus frequencies in both independently performed experiments. In this study, in the absence and presence of metabolic activation the frequency of micronucleated cells (2.4 - 12.8% micronucleated cells) was clearly above the range of our historical negative control data range (0.1 - 1.8% micronucleated cells) and within our historical positive control data range (2.3 - 26.6% micronucleated cells). However, in the 1st Experiment in the absence of S9-mix after exposure with 300 µg/mL EMS a micronucleus rate (0.6%) was obtained that was still within our historical negative control data range.

To fulfil the acceptance criteria of the concurrent guideline, an additional positive control test group treated with 400 µg/mL EMS was scored. The value of this test group (2.4% micronucleated cells) was clearly above our historical negative control data range and within our historical positive control data range.

Table 5.4.1-13: Results of in-vitro micronucleus test in V79 cells – with S9-mix

With metabolic activation (S9-mix): 4-hour exposure period, harvest at 24 hours						
Experiment I			Cytotoxicity			Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	RICC [%]	CBPI [%]	RI [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO)		n.d.	100.0	0.0	100.0	0.9
BAS 750 F	1.56	-	118.7	n.d.	n.d.	0.9
	3.13	-	109.3	n.d.	n.d.	n.d.
	6.25	-	103.5	-0.6	100.6	0.5
	12.5	-	114.3	27.8	72.2	0.3
	25.0	+	61.3	38.6	61.4	0.2
	50.0	+	-48.0	n.d.	n.d.	n.d.
Pos. ctrl. (CPP)	1.0	n.d.	99.7	52.9	47.1	**12.8****
With metabolic activation (S9-mix): 4-hour exposure period, harvest at 44 hours						
Experiment II			Cytotoxicity			Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	RICC [%]	CBPI [%]	RI [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO)		n.d.	100.0	0.0	100.0	0.5
BAS 750 F	1.56	-	138.8	n.d.	n.d.	n.d.
	3.13	-	154.1	n.d.	n.d.	n.d.
	6.25	-	154.5	-0.6	100.6	0.8
	12.5	-	170.4	17.4	82.6	0.5
	25.0	-	94.3	23.0	77.0	0.5
	50.0	+	-10.0	n.d.	n.d.	n.d.
Pos. ctrl. (CPP)	1.0	n.d.	122.0	0.0	100.0	**7.9****

Statistical evaluation: *: p≤0.05; **: p≤0.01 (Fisher's Exact test (1-sided), with Bonferroni-Holm correction); statistically significantly increased over corresponding control values

n.d. = not determined

III. CONCLUSION

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the in vitro micronucleus test in V79 cells. Therefore, BAS 750 F is considered to be non-mutagenic in this in vitro micronucleus test when tested up to cytotoxic concentrations.

Report:	CA 5.4.1/7 Sokolowski A., 2015 a BAS 750 F: Micronucleus test in human lymphocytes in vitro 2015/1101907
Guidelines:	OECD 487 (2014)
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

EXECUTIVE SUMMARY

BAS 750 F (Batch: 01651-181; Purity: 97.9%) was tested for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of hepatic S9-mix from induced rats (metabolic activation). Two independent experiments were performed in which the cells were incubated for 4 (±S9-mix) or 20 hours (-S9-mix) with the test substance at concentrations in the range of 0.3 to 2094 µg/mL; concentrations from 2.0 to 8.2 µg/mL were evaluated. The vehicle DMSO served as negative control, mitomycin C (4 h) and demecolcin (20 h) as positive controls in the absence of metabolic activation and cyclophosphamide as positive control in the presence of metabolic activation. Exposure was started after a 48 hour stimulation period with phytohemeagglutinine. Thereafter, cytochalasin B was added and the cultures were fixed and stained finally after another 20 hours. Cytokinesis-block proliferation index (CBPI) and cytostasis were determined in 500 binucleated cells/culture as cytotoxicity parameters and number of micronucleated cells were determined in 1000 binucleated cells/culture for evaluation of mutagenicity.

Cytotoxicity was observed in both experiments either with or without addition of S9-mix at the highest evaluated concentration of 8.2 µg/mL. Osmolarity and pH values were not influenced by test substance treatment. Precipitation of the test item in the culture medium at the end of treatment was observed at 32.7 and 130.9 µg/mL and above in the absence and the presence of S9-mix, respectively.

The test substance BAS 750 F did not lead to a relevant increase in the number of micronucleated cells either without S9-mix or after the addition of a metabolizing system in two experiments performed independently of each other. In both experiments, either CPA or demecolcin and MMC showed distinct increases in cells with micronuclei, and thus demonstrating sensitivity of the test system towards known mutagens that do or do not require metabolic activation, respectively. Furthermore, the number of micronucleated cells induced by the vehicle control DMSO was within the range of the historical control data.

In conclusion, BAS 750 F is considered to be non-mutagenic in this *in vitro* micronucleus test when tested up to cytotoxic concentrations.

(DocID 2015/1101907)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description:	Solid, white
Lot/Batch #:	01651-181
Purity:	97.9%
Stability of test compound:	The test substance was stable over the study period under the storage conditions (Expiration date: 01-Jun-2018). The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified using a comparable batch.
Vehicle used:	Dimethylsulfoxide (DMSO)

2. Control Materials:

Vehicle/solvent control:	DMSO, 0.5% v/v in culture medium
Positive control:	<u>Without metabolic activation:</u> Mitomycin C (MMC, 2 µg/mL; 4 h treatment) dissolved in deionized water; Demecolcin (50 ng/mL; 20 h treatment) dissolved in deionized water <u>With metabolic activation:</u> Cyclophosphamide (CPP, 15.0 and 17.5 µg/mL) dissolved in saline

3. Activation: S9 was produced from the livers of rats pretreated with phenobarbital/β-naphthoflavone. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction (protein content: 35 mg/mL) was thawed at room temperature and mixed with a sufficient amount of S9-supplement (cofactors). This preparation, the so called S9-mix, was kept on ice until used.

The concentrations of the co-factors in the S9-mix were as follows:

Component	*Concentration*
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

50 µL S9-mix per mL culture medium were added yielding a final protein concentration of 0.75 mg/mL in the cultures.

4. Test organism: Human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication: male donor (31 years old) for experiment I, and female donor (32 years old) for experiment II.

5. Culture media: Dulbecco´s Modified Eagles medium/Ham´s F12 (1:1) with GlutaMAX™ (200 mM) supplemented with 10% (v/v) fetal bovine serum (FBS), Pen/Strep (100 U/mL / 100 µg/mL), HEPES (10 mM), heparin (125 U.S.P.-U/mL) and phytohemeagglutinine (PHA, 3 µg/mL).

6. Test concentrations:

Micronucleus assay

Experiment I

(4-h exposure, ±S9): 2.0, 4.1, 8.2 µg/mL

Experiment II

(4-h exposure, +S9): 2.0, 4.1, 8.2 µg/mL

(20-h exposure, -S9): 2.0, 4.1, 8.2 µg/mL

B. TEST PERFORMANCE

1. Dates of experimental work: 20-May-2015 to 15-Jul-2015

2. Dose selection

Dose selection was performed according to the current OECD Guideline for the *in vitro* micronucleus test. The highest test item concentration should be 2000 µg/mL, 2 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

With regard to the purity (95.5% preliminary purity data on study start) of the test item, 2094.0 µg/mL were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations ranging from 2.0 to 2094 µg/mL (with and without S9-mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, precipitation of the test item was observed at the end of treatment at 32.7 and 130.9 µg/mL in the absence and the presence of S9-mix, respectively. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity clear toxic effects were observed after 4 hours treatment with 4.1 µg/mL and above in the absence of S9-mix and with 8.2 µg/mL and above in the presence of S9-mix. Considering the toxicity data and precipitation, 130.9 µg/mL (with and without S9-mix) were chosen as top concentration in Experiment II.

Preliminary cytotoxicity assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9-mix). The preparation interval was 40 hrs after start of the exposure.

3. Micronucleus test

The cultures were treated according to the following scheme:

	Without S9-mix		With S9-mix	
	Experiment I	Experiment II	Experiment I	Experiment II
Stimulation period	48 h	48 h	48 h	48 h
Exposure time	4 h	20 h	4 h	4 h
Recovery time	16 h	-	16 h	16 h
Cytochalasin B exposure	20 h	20 h	20 h	20 h
Harvest time	40 h	40 h	40 h	40 h
Total culture period	88 h	88 h	88 h	88 h

Pulse exposure (4 h):

About 48 h after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9-mix per mL culture medium was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period, Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous (20 h) exposure:
About 48 h after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10% FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Preparation of cells:
The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37°C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

4. Cytotoxicity and genotoxicity evaluation

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 1000 binucleate cells per culture were evaluated for cytogenetic damage on coded slides, except for the positive control in Experiment I in the absence of S9-mix, where only 500 binucleated cells were evaluated. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI (Cytokinesis-block proliferation index) was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$\text{CBPI} = \frac{(\text{MONC} \times 1) + (\text{BINC} \times 2) + (\text{MUNC} \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mono-nucleated cells
BINC	Bi-nucleated cells
MUNC	Multi-nucleated cells

$$\text{Cytostasis (\%)} = 100 - 100 \times \frac{(\text{CBPI}_T - 1)}{\text{CBPI}_C - 1}$$

T	Test substance
C	Solvent control

6. Acceptability criteria

- The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significant increased.
- The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.

7. Assessment criteria

A test item can be classified as **non-clastogenic** and **non-aneugenic** if:

- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data and
- no statistically significant or concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as **clastogenic** and **aneugenic** if:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.

An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS AND TREATMENT CONDITIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance (comparable batch) at room temperature in the vehicle DMSO was verified analytically (Project No. 01Y0741/11Y125, see DocID 2015/1040886).

Osmolarity and pH values were not influenced by test substance treatment. Precipitation of the test item in the culture medium at the end of treatment was observed at 32.7 and 130.9 µg/mL and above in the absence and the presence of S9-mix, respectively.

B. CYTOTOXICITY

In Experiment I and II in the absence and presence of S9-mix, clear cytotoxicity was observed at the highest evaluated concentrations of 8.2 µg/mL.

C. MICRONUCLEUS ASSAY

In both experiments in the absence and presence of S9-mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item.

In both experiments, either CPA (15.0 or 17.5 µg/mL) or Demecolcin (50.0 ng/mL) and MMC (2.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, and thus demonstrating sensitivity of the test system towards known mutagens that do or do not require metabolic activation, respectively. Furthermore, the number of micronucleated cells induced by the vehicle control DMSO was within the range of the historical control data in both experiments.

Table 5.4.1-14: Results of in-vitro micronucleus test in human lymphocytes – without S9-mix

Without metabolic activation (S9-mix): 4-hour exposure period, harvest at 40 hours					
Experiment I			Cytotoxicity		Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	CBPI [%]	Cytostasis [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO) [1]		-	1.95		0.50
BAS 750 F	2.0	-	1.77	18.7	0.40
	4.1	-	1.50	47.8	0.35
	8.2	-	1.26	72.7	0.20
Pos. ctrl. (MMC) [2]	2.0	-	1.41	56.5	**5.60***
Without metabolic activation (S9-mix): 20-hour exposure period, harvest at 40 hours					
Experiment II			Cytotoxicity		Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	CBPI [%]	Cytostasis [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO) [1]		-	1.82		0.95
BAS 750 F	2.0	-	1.72	13.0	0.70
	4.1	-	1.63	23.3	0.75
	8.2	-	1.39	52.7	0.75
Pos. ctrl. (DMC)[3]	0.05	-	1.85	n.c.	**3.30***

[1] DMSO = Dimethyl sulfoxide: 0.5 % (v/v); [2] MMC = Mitomycin: 2.0 µg/mL; [3] DMC = Demecolcin: 50 ng/mL
n. c.: not calculated as the CBPI is equal or higher than the solvent control value
Statistical evaluation: *: $p \leq 0.05$ (Chi square test)

Table 5.4.1-15: Results of in-vitro micronucleus test in human lymphocytes – with S9-mix

With metabolic activation (S9-mix): 4-hour exposure period, harvest at 40 hours					
Experiment I			Cytotoxicity		Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	CBPI [%]	Cytostasis [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO) [1]		-	2.01		0.30
BAS 750 F	2.0	-	1.89	12.2	0.60
	4.1	-	1.63	37.6	0.20
	8.2	-	1.30	70.5	0.55
Pos. ctrl. (CPA) [2]	17.5	-	1.66	34.4	**3.95***
Experiment II			Cytotoxicity		Genotoxicity
Test item	Conc. [µg/mL]	Precipi–tation	CBPI [%]	Cytostasis [%]	Micronucleated cells (%)
Neg. ctrl. (DMSO) [1]		-	2.02		0.75
BAS 750 F	2.0	-	1.97	4.9	0.70
	4.1	-	1.81	20.4	0.80
	8.2	-	1.59	42.0	0.65
Pos. ctrl. (CPA) [2]	15.0	-	1.66	35.9	**5.50***

[1] DMSO = Dimethyl sulfoxide: 0.5 % (v/v); [2] CPA = Cyclophosphamide: 17.5 / 15.0 µg/mL
Statistical evaluation: *: p≤0.05 (Chi square test)

III. CONCLUSION

Under the experimental conditions reported, the test substance did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, BAS 750 F is considered to be non-mutagenic in this in vitro micronucleus test when tested up to cytotoxic concentrations.

CA 5.4.2 In vivo studies in somatic cells

Report: CA 5.4.2/1
[redacted] 2014 a
BAS 750 F - Micronucleus test in bone marrow cells of the mouse
2014/1043159

Guidelines: OECD 474 (1997), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.12, EPA 870.5395

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch: COD-001740; Purity: 98.8%) was tested for chromosomal damage (clastogenicity) in NMRI mice using the micronucleus test method. For this purpose, the test substance, suspended in DMSO/corn oil (ratio 2:3), was administered once orally to groups of 5 male mice at dose levels of 375, 750 and 1500 mg/kg body weight in a volume of 20 mL/kg body weight. The vehicle served as negative and cyclophosphamide as positive control. The animals were sacrificed 24 or 48 (additional high dose group) hours after the administration, the bone marrow of the two femora was prepared from each animal. After staining of the preparations, 2000 polychromatic erythrocytes were evaluated per animal and investigated for micronuclei. The normocytes occurring per 2000 polychromatic erythrocytes were also recorded. In addition, blood samples taken immediately after sacrifice were analyzed to verify the bioavailability of the test substance.

The oral administration of BAS 750 F did not lead to any biologically relevant increase in the number of polychromatic erythrocytes containing micronuclei. The rate of micronuclei was mostly close to the concurrent negative control and was within the range of the historical control data. A slight inhibition of erythropoiesis induced by the treatment of mice with BAS 750 F was detected at the top dose of 1500 mg/kg body weight at 48-hour sacrifice interval but not in other dose groups.

The bioavailability of the test substance in blood after oral administration was clearly confirmed in plasma samples from all test group animals. Clinical signs observed in all dose groups included piloerection, hunched posture, reduced general condition, irregular respiration and duration of clinical signs were comparable between the different dose groups. Lacrimation was observed after 1 d in all dose groups examined. No signs of systemic toxicity were observed in any of the animals treated with the positive control substances, the vehicle or the test substance at the low/mid dose level. The positive control chemical cyclophosphamide led to the expected increase in the rate of polychromatic erythrocytes containing micronuclei, thus demonstrating the sensitivity of the test system.

Thus, under the experimental conditions of this study, the test substance BAS 750 F does not induce cytogenetic damage in bone marrow cells of NMRI mice *in vivo*.

(BASF DocID 2014/1043159)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material BAS 750 F

Description:	Solid, white
Lot/Batch #:	COD-001740
Purity:	98.8%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The homogeneity of the test substance was given on account of its high purity and moreover ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in DMSO over a period of 4 hours was verified analytically. The stability of the test substance in the vehicle DMSO/corn oil was determined indirectly by concentration control analysis.
Vehicle used:	DMSO in corn oil (2:3, v/v)

2. Control Materials:

Solvent control:	DMSO/corn oil (2:3, v/v)
Positive control:	Cyclophosphamide (CPP) 20 mg/kg bw (dissolved in deionized water); Vincristine sulfate (VCR) 0.15 mg/kg bw (dissolved in deionized water)

3. Test animals:

Species:	Mouse
Strain:	Crl:NMRI
Sex:	Male for the main study; male and female for the range finding study
Age:	5-8 weeks
Mean body weight at dosing:	28.0 g
Source:	Charles River Laboratories, Research Models and Services Germany GmbH, Sulzfeld, Germany
Number of animals per dose:	
Range finding study:	3 per sex and dose
Micronucleus assay:	5 males per control and treatment group
Acclimation period:	At least 5 days
Diet:	Standardized pelleted feed (Maus/Ratte Haltung “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland)
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cages, type II with dust-free wooden bedding

4. Environmental conditions:

Temperature:	20-24 °C
Humidity:	30-70%
Air changes:	not indicated (central air-conditioning)
Photo period:	12-hour light-dark cycle (06:00 - 18:00, 18:00 - 06:00)

5. Test compound doses:

Range finding test:	2000 mg/kg bw
Micronucleus assay:	375, 750 and 1500 mg/kg bw
	The test substance was administered once by oral gavage using an application volume of 10 mL/kg bw.

B. TEST PERFORMANCE:

1. Dates of experimental work: 09-Dec-2013 to 23-July-2014

2. Preliminary range finding test

In a pretest for the determination of the acute oral toxicity, 3 male and 3 female animals were treated once by oral gavage with a test substance dose of 2000 mg/kg bw.

3. Micronucleus test

Treatment and sampling:
Groups of 5 male mice were treated once with either the vehicle or 375, 750 or 1500 mg test substance per kg bodyweight by oral gavage (24 h sacrifice). An additional test group was used for the 48 h sacrifice period, where 5 animals were treated with vehicle or 1500 mg/kg bw of the test substance. The application volume was 10 mL/kg bw and the volume to be applied was calculated based on actual weight on the day of administration. The positive control substance CPP and VCR were administered once by oral gavage and the mice sacrificed after 24 h. The animals were observed for evident clinical signs of toxicity throughout the study.

24 or 48 hours after the administration the mice were anesthetized with isoflurane and afterwards killed by cervical dislocation. From each animal, both femora were prepared free of all soft tissue. After cutting the epiphyses the bone marrow was flushed out in a centrifugation tube with fetal calf serum (FCS) and subsequently centrifuged at 300 x g for 5 minutes. The supernatant was discharged and the pellet resuspended in FCS.

Slide preparation
One drop of the suspension was applied on a clean microscopic slide and smears were prepared. After air drying the smears were stained with May-Grünwald solution or Wrights solution, rinsed, and finally stained with Giemsa solution. Cover slips were mounted with Corbit-Balsam. The slides were coded prior to microscopic evaluation.

Slide evaluation

In general, 2000 polychromatic erythrocytes (PCEs) from each male animal of every test group were evaluated and investigated for micronuclei (MN). The normochromatic erythrocytes (NCEs) that occurred were also scored.

The increase in the number of micronuclei in polychromatic erythrocytes of treated animals as compared to the vehicle control group provides an index of a chromosome-breaking (clastogenic) effect or of a spindle activity (aneugenic) of the substance tested.

The ratio of polychromatic to normochromatic erythrocytes was calculated. An alteration of this ratio indicates a toxic effect on erythropoiesis and thus, that the test substance actually reached the target organ.

4. Plasma analysis

The bioavailability of the test substance in blood after oral administration was investigated by LC/MS analysis of plasma samples taken at the respective sampling intervals. The presence of the test substance in plasma was confirmed by the following criteria:

- Comparable retention times (± 5%) of the test substance in the sample and in the calibration solution (approx. 2.4 min)
- Ion ratios of the quantifier ion and the qualifier ion have to be comparable between test substance in sample and calibration solution (± 20% relative)

5. Statistics

The number of polychromatic erythrocytes with micronuclei was analyzed by comparing the dose groups with the vehicle control using the nonparametric Mann-Whitney test.

6. Evaluation criteria

The test substance is considered “positive” in this assay if the following criteria are met:

- A statistically significant and dose-related increase in the number of PCEs containing micronuclei.
- The number of PCEs containing micronuclei has to exceed both the concurrent vehicle control value and the range of the historical vehicle control data.

A test substance is generally considered “negative” in this test system if:

- The number of cells containing micronuclei in the dose groups is not statistically significant increased above the concurrent vehicle control value and is within the range of the historical vehicle control data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Stability, homogeneity and concentration control analyses

The stability of the test substance in the vehicle DMSO was verified analytically. The homogeneity of the test substance in the vehicle DMSO/corn oil (2:3 v/v) was guaranteed by constant stirring during the removal and administration of the test substance preparation and indirectly by analytical determination of 3 individual samples of each concentration. The mean concentrations were determined as 40.864, 73.636, and 148.757 mg/mL at nominal concentrations of 37.5, 75 and 150 mg/mL, respectively. This corresponds to a recovery rate in the range of 98-109% and is thus, within the expected range (90-110%).

Based on the analytical data the test substance was stable at room temperature in the vehicle DMSO/corn oil at least over a period of 35 minutes.

Plasma analyses

The bioavailability of the test substance in blood after oral administration was clearly confirmed in all plasma samples investigated by LC/MS analysis. The retention time of 2.42 min and the measured ion ratios were comparable to data obtained in reference chromatograms of the BAS 750 F calibration solution. No test substance could be detected in the vehicle control mouse samples.

B. PRELIMINARY RANGE FINDING TEST

In the pretest the recommended highest dose of 2000 mg/kg body weight was survived by all animals. However, severe signs of toxicity were observed from administration of the test substance until sacrifice about 48 hours later. Clinical signs included piloerection, abdominal position, hunched posture, reduced general condition, irregular respiration, eyelid closure and tremor. Besides, there were no distinct differences in clinical observations between male and female animals. Thus, only male animals were used in the main experiment as requested by the current OECD Guideline 474. Based on the data of the pretest a dose of 1500 mg/kg body weight was defined as MTD (maximum tolerated dose) and was administered as the highest dose in the present cytogenetic study. 750 mg/kg and 375 mg/kg body weight were administered as further doses.

C. MICRONUCLEUS ASSAY

Clinical examinations

The single oral administration of the vehicle in a volume of 10 mL/kg body weight was tolerated by all animals without any clinical observations. The administration of the test substance led to distinct clinical signs of toxicity in all exposed test groups from start of exposure until sacrifice (see Table 5.4.2-1). Unfortunately, the clinical observations of the animals of mid dose group were not recorded at time point 24 hours after oral administration.

Table 5.4.2-1: Mouse micronucleus test: clinical findings

Clinical observations	Number of animals with signs (number examined: 5)												
Dose:	375 mg/kg bw				750 mg/kg bw				1500 mg/kg bw				
Time point of observation:	1h	2h	4h	1d	1h	2h	4h	1d	1h	2h	4h	1d	2d
piloerection	5	5	5	5	5	5	5	ND	5	5	5	5	5
hunched posture	5	5	5	5	5	5	5	ND	5	5	5	5	5
reduced general condition		5	5	5	5	5	5	ND	5	5	5	5	5
irregular respiration				5				ND				5	
lacrimation				5				ND				5	

ND: not determined

Neither the single administration of the positive control substance cyclophosphamide in a dose of 20 mg/kg body weight nor that of vincristine sulfate in a dose of 0.15 mg/kg body weight caused any evident signs of toxicity.
Clinical signs observed in all dose groups comprised piloerection, hunched posture, reduced general condition, irregular respiration and duration of clinical signs were comparable between the different dose groups. Lacrimation was observed after 1 d in all dose groups examined.

Micronucleus test results
The single oral administration of the vehicle DMSO/corn oil in a volume of 10 mL/kg body weight led to 0.6‰ polychromatic erythrocytes containing micronuclei after the 24-hour sacrifice interval or to 1.5‰ after the 48-hour sacrifice interval, respectively. After the single administration of the highest dose of 1500 mg/kg body weight, 1.6‰ polychromatic erythrocytes containing micronuclei were found after 24 hours and 1.3‰ after 48 hours. In the two lower dose groups, rates of micronuclei of 1.0‰ (750 mg/kg group) and 1.2‰ (375 mg/kg group) were detected at a sacrifice interval of 24 hours in each case.

The positive control substance for clastogenicity, cyclophosphamide, led to a statistically significant increase (18.9‰) in the number of polychromatic erythrocytes containing exclusively small micronuclei, as expected.

Vincristine sulfate, a spindle poison, produced a statistically significant increase (42.0‰) in the number of polychromatic erythrocytes containing micronuclei. A significant portion increase, 10.1‰ was attributable to large micronuclei.

The number of normochromatic erythrocytes containing micronuclei did not differ to any appreciable extent in the vehicle control group or in the various dose groups at any of the sacrifice intervals.

A slight inhibition of erythropoiesis induced by the treatment of mice with BAS 750 F was detected at the top dose of 1500 mg/kg body weight at 48-hour sacrifice interval.

Table 5.4.2-2: Induction of micronuclei in bone marrow cells

Sampling: 24 h post-dosing		Scored	PCE With MN Total [‰]	PCE With MN Small [‰]	PCE With MN Large [‰]	NCE No.	NCE With MN [‰]	PCE / NCE ratio
BAS 750 F:	0 mg/kg bw	10000	0.6	0.6	0.0	5077	0.8	1.97
BAS 750 F:	375 mg/kg bw	10000	1.2	1.2	0.0	5202	0.6	1.92
BAS 750 F:	750 mg/kg bw	10000	1.0	1.0	0.0	5097	0.4	1.96
BAS 750 F:	1500 mg/kg bw	10000	1.6	1.5	0.1	4591	0.7	2.18
CPA:	20 mg/kg bw	10000	18.9**	18.9**	0.0	4895	0.6	2.04
Vincristine:	0.15 mg/kg bw	10000	42.0**	31.9**	10.1**	6361	1.4	1.57
Sampling: 48 h post-dosing		Scored	PCE With MN Total [‰]	PCE With MN Small [‰]	PCE With MN Large [‰]	NCE No.	NCE With MN [‰]	PCE / NCE ratio
BAS 750 F:	0 mg/kg bw	10000	1.5	1.5	0.0	4421	1.6	2.26
BAS 750 F:	1500 mg/kg bw	10000	1.3	1.3	0.0	6799	1.0	1.47

Statistical analysis: ** = $p \leq 0.01$ (Wilcoxon-test, 1-sided); MN = Micronucleated cells;
PCE = Polychromatic erythrocytes; NCE = normochromatic erythrocytes;
CPA = Cyclophosphamide

III. CONCLUSION

Based on the result of this study BAS 750 F did not induce micronuclei in mouse polychromatic erythrocytes under the conditions of the study, i.e. is devoid of clastogenic activity *in vivo*.

CA 5.4.3 In vivo studies in germ cells

According to Comm Reg. (EU) 283/2013, an *in vivo* study in germ cells may be considered in some specific cases to investigate whether a somatic cell mutagen is or is not a germ cell mutagen. In the case of BAS 750 F, the available data base on genotoxicity of BAS 750 F did not give rise to any concern. Therefore, an additional genotoxicity study in germ cells is not required. The absence of this study does not constitute a data gap.

CA 5.5 Long-Term Toxicity and Carcinogenicity

In long-term toxicity and carcinogenicity studies with BAS 750 F (2-yr combined chronic toxicity / carcinogenicity study in Wistar rats and 18-month dietary carcinogenicity study in C57BL/6JRj mice), there was no evidence of treatment-related tumorigenicity in either the rat or mouse. In both species, the liver was identified as target organ. In the rat, changes in several clinical chemistry parameters were seen at 600 ppm and 3600 ppm, which at the high-dose level were accompanied by liver weight increases in combination with liver cell hypertrophy. Liver effects in the mouse comprised increased liver weights and at the high dose increased incidence and severity of fatty change which in males was accompanied by eosinophilic inclusions in the cytoplasm (assessed as (pre)degenerative change), while in females single cell necroses were significantly increased. In addition, in the mouse cancer study, treatment-related effects at the high-dose level were noted in the thyroid (increased incidence of follicular cell hyperplasia in males), adrenal gland (increased absolute weight in males and relative weight in both sexes, and eosinophilic cytoplasm in females) and the kidney (decreased vacuolation in males with slightly decreased weights), which however were considered to be non-adverse due to the low incidence or low severity of the change compared to the concurrent control group.

The NOAEL in the rat combined chronic toxicity / carcinogenicity study was 5 mg/kg bw/d (100 ppm) based on two, slight clinical chemistry changes in male rats indicating possible impaired liver function at 29 mg/kg bw/d (600 ppm).

In the mouse carcinogenicity study, the NOAEL was 9.1 mg/kg bw/d (50 ppm) based on reduced body weight gain and adverse liver changes in males at 36 mg/kg bw/d (200 ppm).

Table 5.5-1: Summary of long-term toxicity studies with BAS 750 F

Study **Species,** **Dose levels** **(batch / purity)**	**NOAEL (mg/kg bw/d)**	**LOAEL (mg/kg bw/d)**	**Main adverse effects**	**Reference (BASF DocID)**
24-month combined chronic toxicity / carcinogenicity diet **Wistar rat** Batch: COD-001740 / 98.8% 0 – 100 – 600 – 3600 ppm Intake, 12-months: ♂: 0 – 6 – 34 – 216 mg/kg bw/d ♀: 0 – 8 – 45 – 322 mg/kg bw/d Intake, 24-months: ♂: 0 – 5 – 29 – 185 mg/kg bw/d ♀: 0 – 6 – 41 – 312 mg/kg bw/d	Chronic tox. ♂: **5** [100 ppm] ♀: **41** [600 ppm] Carcinogenicity ♂: **185** ♀: **312** [3600 ppm]	Chronic tox. ♂: **29** [600 ppm] ♀: **312** [3600 ppm] Carcinogenicity ♂: > **185** ♀: > **312** [3600 ppm]	≥ 600 ppm: altered clinical chemistry parameters in males 3600 ppm decreased body wt / gain, altered clinical chemistry parameters, increased liver weight with minimal-slight centrilobular hypertrophy in both sexes Not carcinogenic in rats.	[redacted] 2016b (2015/1000531) Kuettler, 2015a (2015/1261375)

Table 5.5-1: Summary of long-term toxicity studies with BAS 750 F

Study Species, Dose levels (batch / purity)	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Main adverse effects	Reference (BASF DocID)
18-month carcinogenicity **Oral diet** **Mouse (C57BL/6JRj)** Batch: COD-001740 / 98.8% ♂: 0 – 20 – 50 – 200 ppm = 0 – 3.5 – **9.1** – 36 mg/kg bw/d ♀: 0 – 20 – 50 – 250 ppm = 0 – 4.9– **12.6** – 61.5 mg/kg bw/d	♂: **9.1** [50 ppm] ♀: **12.6** [50 ppm] Carcinogenicity ♂: **36** [200 ppm] ♀: **61.5** [250 ppm]]	♂: **36** [200 ppm] ♀: **61.5** [250 ppm] Carcinogenicity ♂: > **36** [200 ppm] ♀: **> 61.5** [250 ppm]	200 ppm (♂) / 250 ppm (♀) decreased body wt / gain, increased rel. liver wt, increased incidence/severity of fatty change and signs of (pre)degeneration in liver cells (♂: eosinophilic inclusions, ♀: single cell necrosis) Not carcinogenic in mice	[redacted] 2015b (2015/1000532)

Comparison with CLP criteria:

Carcinogenicity

Two long term studies are available with BAS 750 F: One combined chronic and carcinogenicity study in rats and one carcinogenicity study in mouse.

There were no treatment-related increased incidences of tumors in these studies. Thus no classification for carcinogenicity is warranted for BAS 750 F.

Target organ toxicity, repeated exposure

Rats: In the 2-yr combined chronic toxicity / carcinogenicity rat study, treatment-related adverse effects occur at doses of 34 mg/kg bw in male rats and at the high dose level (216-322 mg/kg bw/d) in both sexes. The liver was identified as only target organ. Treatment-related effects in male rats at 34 mg/kg bw/d were confined to altered hematology or clinical-chemistry parameters (reduced activated thromboplastim time after 12-month treatment, increased serum alkaline phosphatase seen after 3, 6 and 12 months, increased urea after 12 months); there were no corresponding adverse liver weight or histopathological changes at this dose level after 12- or 24-month treatment. At the high dose level, additional clinical chemistry changes were seen and accompanied by slight liver weight increases and hepatocellular hypertrophy (of slight severity at most). Overall, while the observed clinical chemistry changes seen at 34 mg/kg bw/d might be considered significant, they are not considered to be severe effects. By comparison, guidance values for STOT RE 2 classification for 3-month treatment are 100 mg/kg bw/d, and applying Haber's rule, 6-mo: 50 mg/kg bw/d, 12-mo: 25 mg/kg bw/d. After 3- or 6-month treatment only one clinical chemistry parameter was changed. On the basis of expert judgement and consideration of classification practice, the effects on the rat liver induced are not regarded to constitute serious toxicity therefore do not warrant STOT RE classification.

Mouse: In the 18-month carcinogenicity study, the liver was identified as target organ. At dose levels of 36 mg/kg bw/d (200 ppm) in males and 61 mg/kg bw/d (250 ppm), markedly increased liver weights (rel bw, ♂: +42%, ♀+57%), increased incidence/severity of fatty change and signs of (pre)degeneration in liver cells (♂: eosinophilic inclusions in 38/50 mice, ♀: minimal grade single cell necrosis in 10/50 mice) were observed. However, no cases of liver cell necrosis were seen in male mice at the top dose level of 200 ppm. At the next lower dose level of 50 ppm (intakes of 9.1 and 12.6 mg/kg bw/d in male and female mice, respectively), slight effects on the liver were observed but not considered to be adverse. Overall, the liver toxicity seen at 200 and at 250 ppm is considered to be both significant and severe. However, by comparison with guidance values for STOT RE 2 classification (17 mg/kg bw/d, applying Haber's rule for an 18-month treatment period), significant/severe effects occurred only at dose levels that were at least 2-fold higher than the calculated guidance classification threshold value, while the next lower tested (non-adverse) dose level was up to 2-fold lower than the guidance value. Overall, applying expert judgement and considering the classification practice, the effects in the 18-month mouse study do not trigger a STOT RE 2 classification.

Report:	CA 5.5/1 [redacted], 2016 b BAS 750 F - Combined chronic toxicity/carcinogenicity study in Wistar rats - Administration via the diet up to 24 months 2015/1000531
Guidelines:	(EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.33, EPA 870.4300, OECD 453, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Report:	CA 5.5/2 Kuettler K., 2015 a Historical control data - Compilation from 24-month studies in Wistar rats (CrI:WI (Han)) - Performed at BASF SE Experimental Toxicology and Ecology, Ludwigshafen, Germany 2015/1261375
Guidelines:	OECD 453; EPA 870.4300; Commission Regulation (EC) No 440/2008; JMAFF No 12 Nosan No 8147
GLP:	no
Note:	The summarized historical control data refers to the same studies as in the BAS 750 F study report, but includes additional study details such as survival data.

EXECUTIVE SUMMARY

BAS 750 F (batch: COD-001740, purity: 98.8%) was orally administered via the diet to Wistar rats over a period of either 12 or 24 months, at dietary concentrations of 0, 100, 600 and 3600 ppm (corresponding to mean intakes 0, 5, 29 or 185 mg/kg bw/d in male rats and 0, 6, 41 or 312 mg/kg bw/d after 24-month treatment). The study design followed OECD Test Guideline 453, with groups of 10 rats/sex assessed for chronic toxicity after 12-month treatment and groups of 50 rats/sex evaluated for carcinogenicity following a 24-month exposure period. Treatment resulted in reduced body weight, body weight gain and changed clinical pathology parameters, which at the high dose level were associated with liver weight increases and liver cell hypertrophy of minimal to slight severity. Thus, signs of systemic toxicity were determined at 3600 ppm and 600 ppm (about 29 mg/kg bw/d) in males and at 3600 ppm (about 312 mg/kg bw/d) in females.

The no observed adverse effect level (NOAEL) was considered to be 100 ppm (about 5 mg/kg bw/d) in males and 600 ppm (41 mg/kg bw/d) in females.

Under the conditions of the study, BAS 750 F demonstrated no carcinogenic potential in Wistar rats up to the highest dose level tested.

(DocID 2015/1000531)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: Rodent diet

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Source:	Charles River Laboratories, Research Models and Services GmbH, Germany
Sex:	male and female
Age (day 0):	42 ± 1 days
Weight (day 0, means):	chronic tox. groups: ♂: 155.3 - 158.9 g; ♀: 125.4 - 127.8 g carcinogenicity gr. ♂: 158.3 - 159.6 g; ♀: 126.0 - 128.4 g
Acclimatization period:	at least 7 days
Diet:	Kliba maintenance diet rat/mouse "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water from water bottles, ad libitum
Housing:	Group housing (5 animals/cage) housed in H-Temp (PSU) cages (TECNIPLAST Deutschland GmBH, Hohenpeißenberg, Germany, floor area about 2065 cm² with dust free wooden bedding.; wooden gnawing blocks as enrichment ((Typ NGM E-022), Abedd®, Lab. and Vet. Service GmbH, Vienna, Austria)
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 12-Mar-2013 to 28-Jan-2016 [in life phase: 19-Mar-2013 (start treatment of male rats) to 06-Apr-2015 (necropsy of female rats)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 60 male and 60 female Wistar rats at dietary concentrations of 0, 100 (low dose), 600 (intermediate dose), and 3600 ppm (top dose) for about 12 months (chronic toxicity groups; 10 animals/sex/dose) and 24 months (carcinogenicity groups; 50 animals/sex/dose). The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights. At the end of the administration period the animals were sacrificed after a fasting period of at least 16 hours.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diet preparations were usually performed every 2 weeks.

Analyses performed with a similar batch (L84-176) prior to the start of the administration period revealed that the test-substance was stable in the diet for at least 35 days.

Concentration control analyses of all dose levels were determined after about 3, 6, 9, 12, 15, 18 and 21 months as well as towards the end of the administration period. Homogeneity was demonstrated using 3 samples of one concentration taken at separate levels from the bulk container (nominally top middle bottom), and concentration control analyses were performed with 1 random sample. The samples were taken from the specific food containers. The homogeneity analyses also served as concentration controls for the respective concentration. The analyses from 3 months onwards were performed with samples at the end of the time period for which the respective test substance preparations were used. The samples were taken out in an ascending sequence, beginning with the lowest concentration after about 3 months, taken from replacement food containers being stored in the animal room. Thus, the stability of the test substance in the diet was proven under test conditions.

Table 5.5-2: Homogeneity and concentration control analyses of diet preparations

Dose level [ppm]	Sampling	Analysis	Concentration [ppm] Mean ± SD	Relative standard deviation [%]	Mean % of nominal concentration
100 ppm	18.03.13	27.03.13 03.04.13	93.4 ± 2.1#	2,1	93.4
	24.06.13	02.07.13	91.3 ± 0.9#	0.9	91.3
	16.09.13	02.10.13	93.9		93.9
	23.12.13	16.01.14	92.7		92.7
	31.03.14	09.04.14	93.1 ± 3.2#	3.5	93.1
	07.07.14	07.07.14	94.5		94.5
	22.09.14	22.09.14	98.5		98.5
	15.12.14	16.12.14	98.7 ± 0.2#	0.2	98.7
	26.03.15	27.03.15	91.9		91.9
average			**94.2 ± 3.0**		**94.2 ± 3.0**
600 ppm	18.03.13	27.03.13	647		107.9
	24.06.13	02.07.13	564		93.9
	16.09.13	02.10.13	589 ± 11#	1.9	98.2
	23.12.13	16.01.14	540		90.0
	31.03.14	09.04.14	611		101.8
	07.07.14	07.07.14	553 ± 15#	2.7	92.2
	22.09.14	22.09.14	575		95.9
	15.12.14	16.12.14	588		97.9
	26.03.15	27.03.15	551 ± 24#	4.4	91.8
average			**574 ± 31**		**95.6 ± 5.2**
3600 ppm	18.03.13	27.03.13 03.04.13	3630 ± 61#	1.7	100.8
	24.06.13	02.07.13	3900		108.3
	16.09.13	02.10.13	3948		109.7
	23.12.13	16.01.14	3589 ± 64#	1.8	99.7
	31.03.14	09.04.14	3598		99.9
	07.07.14	07.07.14	3531		98.1
	22.09.14	22.09.14	3800 ± 153#	4.0	105.6
	15.12.14	16.12.14	3919		108.9
	26.03.15	27.03.15	3768		104.7
average			**3715 ± 155**		**103.2 ± 4.3**

No test article was detectable in control diets. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC analysis with an external standard.

Relative standard deviations of maximum 5.2% indicated the homogenous distribution of BAS 750 F in the diet preparations. The actual nominal test-item concentrations were in the range of 90 to 110% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
Food consumption (main groups), body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means

Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Urinalysis parameters (except pH, urine volume, specific gravity, color and turbidity	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians. In case of exactly the same values of the dose group and the control, no statistical test is performed.
Urine pH, volume, and specific gravity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Urine color and turbidity	Urine color and turbidity are not evaluated statistically.

Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Histopathological findings of protocol organs examined in all test groups (main groups	Comparison of all test groups with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Histopathological findings of protocol organs examined only in control and high-dose groups (main groups)	Comparison of high-dose with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the conduct of the study, body weight was determined on day 0 (start of the administration period), at weekly intervals during the first 13 weeks of the study, at 4-weeks intervals thereafter, and prior to start of necropsy. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly over a period of 1 day during the first 13 weeks of the administration period, thereafter at 4-week intervals and prior to the start of necropsy. The average food consumption/cage was used to estimate the mean food consumption in grams per animal and day.

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and food consumption, according to the following equation:

$$\text{Substance intake for Day}_x = \frac{FC_x \times C}{BW_x}$$

with FC_x as the mean daily food consumption (in g/day) on Day_x, C as the dose in ppm and BW_x as body weight on Day_x of the study (in g).

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any overt changes in volume.

5. Ophthalmoscopy

Prior to the start of the administration period the eyes of all satellite animals, and at the end of the administration period (12 months) the eyes of the control (0 ppm) and high dose animals (3600 ppm) were examined for any changes using an ophthalmoscope after administration of a mydriatic agent.

6. Hematology and clinical chemistry

Blood was withdrawn in the morning from fasted, isoflurane-anesthetized animals from the retro-orbital plexus. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for 10 animals per test group (satellite animals) after 3, 6 and 12 months of the administration period:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
✓	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

Additionally, differential blood smears were prepared and stained according to Wright from all surviving carcinogenicity group animals without being evaluated.

7. Urinalysis

For urinalysis the individual animals were transferred to metabolism cages (withdrawal of food and water) and urine was collected overnight. Urine samples were evaluated in a randomized sequence. The following parameters were determined in 10 animals per test group and sex (satellite groups) after 3, 6 and 12 months of the administration period:

Urinalysis					
	Quantitative parameters		*Semi-quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilirubin
		✓	Glucose	✓	Sediment (microscop. examination)
		✓	Ketones		

8. Sacrifice and pathology

The animals – if not found dead – were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining:

Pathology:											
S	**W**	**H**		**S**	**W**	**H**		**S**	**W**	**H**	
✓	✓	1	adrenals	✓	✓	1	kidneys	✓		1	skin
✓		1	aorta	✓		1	lachrymal glands[%]	✓		1	spinal cord (3 levels)[@]
✓		1	bone marrow[§]	✓		1	larynx	✓	✓	1	spleen
✓	✓	1	brain	✓	✓	✓	liver	✓		1	sternum w. marrow
✓		1	caecum	✓		1	lung	✓		1	stomach (fore- & glandular)
✓		1	cervix	✓		1	lymph nodes[#]	✓			teeth
✓		1	coagulating gland	✓		1	mammary gland (♀+♂)	✓	✓	1	testes
✓		1	colon	✓		1	muscle, skeletal	✓		1	thymus
✓		1	duodenum	✓		1	nerve, peripheral (sciatic n.)	✓	✓	✓	thyroid [‡‡]
✓	✓	1	epididymides	✓		1	nose/nasal cavity[‡]	✓		1	parathyroid
✓		1	esophagus	✓			olfactory bulb	✓			tongue
✓		1	eyes (with optic nerve)	✓	✓	#	ovaries and oviduct**	✓		1	trachea
✓		1	femur (with joint)	✓		1	pancreas	✓			ureter
			gall bladder	✓		1	pharynx	✓			urethra
✓		✓	gross lesions	✓		1	pituitary	✓		1	urinary bladder
✓		1	Harderian gland	✓		1	prostate	✓	✓	#	uterus
✓	✓	1	heart	✓		1	rectum	✓		#	vagina
✓		1	ileum	✓		1	salivary glands*				
✓		1	jejunum	✓		1	seminal vesicles				

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, **#**: control and top dose groups plus animals died pre-schedule. **1** =**#** plus all males from low- and mid-dose 24-month study groups.

[§] from femur; [#] axillary and mesenteric; [@] cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; [%] extraorbital, [‡] histopathology at level III; [‡‡] thyroid wts were determined only in satellite groups

All animals which died intercurrently or were sacrificed in a moribund state were processed histopathologically and assessed like control animals.

In addition, the following special stains and immunohistochemical investigations were carried out in livers and pituitary glands of selected animals:

- Cresyl violet stain for classification of basophilic foci
- Glutathione S-transferase P (GSTP) immunohistochemistry for detection of eosinophilic foci

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

Chronic toxicity groups

No animals of the satellite groups died during the administration period.

Carcinogenicity groups

The mortality rates of main groups until study day 728 (equivalent to 24 months of treatment) are given in the following table:

Table 5.5-3: Mortality within 24 months

Dose level (ppm)	Mortality (%)	
	Males	Females
0	0	24
100	24	20
600	22	18
3600	6	10

No treatment-related increase of mortality incidences was observed. For male animals, the highest mortality occurred in the low-dose group (100 ppm) and for females in the control group (0 ppm). The lowest mortality rates were obtained in males of the control group (0 ppm) and in females of the high-dose test group (3600 ppm). The obtained mortality rates were within the biological range typical for this strain of rats.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals.

3. Ophthalmoscopy

Chronic toxicity groups

No test substance-related effects were obtained. The findings which occurred in animals of the control and high-dose groups after one year of exposure, i.e. corneal stipplings, are common findings in animals of this strain and age. The findings were often observed in the same animals at both time points. Therefore, a relation to treatment was excluded.

C. BODY WEIGHT AND BODY WEIGHT GAIN

1. Body weight [Figure 5.5-1, Table 5.5-4]

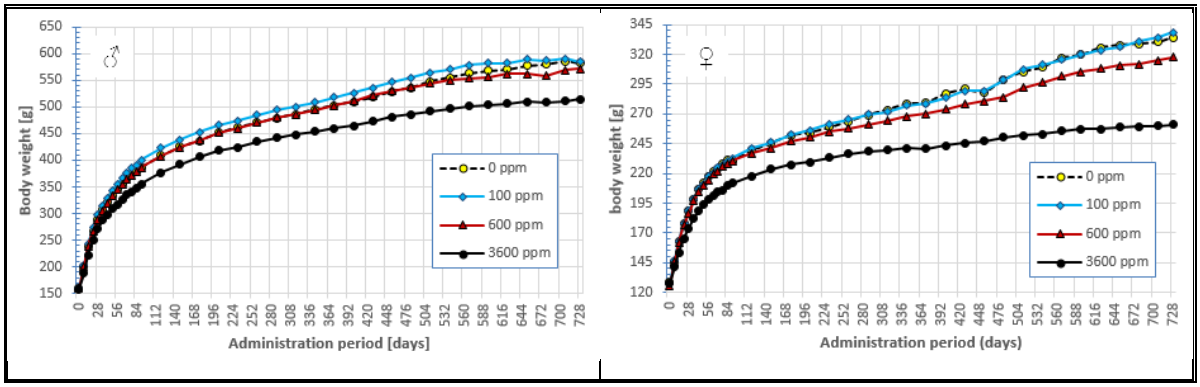
Chronic toxicity groups

A trend to lower mean body weights in male and female animals of the high-dose group (3600 ppm) was observable which showed a statistical significance on study days 14 and 63 in males and from study day 14 until the end of the administration period in females, with a maximum of -9.1% on study day 63 in male and -15% on study day 343 in female animals. No relevant effects on body weight were observed in male and female animals of the low- and mid-dose groups (100 and 600 ppm).

Carcinogenicity groups

In male and female animals of the high-dose groups (3600 ppm) the mean body weight values were significantly lower from study day 7 onwards during the entire administration period, with a maximum of -13% in males on study day 707 and -22% in females on study day 728. Again, male and female animals of the low- and mid-dose groups (100 and 600 ppm) did not show differences in mean body weights to the control groups which were assessed as being relevant.

Figure 5.5-1: Body weight development of rats administered BAS 750 F for 2 years



2. Body weight gain [Table 5.5-4]

Chronic toxicity groups

Mean body weight change values of male and female animals of satellite test group 13 (3600 ppm) were significantly lowered during the entire administration period, with a maximum of -28% in males on study day 7 and -45% in females on study day 14. The slightly lower body weight change values observed for male and female animals administered 100 or 600 ppm were assessed to be of no toxicological relevance.

Carcinogenicity groups

In male and female animals of the high-dose group (3600 ppm) the body weight gain values were significantly lower from study day 7 onwards until the end of the administration period, with a maximum of -29% in males on study day 7 and -35% in females on study day 728. The slightly lower body weight gains observed for male and female animals of low and mid-dose test groups (100 and 600 ppm) were considered to be of no biological relevance.

Table 5.5-4: Body weight development (main groups)

	Males				**Females**			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Body weight [g]								
- Day 0	159.1	159.6	159.2	158.3	128.4	128.1	126.0	127.5
- Day 91	386.3	400.0	386.0	**355.4****	230.8	232.8	230.3	**211.6****
Δ% (compared to control[#]		+3.5	-0.1	**-8.0**		+0.9	-0.2	**-8.3**
- Day 371	502.0	518.0	502.4	**460.2****	279.4	278.6	270.1	**240.9****
Δ% (compared to control[#]		+3.2	+0.1	**-8.3**		-0.3	-3.3	**-13.8**
- Day 728	582.1	584.3	572.0	**519.9****	334.1	338.5	317.8	**260.9****
Δ% (compared to control[#]		+0.4	-1.7	**-11.6**		+1.3	-4.9	**-21.9**
Overall body weight gain (g)								
- Day 91	227.2	240.3	226.7	**197.1****	102.4	104.7	104.3	**84.0****
Δ% (compared to control[#]		+5.8	-0.2	**-13.3**		+2.2	+1.8	**-18.0**
- Day 371	342.9	358.2	343.2	**301.9****	158.5	155.4	147.9	**115.6****
Δ% (compared to control[#]		+4.5	+0.1	**-12.0**		-2.0	-6.7	**-27.1**
- Day 728	423.0	424.8	413.3	**356.8****	206.3	210.6	192.5	**133.8**
Δ% (compared to control[#]		+0.4	-2.3	**-15.7**		+2.1	-6.7	**-35.1**

[#] Values may not calculate exactly due to rounding of mean values

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

No test substance-related changes were observed in male and female animals.

2. Water consumption

No test substance-related, adverse changes were observed.

3. Test substance intake

The approximate, mean daily test-substance intake in mg/kg bw/d over the entire study period is shown in the following table, i.e. separately for the satellite as well as for the main groups:

Table 5.5-5: Mean test substance intake

	Males			Females		
Dose level [ppm]	**100**	**600**	**3600**	**100**	**600**	**3600**
BAS 750 F (mg/kg bw/d)						
- Satellite groups (Day 0-371)	6	34	216	8	45	322
- Main groups (Day 0-728)	5	29	185	6	41	312

E. BLOOD ANALYSES (Satellite groups)

1. Hematology

The following statistically significant changes of hematology parameters were assessed to be treatment-related:

≥ 600 ppm

- ↓ Activated partial thromboplastin time (PTT) in males after 6- and 12-month treatment

3600 ppm

- ↓ Activated partial thromboplastin time (PTT) in both sexes after 3, 6, 12-month treatment (not statistically significant in females after six months)
- ↓ Platelet counts (PLT) in males after 12-month treatment

The following statistically significant changes of hematology parameters were assessed to be incidental, i.e. not considered treatment-related:

3-months

- ↑ Hemoglobin (HGB) and hematocrit (HCT) in males at 3600 ppm (within the historical control range, HCR)
- ↑ Abs. basophil counts (BASOA) in males at 3600 ppm (within the HCR)
- ↑ Rel. neutrophil counts (NEUT) in females at 100 ppm (no dose-dependent change)
- ↓ Rel. lymphocyte counts (LYMPH) in females at 100 ppm (no dose-dependent change)

6-months

- ↑ Abs. large unstained cell (LUC) counts in males at 3600 ppm (within the HCR)
- ↑ Rel. large unstained cell (LUC) counts in both sexes at 3600 ppm (within the HCR)
- ↑ Rel. neutrophil counts (NEUT) in females at 100 ppm (no dose-dependent change)
- ↓ Rel. lymphocyte counts (LYMPH) in females at 100 ppm (no dose-dependent change)
- ↓ Rel. monocyte counts (MONO) in males at 100 and 3600 ppm (no dose-dependent change)

12-months

- ↓ Abs. and rel. eosinophilic counts in females at 600 ppm (no dose-dependent change)

Table 5.5-6: Selected hematology parameters (statistically significant)

Dose level [ppm]		Males				Females			
		0	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Parameter	**Month**								
PTT [s]	3	21.4	21.3	20.1	**18.8****	19.9	19.7	19.6	**18.5***
	6	19.7	19.3	**18.8***	**17.9****	18.5	18.7	18.4	**17.3**
	12	20.3	19.8	**19.2***	**18.4****	19.1	19.6	19.3	**17.9***
PLT [giga/L]	3	771	727	719	667	819	796	750	781
	6	693	661	663	663	747	724	691	709
	12	724	661	680	**640****	716	717	666	691
LUCA [giga/L]	3	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02
	6	0.02	0.01	0.01	0.02*	0.01	0.01	0.01	0.02
	HCR 6	*range: 0.01–0.03 giga/L; mean: 0.02*							
	12	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01
LUC [%]		0.3	0.3	0.4	0.4	0.5	0.4	0.3	0.5
		0.3	0.2	0.3	0.5*	0.3	0.3	0.3	0.5**
	HCR 6	*range: 0.2–0.5%; mean: 0.4*				*range: 0.3–0.5%; mean: 0.4*			
		0.4	0.4	0.4	0.5	0.4	0.4	0.3	0.4
HGB [mmol/L]	3	8.8	9.0	8.8	9.1*	8.4	8.2	8.2	8.3
	HCR 3	*range: 8.7–9.8 mmol/L; mean: 9.1*							
	6	8.8	8.8	8.8	8.9	8.7	8.5	8.5	8.4
	12	8.8	9.0	8.9	8.9	8.6	8.6	8.5	8.6
HCT [L/L]	3	0.417	0.426	0.419	0.435**	0.401	0.393	0.393	0.393
	HCR 3	*range: 0.411–0.457 L/L; mean: 0.428*							
	6	0.413	0.414	0.416	0.418	0.406	0.398	0.396	0.390
	12	0.415	0.425	0.419	0.421	0.401	0.408	0.398	0.405
BASOA [giga/L]	3	0.02	0.02	0.01	0.02*	0.02	0.02	0.02	0.02
	HCR 3	*range: 0.00–0.02 giga/L; mean: 0.01*							
	6	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	12	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
MONO [%]	3	1.8	1.8	1.8	1.7	1.8	2.1	1.9	1.6
	6	2.3	1.7**	2.0	1.9*	2.1	2.3	2.2	2.0
	12	2.7	2.4	2.3	2.5	2.8	3.4	2.6	2.4
EOSA [giga/L]	3	0.11	0.12	0.10	0.10	0.10	0.10	0.08	0.10
	6	0.10	0.11	0.08	0.10	0.09	0.09	0.07	0.08
	12	0.10	0.08	0.08	0.08	0.08	0.06	0.05**	0.07
EOS [%]	3	2.3	2.2	2.0	1.8	2.9	2.8	2.5	2.7
	6	2.2	2.1	1.9	2.0	2.8	2.8	2.4	2.7
	12	2.2	2.0	2.1	2.2	3.7	2.8	2.1**	2.9
LYMPH [%]	3	75.4	76.1	76.5	78.2	78.1	72.7*	77.9	81.0
	6	70.6	72.7	74.0	74.2	77.0	68.3*	73.6	77.4
	12	64.2	62.0	62.2	67.1	63.8	65.1	64.4	70.2
NEUT [%]	3	19.9	19.3	19.0	17.5	16.1	21.4*	16.6	13.7
	6	24.4	23.2	21.6	21.3	17.7	26.1*	21.4	17.1
	12	30.2	32.9	32.7	27.4	28.8	27.7	30.1	23.7

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test. two sided)
HCR = Historical control data - 3-month: 47/48 studies (2008-2013); - 6-month: 11/12 studies (2004-2014)

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related:

100 ppm, male rats

- ↓ Alanine aminotransferase (ALT) at 100, 600 and 3600 ppm (3 months), decreases were slight (less than 50%), not adverse but considered related to enzyme induction

600 ppm, male rats

- ↓ Alanine aminotransferase (ALT) at 100, 600 and 3600 ppm (3 months), decreases were slight (less than 50%), not adverse but considered related to enzyme induction
- ↑ Alkaline phosphatase (ALP) at 600 ppm and 3600 ppm (3, 6, 12 months); **adverse**
- ↑ Urea at 600 ppm (12 months) and 3600 ppm (6, 12 months); **adverse**

600 ppm, female rats

- ↓ Total bilirubin (TBIL) at 600 ppm (6 months) and 3600 ppm (3, 6, 12 months), likely due to increased bilirubin conjugation and excretion via enzyme induction; not adverse

3600 ppm, male rats

- ↑ Alkaline phosphatase (ALP) at 600 ppm and 3600 ppm (3, 6, 12 months); **adverse**
- ↓ Glucose (GLUC) at 3, 6, and 12 months; **adverse**
- ↓ Alanine aminotransferase (ALT) at 12 months, decreases were slight (less than 50%), not adverse but considered related to enzyme induction
- ↓ Total bilirubin (TBIL) at 3 months; likely due to increased bilirubin conjugation and excretion via enzyme induction; not adverse

3600 ppm, female rats

- ↑ Alkaline phosphatase (ALP) at 3, 6, and 12 months; **adverse**
- ↓ Glucose (GLUC) at 3, 6, and 12 months (not stat. sign. after 6 months); **adverse**
- ↓ Creatinine (CREA) at 3 months; **adverse**
- ↓ Total protein (TPROT) and albumin (ALB) at 3 and 6 months; **adverse**
- ↑ Cholesterol (CHOL) at 3 months; **adverse**
- ↓ Alanine aminotransferase (ALT) at 12 months; decreases were slight (less than 50%), not adverse but considered related to enzyme induction
- ↓ Total bilirubin (TBIL) at 600 ppm (6 months) and 3600 ppm (3, 6, 12 months), likely due to increased bilirubin conjugation and excretion via enzyme induction; not adverse

After three months, alanine aminotransferase (ALT) activities were decreased in males of test groups 11, 12 and 13 (100, 600 and 3600 ppm). ALT activities were also decreased in male and female rats of test group 13 (3600 ppm) after twelve months. However, these decreases were slight (decrease below 50%). They were most probably due to a liver enzyme induction resulting in a decreased synthesis of ALT in favor of other enzymes (PSD Guidance document, 2007) or were not clinically significant (Whalan J.E. (2015): A Toxicologist's Guide to Clinical Pathology in Animals: Hematology, clinical Chemistry, Urinalysis", Switzerland, Springer Intl. Publ., page 68). This was confirmed by decreased total bilirubin levels in males and females of test group 13 (3600 ppm) after three months and in females of test group 12 and 13 (600 and 3600 ppm) after six months and in females of test group 13 after twelve months. Lower total bilirubin levels were most probably due to an increased conjugation rate of bilirubin and a subsequent higher excretion via the bile. Therefore, the decreases of ALT activities and total bilirubin levels in the mentioned individuals were regarded as treatment-related, but not adverse.

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

- ↓ Aspartate aminotransferase (AST) at 100 ppm (3 months); no dose-response
- ↑ Sodium at 600 ppm (6 months); no dose-response
- ↑ Cholesterol (CHOL) at 600 ppm (3 months) and 3600 ppm (3, 6 months); within the HCR

Females

- ↑ Alkaline phosphatase (ALP) at 100 ppm (6, 12 months); no dose-response
- ↑ Aspartate aminotransferase (AST) at 3600 ppm (3 months); within the HCR
- ↓ Total protein (TPROT) at 600 ppm and albumin (ALB) at 600 and 100 ppm (3 months); within the HCR
- ↓ Globulin (GLOB) at 3600 ppm (3 months); within the HCR
- ↑ Inorganic phosphate (INP) at 100 and 3600 ppm (3 months); no dose-response, within the HCR
- ↓ Triglycerides (TRIG) at 600 ppm (12 months); no dose-response

Table 5.5-7: Selected clinical chemistry parameters (statistically significant)

Dose [ppm]		Males				Females			
		0	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Parameter	**Month**								
ALT [µkat/L]	3	0.92	0.68**	0.71*	0.65**	0.53	0.61	0.48	0.55
	HCR 3	*range: 0.53–0.85 µkat/L; mean: 0.70*							
	6	0.72	0.65	0.67	0.61	0.54	0.56	0.49	0.46
	12	0.81	0.71	0.72	0.56*	0.80	0.66	0.73	0.48**
AST [µkat/L]	3	3.44	1.79*	1.89	2.55	1.32	1.47	1.40	1.58**
	HCR 3					*range: 1.43–2.30 µkat/L; mean: 1.75*			
	6	1.76	1.59	1.68	1.78	1.53	1.56	1.49	1.78
	12	1.48	1.47	1.56	1.49	2.40	1.63	2.09	1.71
ALP [µkat/L]	3	1.08	1.12	**1.38****	**1.59****	0.48	0.61	0.56	**0.88****
	6	0.92	0.98	**1.24****	**1.33****	0.34	0.47**	0.43	**0.63****
	12	0.92	1.00	**1.28****	**1.36****	0.33	0.50*	0.44	**0.72****
SGGT [nkat/L]	3	0	1	1	0	8	8	12	17
	6	0	0	0	0	0	0	0	3
	12	0	0	1	2	0	0	0	3
PO_4^{3-}, inorg. [mmol/L]	3	1.71	1.69	1.79	1.95	1.19	1.39*	1.28	1.47**
	HCR 3					*range: 1.14–1.55 mmol/L; mean: 1.33*			
	6	1.56	1.54	1.60	1.68	1.30	1.37	1.22	1.35
	12	1.21	1.22	1.25	1.31	0.88	1.07	0.98	1.11
Sodium [mmol/L]	3	142.1	143.2	143.5	143.4	139.8	138.8	139.3	138.3
	6	142.3	142.6	143.4*	142.7	141.2	141.2	141.1	140.3
	12	141.9	142.8	142.4	142.2	141.1	141.2	141.4	140.1
Glucose [mmol/L]	3	6.95	6.54	6.38	**5.49****	5.46	5.40	5.50	**4.86***
	6	6.88	6.53	6.29	**5.76****	5.63	5.26	5.19	5.05
	12	6.59	6.43	6.22	**5.79****	6.10	5.91	5.64	**4.90****
Bilirubin, tot. [µmol/L]	3	1.96	1.83	1.76	1.28**	2.00	1.90	1.81	1.23**
	6	1.54	1.69	1.56	1.21	3.08	2.98	2.45*	1.71**
	12	1.33	1.51	1.48	1.15	2.39	2.18	1.96	1.28**
Protein, total [g/L]	3	62.48	62.45	61.74	62.00	67.04	65.49	64.67*	**60.68****
	HCR 3					*range: 62.13–70.12 g/L; mean: 66.25*			
	6	63.24	63.32	63.58	61.66	66.53	66.25	64.96	**61.77****
	12	67.26	68.09	68.12	65.99	70.53	71.11	70.31	67.98
Albumin [g/L]	3	34.25	34.41	33.85	34.33	42.52	41.58*	40.76*	**38.30****
	HCR 3					*range: 37.49–43.65 g/L; mean: 41.07*			
	6	38.50	38.70	38.63	38.02	41.79	41.43	40.63	**38.22****
	12	39.55	39.82	39.68	38.97	43.15	43.40	42.75	41.12
Globulin [g/L]	3	28.23	28.05	27.89	27.67	24.52	23.91	23.91	22.38*
	HCR 3					*range: 19.54–29.31 g/L; mean: 25.26*			
	6	24.75	24.62	24.95	23.64	24.74	24.81	24.33	23.54
	12	27.71	28.26	28.45	27.02	27.38	27.71	27.56	26.86
Cholesterol [mmol/L]	3	1.63	1.88	1.92*	2.02*	1.39	1.40	1.29	**1.95****
	HCR 3	*range: 1.48–2.14 mmol/L; mean: 1.82*							
	6	1.79	2.05	2.12	2.29**	1.60	1.53	1.45	1.96
	HCR 6	*range: 1.84–2.32 mmol/L; mean: 2.09*							
	12	2.33	2.47	2.57	2.77	2.18	2.09	1.97	2.37
Triglycerides [mmol/L]	3	0.84	0.86	0.69	0.79	0.46	0.46	0.41	0.73
	6	0.99	1.02	0.86	0.82	0.82	0.72	0.65	1.19
	12	1.65	1.52	1.37	1.33	1.19	1.33	0.81**	1.44
Urea [mmol/L]	3	6.79	6.48	7.20	7.05	6.72	7.19	7.26	7.67
	6	5.33	5.67	5.81	**6.27****	5.86	6.05	5.96	6.78
	12	4.05	4.34	**4.72****	**5.15****	6.02	6.01	5.72	6.78

Table 5.5-7: Selected clinical chemistry parameters (statistically significant)

Dose [ppm]		Males				Females			
		0	100	600	3600	0	100	600	3600
Creatinine [µmol/L]	3	55.9	56.1	57.8	55.6	59.2	58.7	60.2	**54.5****
	6	31.8	31.5	32.6	32.9	36.3	37.2	39.0	36.1
	12	32.1	30.8	33.0	31.8	35.0	35.1	35.6	34.6

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test. two sided)
HCR = Historical control data - 3-month: 48 studies (2008-2013); - 6-month: 11 studies (2004-2014)

F. URINALYSES (Chronic toxicity groups)

No treatment-related adverse changes among urinalysis parameters were observed.

After three months of administration in females of the mid-dose group (600 ppm) urine volume was higher compared to controls, but the values were not dose-dependently changed.

After six months in males of the high-dose group (3600 ppm) urine urobilinogen values were higher compared to controls, but this was an isolated finding, which was neither observed after twelve months in males of this test group nor in females throughout the study.

Therefore, the mentioned alterations in the urine were regarded as incidental and not treatment-related.

G. NECROPSY

1. Organ weight

12-month chronic toxicity groups [Table 5.5-8]

The terminal body weight was significantly decreased in top-dose females (3600 ppm) resulting in significantly decreased mean absolute weights of the adrenal glands and the kidneys as well as in increased mean relative weights of the brain, heart, and spleen. In these organs, treatment-related histopathological findings were not observed. In males there were no statistically significant differences between test and control groups in regard to terminal body or absolute organ weights.

The mean relative liver weights were significantly increased in top-dose males and females (3600 ppm). The increased liver weights were considered to be treatment-related.

24-month carcinogenicity groups [Table 5.5-9]

The terminal body weight was significantly decreased in females of the mid-dose group (600 ppm) as well as in top-dose group males and females (3600 ppm). These decreases resulted in significantly reduced mean absolute adrenal, heart, and kidney weights of top-dose males and females as well as in decreased absolute weights of the liver and the spleen in top-dose females. In addition, decreased terminal body weights resulted in increased relative weights of the brain, epididymides, heart, and kidneys in top-dose males.

In females of the mid- and top-dose groups (600 and 3600 ppm) the increased relative weights of the adrenal glands, brain, the heart, and the kidneys were related to the reduced terminal body weight. In these organs, there were no histopathological treatment-related findings.

The mean values of the relative ovarian weights of control and high-dose females were identical. The statistical significance based on the comparison of the median values, i.e. a slightly higher value in the high-dose group. The statistically significant deviation was considered to be of no factual relevance.

The increased mean relative liver weights in mid-dose group females and top-dose group males and females might also be related to the decreased terminal body weight, but because there was a histopathological correlate in high-dose male and female animals, a treatment-related effect seemed rather likely.

Because there was no dose-response relationship, the increased absolute adrenal weight in males of the low-dose group (100 ppm) and the increased relative adrenal weights in males of low- and mid-dose test groups (100 and 600 ppm) were regarded to be incidental.

All other non-statistical differences in organ weights between treatment and control groups were regarded as incidental.

Table 5.5-8: Organ weights (12-month chronic toxicity group)

Sex		Males				Females			
Organ weight	**Dose [ppm]**	**Absolute weight**	**Δ%**	**Relative weight [% of bw]**	**Δ% #**	**Absolute weight**	**Δ%**	**Relative weight [% of bw]**	**Δ% #**
Terminal body	**0**	472.75				263.93			
weight [g]	**100**	461.58	(-2)			264.95	(±0)		
	600	462.84	(-2)			255.29	(-3)		
	3600	442.41	(-4)			**224.89****	**(-15)**		
Adrenal glands	**0**	51.2		0.011		59.2		0.023	
(mg)	**100**	51.6	(+1)	0.011	(+3)	58.5	(-1)	0.022	(-1)
	600	47.9	(-6)	0.010	(-5)	62.2	(+10)	0.026	(+14)
	3600	47.8	(-7)	0.011	(±0)	**51.6***	**(-13)**	0.023	(+3)
Brain	**0**	2.195		0.466		2.028		0.774	
(g)	**100**	2.233	(+2)	0.488	(+5)	2.024	(±0)	0.769	(-1)
	600	2.210	(+1)	0.480	(+3)	2.088	(+3)	0.823	(+6)
	3600	2.185	(±0)	0.499	(+7)	1.965	(-3)	**0.880***	**(+14)**
Epididymides (g)	**0**	1.165		0.247		79.6		0.030	
	100	1.183	(+2)	0.258	(+5)	75.5	(-5)	0.029	(-4)
Ovaries (mg)	**600**	1.130	(-3)	0.247	(±0)	88.7	(+11)	0.035	(+14)
	3600	1.086	(-7)	0.248	(±0)	105.7	(+33)	0.047	(+56)
Heart	**0**	1.128		0.239		0.795		0.303	
(g)	**100**	1.112	(-1)	0.241	(+1)	0.827	(+4)	0.314	(+3)
	600	1.106	(-2)	0.239	(±0)	0.801	(+1)	0.314	(+3)
	3600	1.071	(-5)	0.243	(+1)	0.779	(-2)	**0.349****	**(+15)**
Kidneys	**0**	2.326		0.492		1.727		0.657	
(g)	**100**	2.437	(+5)	0.530	(+8)	1.651	(-4)	0.625	(-5)
	600	2.384	(+2)	0.516	(+5)	1.664	(-4)	0.653	(-1)
	3600	2.303	(-1)	0.521	(+6)	**1.465****	**(-15)**	0.654	(-1)
Liver	**0**	9.58		2.031		5.537		2.103	
(g)	**100**	9.746	(+2)	2.114	(+4)	5.951	(+7)	2.239	(+6)
	600	9.581	(±0)	2.068	(+2)	5.777	(+4)	2.270	(+8)
	3600	9.797	(+2)	**2.212***	**(+9)**	5.734	(+4)	**2.559****	**(+22)**
Spleen	**0**	0.667		0.141		0.507		0.192	
(g)	**100**	0.695	(+4)	0.151	(+7)	0.462	(-9)	0.175	(-9)
	600	0.704	(+6)	0.152	(+8)	0.500	(-1)	0.197	(+3)
	3600	0.686	(+3)	0.155	(+10)	0.498	(-2)	**0.222***	**(+16)**
Testes (g)	**0**	4.019		0.855		1.193		0.462	
	100	4.004	(±0)	0.871	(+2)	0.888	(-26)	0.336	(-27)
Uterus (g)	**600**	3.893	(-3)	0.846	(-1)	0.883	(-26)	0.350	(-24)
	3600	3.808	(-5)	0.866	(+1)	0.793	(-34)	0.363	(-21)
Thyroid	**0**	25.0		0.005		18.4		0.007	
(mg)	**100**	23.6	(-4)	0.005	(-4)	18.6	(+1)	0.007	(+1)
	600	23.0	(-8)	0.005	(-6)	18.0	(-2)	0.007	(±0)
	3600	23.1	(-8)	0.005	(-1)	16.5	(-10)	0.007	(+5)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

Table 5.5-9: Organ weights (24-month carcinogenicity group)

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal body	**0**	559.634				320.505			
Weight [g]	**100**	557.305	(±0)			319.898	(±0)		
	600	548.531	(-2)			**296.361****	(-8)		
	3600	**492.340****	**(-12)**			**245.609****	**(-23)**		
Adrenal glands	**0**	57.260		0.010		60.514		0.019	
(mg)	**100**	**61.158***	**(+7)**	**0.011***	**(+7)**	63.675	(+5)	0.021	(+6)
	600	61.231	(+7)	**0.011***	**(+8)**	62.439	(+3)	**0.022***	**(+11)**
	3600	**52.532****	**(-8)**	0.011	(+3)	**55.933***	**(-8)**	**0.023****	**(+18)**
Brain	**0**	2.254		0.412		2.048		0.652	
(g)	**100**	2.265	(±0)	0.414	(±0)	2.085	(+2)	0.670	(+3)
	600	2.272	(+1)	0.423	(+3)	2.087	(+2)	**0.717****	**(+10)**
	3600	2.214	(-2)	**0.453****	**(+10)**	2.047	(±0)	**0.839****	**(+29)**
Epididymides	**0**	1.261		0.229					
(g)	**100**	1.149	(-9)	0.210	(-8)				
	600	1.459	(+16)	0.271	(+18)				
	3600	1.143	(-9)	**0.233****	**(+2)**				
Heart	**0**	1.307		0.235		0.952		0.301	
(g)	**100**	1.276	(-2)	0.230	(-2)	0.946	(-1)	0.302	(±0)
	600	1.287	(-1)	0.237	(+1)	0.942	(-1)	**0.322***	**(+7)**
	3600	**1.217****	**(-7)**	**0.248****	**(+5)**	**0.876****	**(-8)**	**0.358****	**(+19)**
Kidneys	**0**	2.935		0.529		2.002		0.634	
(g)	**100**	2.971	(+1)	0.538	(+2)	2.018	(+1)	0.643	(+1)
	600	2.918	(-1)	0.536	(+1)	1.995	(±0)	**0.682***	**(+8)**
	3600	**2.743***	**(-7)**	**0.559****	**(+6)**	**1.776****	**(-11)**	**0.724****	**(+14)**
Liver	**0**	11.655		2.078		6.674		2.094	
(g)	**100**	11.394	(-2)	2.036	(-2)	6.987	(+5)	2.201	(+5)
	600	12.266	(+5)	2.214	(+7)	7.100	(+6)	**2.435****	**(+16)**
	3600	10.940	(-6)	**2.224****	**(+7)**	**6.337***	**(-5)**	**2.580****	**(+23)**
Ovaries	**0**					142.838		0.047	
(mg)	**100**					117.150	(-18)	0.036	(-23)
	600					113.805	(-20)	0.039	(-15)
	3600					112.224	(-21)	**0.047****	**(±0)**
Spleen	**0**	0.958		0.171		0.649		0.204	
(g)	**100**	0.940	(-2)	0.169	(-1)	0.721	(+11)	0.231	(+13)
	600	1.933	(+102)[i]	0.310	(+81)	0.664	(+2)	0.229	(+13)
	3600	0.913	(-5)	0.185	(+8)	**0.600****	**(-8)**	**0.242**	**(+19)**
Testes	**0**	4.358		0.779					
(g)	**100**	4.137	(-5)	0.747	(-4)				
	600	4.042	(-7)	0.752	(-3)				
	3600	4.229	(-3)	0.863	(+11)				
Uterus	**0**					1.072		0.342	
(g)	**100**					1.158	(+8)	0.388	(+14)
	600					1.323	(+23)	0.462	(+35)
	3600					1.081	(+1)	0.442	(+29)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures
[i] high mean spleen wt in males at 600 ppm were due to 3 males (#112: 24.12 g, #135: 13.58 g, #145: 3.7 g); and arose from a malignant lymphoma (#112) or from infiltration of a histiocytic sarcoma with severe extramedullary hematopoiesis (#135) or from massive extramedullary hematopoiesis(#145)

2. Gross pathology

12-month chronic toxicity groups

Most findings occurred individually. All findings were considered to be incidental or spontaneous in origin and without any relation to treatment.

24-month carcinogenicity groups

In top-dose male animals (3600 ppm), the number of foci in the testes was increased. In female animals of all treatment groups, the number of foci in the adrenal cortex was increased. The incidences are given in the table below:

Table 5.5-10: Gross necropsy findings in rats administered BAS 750 F for two years (carcinogenicity group)

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50	50	50	50	50
ADRENAL GLAND								
Foci in adrenal cortex		2	1	1	4	12	9	11
TESTES								
Foci	2	5	4	10	—			

For all these gross lesions a correlation was attempted. There was no consistent correlate to these macroscopic findings histopathologically. Therefore, they were not regarded to be related to treatment.

All other gross lesions occurred either individually or were biologically equally distributed over control and treatment groups. They were also considered to be incidental or spontaneous in origin and without any relation to treatment.

3. Histopathology

3.1. Neoplastic findings

12-month chronic toxicity groups

All neoplasms were individual findings and most of them were observed in the control group. A squamous cell papilloma occurred in a control male. An adenoma in the adrenal cortex, a C-cell adenoma in the thyroid gland, and an endometrial stromal polyp in the uterus were noted in control females. The only neoplastic finding in the treatment groups was an adenoma in the adrenal cortex that was observed in a female animal of the mid-dose group (600 ppm).

24-month carcinogenicity groups

Slight differences between control and high-dose animals without statistical significance were observed in the incidence of malignant lymphoma (hemolymphoreticular system) and of adenocarcinoma in the uterus:

- Three male animals (all of them decedents) of test group 03 (3600 ppm) showed a malignant lymphoma in comparison to none in the control males. In test groups 01 and 02, malignant lymphoma occurred in 2 males each. The incidence of malignant lymphoma (6%) in this study was within the historical control range (0-6%) and was considered incidental.
- An adenocarcinoma of the uterus was observed in 5 female animals of the top dose group (3600 ppm); one female was a decedent. In the control group, one female with an adenocarcinoma was noted. The incidence of adenocarcinoma of the uterus in high dose females (10%) was within the historical control range (2-30%) and therefore also regarded as incidental.

There were no further relevant differences in decedents and survivors regarding neoplastic findings. All neoplastic findings in this study occurred sporadically in control and treated animals. All neoplasms were considered to be incidental and spontaneous in origin and therefore not related to treatment.

Table 5.5-11: Incidence of selected neoplastic findings in rats administered BAS 750 F for 2 years (carcinogenicity groups)

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50	50	50	50	50
HEMOLYMPHRET SYSTEMex	50	50	50	50	50	10	10	50
Lymphoma, malignant		2	2	3			1	
Sarcoma, histiocytic	1	1	2				1	
PITUITARY GLAND exam	50	49	50	50	50	23	26	50
Adenoma, pars distalis	14	22	18	11	23	14	22	22
Adenoma, pars intermed.						1		
Carcinoma, pars distalis					1	1		1
UTERUS exam	—				50	38	37	50
Adenocarcinoma, endometrial					1	7	3	5
Schwannoma, malignant						1	1	1
Adenoma, endometrial					1	1		2

Statistical analysis: *: $p \leq 0.05$, **: $p \leq 0.01$ (Fisher's Exact test, 1-sided)

HISTORICAL CONTROL DATA (24-month data from 12 studies in Wistar rats, started: Jan-2003 to Feb-2013)
(Survival rate: ♂ 74 – 100%, ♀ 58 – 100%)

♂ Malignant lymphoma:	Mean: 2.5% (15/600);	min.: 0% (0/50);	max. 6% (3/50)
♀ Uterus, Adenocarcinoma, endometrial	Mean: 16.2% (97/600);	min.: 2% (1/50);	max: 30% (15/50)

The total numbers of primary, benign, and malignant neoplasms were comparable between control and high dose females. In males, the total numbers of primary neoplasms (67 versus 42), benign neoplasms (53 versus 35), and malignant neoplasms (14 versus 7) was higher in the control group than in males of the top-dose group (3600 ppm).
The total number of systemic and metastasized neoplasms were comparable between control and high dose males and females. They were biologically equally distributed over the control and treatment groups.

The occurrence of all neoplasms was considered to be incidental and spontaneous in origin and therefore not related to treatment.

Table 5.5-12: Total incidence of neoplastic findings in rats administered BAS 750 F for two years

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50	50	50	50	50
Number of animals with:								
- neoplasms	37	44	43	30	40	36	38	42
- 1 primary neoplasm	14	23	18	19	18	17	22	25
- 2 and > primary neoplasms	23	21	25	11	22	19	16	17
Number of animals with:								
- benign neoplasms	31	40	33	28	37	27	34	37
- benign neoplasms only	25	31	21	23	29	19	29	32
- malignant neoplasms	12	13	22	7	11	17	9	10
- malignant neoplasms only	6	4	10	2	3	9	4	5
- systemic neoplasms	1	3	4	3	0	0	2	0
- metastasized neoplasms	3	3	10	0	4	5	1	5
Total number of:								
- primary neoplasms	67	74	76	42	73	63	60	65
- benign neoplasms	53	61	48	35	62	44	51	53
- malignant neoplasms	14	13	28	7	11	19	9	12
- systemic neoplasms	1	3	4	3	0	0	2	0
- metastasized neoplasms	3	3	10	0	4	5	1	5

3.2. Non-Neoplastic findings

12-month chronic toxicity groups

Liver

A minimal or slight hepatocellular centrilobular hypertrophy was observed in 6 males and 5 females of the high-dose group (3600 ppm, see Table 5.5-13). The hypertrophy in these animals correlated with the increased relative liver weights in the high-dose group and was regarded to be treatment-related.

Table 5.5-13: Incidence of liver cell hypertrophy in rats administered BAS 750 F for 1 year (chronic toxicity groups)

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	10	10	10	10	10	10	10	10
LIVER								
examined	10	10	10	10	10	9	10	10
Hypertrophy, centrilobular				6				5
Grade 1				4				5
Grade 2				2				

Pituitary gland
In 3 females of the top-dose group, a focal hyperplasia was observed in the *pars distalis* of the pituitary gland (see Table 5.5-14). No control female showed this finding. Because the number of adenomas and of focal hyperplasia in the *pars distalis* of the pituitary gland was comparable between control females and females of the top-dose group after 2-yr treatment (see Table 5.5-11 and Table 5.5-15), the increased incidence of focal hyperplasia in the *pars distalis* after 12-month treatment was regarded as incidental.

Table 5.5-14: Incidence of selected findings in the pituitary gland of rats administered BAS 750 F for 1 year (chronic toxicity groups)

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	10	10	10	10	10	10	10	10
PITUITARY GLAND								
examined	10			10	10			10
Hyperplasia, pars distalis	1							3
Cyst(s), pars distalis	3							
Cyst(s), pars intermedia	1				2			

All other findings occurred sporadically in control and treated animals. They were considered to be incidental and spontaneous in origin and therefore not related to treatment

24-month carcinogenicity groups
Statistically significant histopathological changes in test group animals were noted in liver, lungs, colon, and thyroid gland. Only findings in the liver of high-dose group animals were considered to be treatment-related.

Liver
A minimal hepatocellular centrilobular hypertrophy was observed in 15 male and 7 female animals (all of them survivors) of the high-dose group (3600 ppm) that was statistically significant (see Table 5.5-15). The occurrence of hypertrophy in this test group correlated with increased relative liver weights and was considered to be treatment-related.

Lungs
The number of males with congestion of the lungs was significantly increased in the mid-dose group (600 ppm). Congestion (presence of blood in alveolar capillaries) was only observed in animals that died prematurely (passive congestion), independently of the test group. Because all control males survived until scheduled sacrifice, this finding was not seen in controls.

Colon
The number of females with parasites in the colon was increased in the high-dose group: 17 females showed parasites in comparison to 8 control females. In male rats, the incidences of parasites in the lumen of the colon were comparable between the high-dose and the control group (17/50 vs 15/50). In the cecum on the other hand, comparable parasite incidences were found in high-dose group females (12/50 vs 9/50), while decreased incidences of parasites were obtained in high-dose group males compared to controls (10/50 vs 19/50). Single or few cross sections of nematodes were noted in the intestinal lumen with no specific inflammatory response in the intestine. Overall, the occurrence of these parasites was considered to be incidental.

Thyroid gland

The number of male animals with altered colloid was significantly increased in the low- and mid-dose test groups (100 and 600 ppm, see Table 5.5-15). Because there was no dose-response relationship, the occurrence of this finding was regarded to be incidental.

All other non-statistically significant findings occurred sporadically in control and treated animals. They were considered to be incidental and spontaneous in origin and were therefore not related to treatment.

Table 5.5-15: Incidence of selected non-neoplastic findings in rats administered BAS 750 F for 2 years (carcinogenicity groups)

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50	50	50	50	50
LIVER								
examined	50	50	50	50	50	50	50	50
Hypertrophy, centrilobular				**15****				**7****
Grade 1				15				7
LUNGS								
examined	50	50	50	50	50	17	18	50
Congestion		2	**6***	1	2	2		1
PITUITARY GLAND								
examined	50	49	50	50	50	23	26	50
Hyperplasia, pars distalis (m)f	31	12	14	20	12	3	3	13
Hyperpl. p. intermedia, (m) f.	3	1	2	4		1		1
Hyperpl. p. intermedia, diff.	1							
Cyst(s), pars distalis	5	6	4	3	2	1	1	3
Cyst(s), pars intermedia	17	9	11	12	10	6	4	14
THYROID GLAND								
examined	50	50	50	50				
Altered colloid	4	**15****	**18****	7	4	10	9	4

Statistical analysis: *: $p \leq 0.05$, **: $p \leq 0.01$ (Fisher's Exact test, 1-sided)

3.3. Histopathological assessment of gross necropsy findings

The macroscopically diagnosed **foci in the testes** mostly represented Leydig cell adenomas, Leydig cell hyperplasia, multifocal tubular degeneration, or tubular mineralization, histopathologically. There was no relationship to dose for these findings.

Table 5.5-16: Incidence of findings in the testes

	Males			
Dose level [ppm]	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50
TESTES				
examined	50	50	50	50
Adenoma, Leydig cell	1	2	1	3
Hyperplasia, Leydig cell, (multi)focal	8	2	5	8
Hyperplasia, Leydig cell, diffuse		2	3	1
Infiltrates, lymphoma		1		
Arteritis, mutifocal	0	1	0	0
Metastasis	2	1	3	
Tubular degeneration, (multi)focal	12	9	8	14
Tubular degeneration, diffuse	2		4	4
Dilation, tubular, diffuse		2		
Dilation, rete testis				1
Edema	5	3	3	6
Fibrosis, rete testis	1			
Hemorrhage, (multi)focal	1			
Infiltration, lymphoid, (multi)focal	1			
Tubular mineralization	6	2	1	8
Vascular mineralization	1			1
Pigment storage	1			

The macroscopically observed **foci in the adrenal cortex** in females correlated with a variety of histopathologic findings, including cortical adenoma, multifocal cortical hyperplasia, accessory cortical tissue, cystic degeneration, multifocal fatty change, or multifocal hypertrophy. None of these findings showed significant differences between control and high-dose animals.

Table 5.5-17: Incidence of findings in the adrenal cortex

	Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50
ADRENAL GLAND CORTEX				
examined	50	19	13	50
Adenoma, cortical	1	1	1	2
Hemangioma				1
Hyperplasia, cortical, (multi)focal	25	6	4	29
Hyperplasia, subcapsular c.				1
Infiltrates, lymphoma			1	
Metastasis		1		
Accessory cortical tissue	3	4	3	4
Cyst(s)	1			
Degeneration, cystic	45	18	11	37
Fatty change, (multi)focal	4	2	2	
Hematopoiesis, extramedullar	1		1	1
Hypertrophy, (multi)focal	15	4	4	17
Hypertrophy, subcaps., (multi)focal	36	11	6	41
Necrosis, (multi)focal		1		

None of these findings showed significant differences between control and high-dose animals.

4. Decedents

12-month chronic toxicity groups

All animals survived until scheduled sacrifice.

24-month carcinogenicity groups

The number of decedents is given in the following table.

Table 5.5-18: Decedents after 2-year treatment with BAS 750 F

	Males				Females			
Dose level [ppm]	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
No. of animals	50	50	50	50	50	50	50	50
Decedents	0	12	11	3	12	10	9	5

The incidences displayed no dose-response relationship and there were no treatment-related histopathological findings, which could explain an earlier death. Most of these animals showed different kinds of neoplasms in varying organs. None of these tumors were considered treatment-related.

III. DISCUSSION

In a combined chronic toxicity / carcinogenicity study, BAS 750 F was administered to groups of 60 male and 60 female Wistar rats. Each group consisted of 10 animals for assessment of chronic toxicity (administration for 12 months), and 50 animals for assessment of carcinogenicity (administration for 24 months). The test substance was administered via the diet at concentrations of 0, 100, 600 and 3600 ppm.

With regard to clinical observations, the oral administration of BAS 750 F via the diet to male and female Wistar rats over a period of either 12 or 24 months did not cause treatment-related mortality. However, signs of systemic toxicity were observed in male and female animals at 3600 ppm indicated by impaired body weight parameters, which were assessed as being related to the test compound administration. No relevant changes were observed for animals of the low- or mid-dose groups (100 and 600 ppm).

Regarding clinical pathology, the parameter changes were most probably due to an altered liver cell metabolism subsequent to a liver enzyme induction. This was true for the reduced activated thromboplastin time (PTT) seen after 12-month treatment of male and females rats at the top-dose level (3600 ppm) and in males of the mid-dose group (600 ppm) indicating an increased synthesis of coagulation factors in the liver. In males of the high-dose group, platelet counts were additionally reduced. The protein metabolism was affected (increased) in top-dose females (3600 ppm) represented by lower total protein and albumin levels as well as reduced creatinine values, which correlated to the lower terminal body weight in these individuals. In males of the mid- and high-dose test groups (600 and 3600 ppm), the higher protein metabolism of the liver cells resulted in higher urea levels. The provenance of the higher alkaline phosphatase (ALP) activities in rats of both sexes at 3600 ppm and additionally in males at 600 ppm was most probably also the liver. The same was true for lower glucose levels in rats of both sexes of test group 13 (3600 ppm) which was an indication for increased energy consumption due to higher liver cell metabolism.

Regarding pathology, target organ was the liver. After 12 months of treatment, the mean relative liver weights were significantly increased in males and females at 3600 ppm. Liver weight increases correlated histopathologically with a minimal or slight hepatocellular centrilobular hypertrophy, which was observed in 6 out of 10 males and 5 out of 10 females in this test group. These findings at 3600 ppm were regarded to be treatment-related and in combination with alterations in clinical chemistry parameters considered to be adverse.

After 24 months of treatment, the mean relative liver weights were significantly increased in females of the mid-dose group (600 ppm) and in both sexes at 3600 ppm. This increase might be related to the decreased terminal body weight. However, at 3600 ppm, a minimal hepatocellular centrilobular hypertrophy was observed in the liver of 15 male and of 7 female animals that correlated with the increased relative liver weights in this test group. The occurrence of hypertrophy and the increase of the relative liver weight at 3600 ppm were therefore assessed to be treatment-related. As there were comparable findings after 12 months of treatment, these findings were regarded to be adverse.

Because there was no histopathological correlate for the increased relative liver weight in females of the mid-dose group, this weight change was considered to be likely treatment-related but non-adverse.

All other findings recorded were considered to be incidental in nature and not related to treatment.

Under the conditions of the study, BAS 750 F demonstrated no carcinogenic potential up to the highest dose level tested.

IV. CONCLUSION

The oral administration of BAS 750 F via the diet to Wistar rats over a period of either 12 or 24 months resulted in reduced body weight, body weight gain and changed clinical pathology parameters, which at the high dose level were associated with liver weight increases and liver cell hypertrophy of minimal to slight severity. Thus, signs of systemic toxicity were determined at 3600 ppm and 600 ppm (about 29 mg/kg bw/d) in males and at 3600 ppm (about 312 mg/kg bw/d) in females.

The no observed adverse effect level (NOAEL) was considered to be 100 ppm (about 5 mg/kg bw/d) in males and 600 ppm (41 mg/kg bw/d) in females.

Under the conditions of the study, BAS 750 F demonstrated no carcinogenic potential up to the highest dose level tested.

Report:	CA 5.5/3 [redacted] 2015 b 18-month carcinogenicity study with BAS 750 F in male and female C57BL/6JRJ mice 2015/1000532
Guidelines:	OECD 451, EPA 870.4200, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Ministry of Health, Welfare and Sport, Utrecht, The Netherlands)
Report:	CA 5.5/4 Groeters S., 2015 a Historical control data mouse study - Compilation from 18-month studies in male and female C57BL/6JRj mice - Performed at BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany 2015/1261376
Guidelines:	<none>
GLP:	no
Note:	Supplemental data. Historical control data are provided from studies performed at a different test facility (i.e. BASF), but that used the same source of the mouse strain and diet; moreover the same diagnostic criteria as in the BAS 750 F mouse carcinogenicity study were applied.
Report:	CA 5.5/5 Becker M.,Kamp H., 2015a BAS 750 F - Plasma analysis for external studies 2015/1186254
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Groups of 50 male and 50 female C57BL/6JRj mice were treated with BAS 750 F for at least 18 months by dietary administration at dose levels of 0, 20 and 50 ppm (both sexes), 200 ppm (males) and 250 ppm (females). The dietary concentration corresponded to mean intakes of 3.5, 9.1 and 36 mg/kg bw/d in males and 4.9, 12.6 and 61.5 mg/kg bw/d in females.

Treatment did not elicit clinical signs and did not adversely affect survival. At the high-dose level, i.e., 200 ppm in males and 250 ppm in females, the overall body weight gain was significantly decreased (up to -15% in males and up to -33% in females). Relative liver weights were significantly increased in high-dose group animals (+42% in males and +57% in females), which in combination with increased incidence and severity of fatty change and signs of liver cell degeneration (eosinophilic cytoplasmic inclusions in males and single cell necrosis in females), were assessed as adverse. Liver weight increases with a higher degree of hepatocellular fatty change were noted also at 50 ppm in males but not accompanied by degenerative findings and therefore considered to be treatment-related but not adverse.

Similarly, at the high-dose level increased incidences of thyroid follicular cell hyperplasia were considered to be treatment-related in males and potentially treatment-related in females, but not adverse, in the absence of any signs of degenerative change.

In the adrenal gland, weights were increased in male mice at 200 ppm but were not associated with any histopathological change. In females at 250 ppm, the relative adrenal weight was significantly increased, and the only histopathological change was a more eosinophilic aspect of the adrenocortical cell cytoplasm together with a minimal to slight size increase of individual eosinophilic cells. The incidence of cortical hypertrophy without cytoplasmic changes in treated female animals was low and comparable to control incidences.

These changes in males and females were assessed as treatment-related, but not adverse. Finally, decreased incidences in the vacuolation of the kidney tubular cells was noted at 200 and at 50 ppm in males, and were associated with slight kidney weight decreases. Since there were no signs of a degenerative process in the male kidney, the observed change was considered to possibly reflect increased (energy-consuming) excretion activity. The change in the kidney was assessed to be treatment-related, but not adverse.

Under the conditions of the study, BAS 750 F demonstrated no carcinogenic potential in C57BL6J/RJ mice up to the highest dose level tested. The observed tumor incidences were comparable to the concurrent control and/or to historical control ranges of the test facility.

Under the conditions of this 18-month carcinogenicity study in C57BL/6JRj mice, the NOAEL for systemic toxicity was 50 ppm, corresponding to 9.1 mg/kg bw/d in males and 12.6 mg/kg bw/d in females, based on reductions of body weight gain, and increased liver weights in combination with histopathological evidence of liver cell degeneration in both sexes at the high dose level of 200 ppm in males (36 mg/kg bw/d) and 250 ppm in females (61.5 mg/kg bw/d). BAS 750 F was not carcinogenic in mice up to the highest dose level tested.
BAS 750 F did not induce or accelerate life threatening changes and did not have a carcinogenic potential up to the highest doses administered in this study (200 ppm in males and 250 ppm in females).

BAS 750 F is not carcinogenic in mice.

(DocID 2015/1000532)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study (expiry date 1 May 2016).

2. Vehicle: Rodent diet

3. Test animals:

Species:	Mouse
Strain:	C57BL/6JRj
Sex:	male and female
Age:	approx. 7 weeks at start of dosing
Weight (day 1, means):	♂: 22.7 – 23.0 g; ♀: 18.6 – 18.9 g
Source:	Janvier S.A.S., Le Genest Saint Isle, France
Acclimatization period:	at least 5 days
Diet:	Ground Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Male animals individually in Macrolon cages type MIII (height 15 cm); female animals housed 2 per cage (except last female of each satellite group that was housed individually); sterilized sawdust bedding (Lignocel S 8-15, JRS GmbH & Co KG, Rosenberg, Germany); enrichment: paper (Enviro-dri, Wm. Lilico & Son, UK); from study wk 26, additionally wooden sticks (Aspen Environm. Enrichments-Chips, Populus Tremula)

Environmental conditions:

Temperature:	18 – 24°C
Humidity:	40 – 70%
Air changes:	Fully air-conditioned rooms, at least 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. Dates of work: 05-Dec-2013 to 25-Jun-2015 [in life phase: 13-Dec-2013 (start treatment) to 25-Jun-2015 (necropsy)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 50 male and 50 female C57BL/6JRj mice at dietary concentrations of 0, 20 (low dose), 50 (intermediate dose), and 200 ppm (top dose males) or 250 ppm (top dose in females) for 18 months. Additionally satellite groups of 7 male and 7 female mice were administered BAS 750 F at the same dose levels for 12 months for determination of BAS 750 F blood concentration levels. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights. At scheduled necropsy, animals were fasted overnight prior to scheduled necropsy (with a maximum of 15 hours).

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diets were prepared at least once a month based on stability data.

BAS 750 F had previously been shown to be stable under study conditions in the diet over a 30-day period within a concentration range of 10–750 ppm (90-day mouse study, [redacted], DocID 2014/1046542).

Stability analysis

The 30-day stability of the test substance in the diet (highest and lowest concentration) at room temperature was investigated using a sample from the week-1 preparation. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC/UV analysis with an external standard.

Table 5.5-19: Stability

BAS 750 F nominal conc. [ppm]	Date of analysis	Date of analysis	Analytical concentration [ppm]		Relative difference [%]
	t = 0	t = 30 days	t = 0 [a]	t = 30 days	
20	16-Dec-2014	15-Jan-2014	21.6 (108% of nominal)	18.7 [b] (93.5% of nominal)	-13
250	16-Dec-2014	15-Jan-2014	239 (95.6% of nominal)	225 [c] (90.0% of nominal)	-6.1

[a] Mean of six samples at t=0 hours taken at 10%, 50% and 90% height of food container
[b] Mean of two samples at t=30 days taken at random position. Individual results were 18.6 and 18.8 ppm.
[c] Mean of two samples at t=30 days taken at random position. Individual results were 232 and 217 ppm.

At the high-dose level of nominal 250 ppm, the relative difference between the Day 0 and Day samples was about 6% and therefore within the acceptable range. At the low-dose level of 20 ppm, a relative difference in the BAS 750 F concentration of more than 10% was obtained. However, since the initial concentration determined (t=0) was relatively high (i.e. 108% of nominal), the result was accepted and the dietary mixtures concluded to be stable during storage at room temperature in an open contained for at least 30 days.

Homogeneity

Samples of diet preparations were analyzed for homogeneity (highest and lowest test concentration) and accuracy of preparations (all concentrations determined weeks 1, 13, 26, 52 and 78). The coefficients of variation for diets of low- and top-dose groups were usually well within the acceptable range, except for the samples prepared for use in Week 1, which were outside the 5% target for homogeneity (values were 10 and 6.2% respectively). However, the results were accepted based on the minimal deviation and isolated occurrence.

Dietary concentration control analysis

The concentrations analyzed in the diets of the low-, mid- and high-dose groups were generally in agreement with target concentrations (i.e. mean accuracies between 90% and 110%). For the diet of high-dose males (200 ppm) and high-dose females (250 ppm), the mean accuracies were slightly below the criterion of 90-110%. For the 50-ppm diet prepared for use in Week 78, the mean accuracy was slightly below the criterion of 90-110% (i.e., 86%). Since the analyzed concentrations at the other time points in these groups were within the acceptable range, the slight and isolated deviations from the target concentrations were considered to be acceptable.

Table 5.5-20: Homogeneity and concentration-control

BAS 750 F nominal conc. [ppm]	Week	BAS 750 F Mean analysed conc. [ppm]	[% of nominal]	Homogeneity (coeeficient of variation) [ppm]
20	1	21.6	108	10
	13	19.9	100	2.0
	26	19.5	97	2.3
	52	20.4	102	2.1
	78	18.5	92	4.7
50	1	49.5	99	n.a.
	13	47.7	95	
	26	46.2	92	
	52	47.4	95	
	78	43.0	86	
200	1	196	98	n.a.
	13	195	97	
	26	174 / 163R	87 / 81R	
	52	189	94	
	78	183	91	
250	1	239	96	6.2
	13	240	96	2.1
	26	209 / 207R	83 / 83R	3.7
	52	236	95	2.9
	78	229	92	2.4

R = Re-analyzed sample

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
body weight, body weight change, food consumption, relative food consumption	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means, based on pooled variance In case the data did not follow a normal distribution, the STEEL-test (two-sided) was applied instead of the Dunnett's test.
Hematology	STEEL-test (two-sided)
Total mortality	FISHER-EXACT test applied to frequency data
Statistics for pathology	
Parameter	**Statistical test**
Terminal body weight, absolute and relative organ weights	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means, based on pooled variance
Macroscopic and histopathological findings	FISHER-EXACT test (one-sided) for the hypothesis of equal proportions applied

C. METHODS

1. Observations

The animals were examined for mortality at least twice daily. Animals showing pain, distress or discomfort which was considered not transient in nature or was likely to become more severe, were sacrificed for humane reasons. The circumstances of any death were recorded in detail.

All animals were observed for clinical signs once daily. The time of onset, grade and duration of any observed sign was recorded in the computer once weekly, unless major significant changes in health status were seen between daily observations.

2. Body weights

Body weights were determined weekly during the first three months of the study, and every four weeks thereafter. Animals displaying signs of ill health were weighed more frequently to monitor health status more closely. These data were recorded in the raw data and not reported.

3. Food consumption and compound intake

Food consumption was determined weekly during the first 3 months and every 4 weeks thereafter. Test article intake was calculated. Food consumption values of ≥ 10 gram/animal/day were excluded from summary and statistics, as these values were regarded as unrealistically high for mice in this type of study, and due to spillage. The individual data remain available in the tables.

4. Water consumption

The water consumption was monitored by subjective appraisal throughout the study period, but no quantitative investigation was introduced as no effect was suspected.

5. Blood concentration analyses

In the morning (between 8:00 - 10:00) of study days 7, 28, 85 (wk 13), 176 (wk 26) and 357 (wk 52), a blood sample (about 100 µL) was taken from all satellite animals from the retro-orbital sinus under isoflurane anaesthesia, and collected into tubes prepared with K3-EDTA for determination of plasma concentration of parent compound. Animals were not fasted overnight prior to sampling. After plasma preparation (40 µL) the samples were stored at ≤ 75°C prior to LC-MS analysis for determination of the BAS 750 F concentration.

6. Hematology

A blood smear for microscopic observation of blood cells, including differential leucocyte count was prepared for all main group animals after 12 and after 18 months and for all main group animals killed *in extremis*. Samples (0.1 mL) were collected from the retro-orbital sinus when the animal was deeply anaesthetised using isoflurane vapour and collected into tubes prepared with K3-EDTA. Smears (2 slides per animal) were prepared on the day of sampling. For the blood sampling after 18 months, the animals were fasted overnight (with a maximum of 15 hours) before blood sampling, but water was provided. The blood samples were taken in the necropsy unit and just prior to scheduled necropsy. Then, while under anaesthesia, the animal was exsanguinated via the thoracic aorta and necropsied.

The following haematology parameters were determined from the blood smears by manual count (100 leucocytes) or severity scoring (not detected / slight / moderate / severe):

Hematology:					
	Differential leucocyte count (%WBC)		*Red blood cells (Severity score)*		*Other (‰ RBC)*
✓	Neutrophils	✓	Polychromasia	✓	Erythroblasts
✓	Lymphocytes	✓	Hyperchromasia	✓	Normoblasts
✓	Monocytes	✓	Hypochromasia	✓	Howell-Jolly bodies
✓	Eosinophils	✓	Anisocytosis	✓	Basophilic punctuation
✓	Basophils	✓	Poikilocytosis		
✓	Others	✓	Microcytosis		
		✓	Macrocytosis		

Blood smears were fixed and stained for all animals, but only examined for control and high-dose groups and for intercurrently sacrificed animals.

7. Sacrifice and pathology

Terminal necropsy of the main group animals was performed after 78 weeks. Following fasting overnight and blood sampling, all animals surviving to the end of the observation period were deeply anaesthetized using isoflurane and subsequently exsanguinated via the thoracic aorta and subjected to a full post mortem examination. Animals found dead were subjected to a full post mortem examination as soon as possible after death and always within 24 hours. Descriptions of all macroscopic abnormalities seen at post mortem were recorded.

Histopathologic examination was performed on an extensive list of organs and tissues from all control and high-dose group animals. Livers and thyroid glands were examined from the males and females of all test groups, and additionally kidneys from all the males and adrenal glands of all the females. Histopathology was also carried out on organs with macroscopic findings and all tissue masses from all mice. Finally, all unscheduled sacrificed animals were subjected to complete necropsy and full histopathologic evaluation.

Pathology:

S	W	H		S	W	H		S	W	H	
✓	✓	2	Adrenal glands*	✓		#	Eyes with optic nerve	✓		#	Rectum
✓	✓	#	Brain	✓		#	Harderian gland	✓		#	Salivary gland, mandibular
✓	✓	#	Epididymides	✓		#	Mammary gland (♂ + ♀)	✓		#	Salivary gland, sublingual
✓	✓	#	Heart	✓		#	Ileum	✓		#	Seminal vesicles
✓	✓	1	Kidneys	✓		#	Jejunum	✓		#	Skeletal muscle
✓	✓	✓	Liver (with gall bladder)	✓		#	Lacrimal gland, exorbital	✓		#	Skin
✓	✓	#	Ovaries (with oviducts)	✓		#	Larynx	✓		#	Spinal chord
✓	✓	#	Spleen	✓		#	Lungs	✓		#	Sternum (w. bone marrow)
✓	✓	#	Testes	✓		#	Lymph nodes, mesenteric	✓		#	Stomach
✓	✓	#	Uterus (including cervix)	✓		#	Lymph nodes, axillary	✓		#	Thymus
✓		✓	Thyroid	✓		#	Nerve, sciatic	✓			Tongue
✓		✓	Gross lesions / tissue mass	✓		#	Nose, nasal cavity, level III	✓		#	Trachea
✓		#	Aorta	✓		#	Esophagus	✓			Ureter
✓		#	Bone marrow (femur)	✓		#	Pancreas	✓			Urethra
✓		#	Caecum	✓		#	Parathyroid	✓		#	Urinary bladder
✓		#	Coagulating glands	✓		#	Peyer's patches	✓		#	Uterus
✓			Clitoral gland	✓		#	Pharynx	✓		#	Vagina
✓		#	Colon	✓		#	Pituitary gland	✓			Head incl. olfactory bulb
✓		#	Duodenum	✓			Preputial gland				and teeth
✓		#	Femur with joint	✓		#	Prostate gland	✓			Remaining tissue / carcass

* after fixation; **S**: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose + animals died pre-schedule. **1 / 2** =all male / female groups plus control and high-dose female / male groups.

II. RESULTS

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

In the main test groups, the mortality rate in treated males and females was not different from that in control animals. Total mortality rates ranged from 0 – 4% in males and from 8 – 18% in females. Causes of death, or factors contributory to death or moribundity, identified by microscopy, showed no evidence of a test substance-relationship. In some cases, females were sacrificed for humane reasons due to skin lesions, which are known to occur as a background finding in this strain of mice.

Table 5.5-21: Mortality

BAS 750 F Dose level [ppm]	Spontaneous death	Killed *in extremis*	Other cause of death	Mortality total	Mortality Corrected [a]
MALES					
0		1		1 / 50 (2%)	1 / 50 (2%)
20		2		2 / 50 (4%)	2 / 50 (4%)
50				0 / 50 (0%)	0 / 50 (0%)
200				0 / 50 (0%)	0 / 50 (0%)
FEMALES					
0		3	1 [b]	4 / 50 (8%)	3 / 49 (6%)
20	1	1	2 [b]	4 / 50 (8%)	2 / 48 (4%)
50		2	7 [b]	9 / 50 (18%)	2 / 43 (5%)
250	1	4	3 [b]	8 / 50 (16%)	5 / 47 (11%)

[a] "Other causes of death" were disregarded for the calculation of corrected mortality
[b] Animals were sacrificed for humane reasons due to skin lesions

No statistically significant changes were noted in Total or Corrected Mortality vs. control (Fisher's Exact test)

2. Clinical signs of toxicity

No treatment-related clinical signs were evident up to a dose level of 200 ppm in males and 250 ppm in females. All findings were within the normal range of biological variation for mice of this age and strain used in this type of study. Apparent was the relatively high incidence of alopecia and wounds in female mice of all groups including control. These skin lesions are known to commonly occur in this mouse strain.

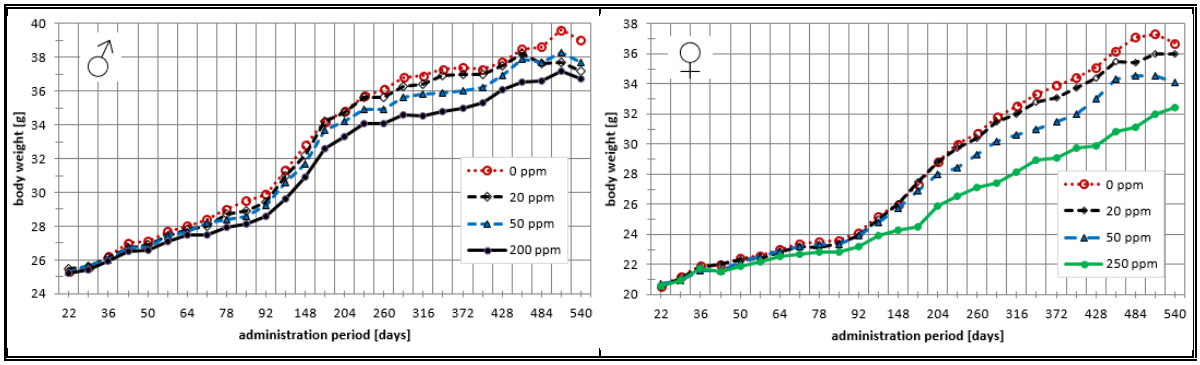
3. Nodules and masses

Treatment with BAS 750 F did not induce increased incidences of palpable nodules and masses. The incidences and distribution of nodules and masses remained within the normal background variation for mice of this strain in this type of study.

C. BODY WEIGHT AND BODY WEIGHT GAIN

A treatment-related effect was observed for males at 200 ppm, and females at 50 and 250 ppm.

Figure 5.5-2: Body weight development



In males at 200 ppm, a decrease in group mean body weight was seen from Week 11 of treatment onwards. Final absolute body weights in this group amounted to 94% of mean control weights for males. Statistical significance was attained on most occasions. Body weight gain values in this group followed the same trend as for absolute weights with statistically significant differences from Week 12 onwards when compared with control males. Mean body weight gain was -14%, -10%, -13% and -11% for males at 200 ppm in Weeks 13, 26, 54 and 78 respectively, as compared to controls.

Table 5.5-22: Body weight development (main groups)

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
Body weight [g]								
- Week 0	23.0	22.9	22.7	22.7	18.9	18.6	18.8	18.9
- Week 13	29.5	28.9	28.6*	28.1**	23.6	23.4	23.3	**22.8****
Δ% (compared to control[#]		-2	-3	-5				**-3**
- Week 26	34.2	34.2	33.7	32.6*	27.3	27.5	26.9	**24.5****
Δ% (compared to control[#]		±0	-1	-5		+1	-1	**-10**
- Week 54	37.4	37.0	36.0	35.0**	33.9	33.1	**31.5***	**29.1***
Δ% (compared to control[#]		-1	-4	-6		-2	**-7**	**-14**
- Week 78	39.0	37.2	37.7	36.7	36.7	36.0	34.1	**32.4****
Δ% (compared to control[#]		-5	-3	-4		-2	-7	**-12**
Overall body weight gain (%)								
- Week 13	28	26	26	**24****	25	25	24	**21***
Δ% (compared to control[#]		-7	-7	**-14**		±0	-4	**-16**
- Week 26	49	50	48	44	45	48	43	**30****
Δ% (compared to control[#]		+2	-2	-10		+7	-4	**-33**
- Week 54	63	61	58	**55***	79	77	**68***	**54****
Δ% (compared to control[#]		-3	-8	**-13**		-3	**-13**	**-32**
- Week 78	70	63	66	62	94	93	82	**71****
Δ% (compared to control[#]		-10	-6	-11		-1	-13	**-24**

[#] Values may not calculate exactly due to rounding of mean values
Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

In **females** at 250 ppm, a decrease in group mean body weight was observed from Week 7 onwards. Final absolute body weights in this group amounted to 88% of mean control weights for females. At the mid dose of 50 ppm, female body weight was decreased from Week 34 onwards. Final body weights at the female mid dose were 93% of mean control female weights. Statistical significance was attained in most cases. Body weight gain values followed again the same trend as for absolute weights with statistically significant differences from Week 7 and 34 onwards for females at 250 and 50 ppm, respectively. Mean body weight gain was -16%, -33%, -32% and -24% for females at 250 ppm in Weeks 13, 26, 54 and 78 respectively, and -14% and -13% in Weeks 54 and 78 respectively for females at 50 ppm, as compared to controls.

D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

In males treated up to 200 ppm, treatment with the test substance had no effect on food consumption.

In females at 250 ppm, food consumption (g diet/animal/d) was decreased in Weeks 6-26 of treatment, with statistical significance on most occasions, followed by normal food intake for the remainder of the study. Relative food consumption (g diet/kg bw/d)) in females of this dose group was decreased over Weeks 6-13 and was increased in Weeks 42 and 66-73. No effects were noted on food intake for females treated up to 50 ppm.

2. Water consumption

No overt changes of water consumption were noted by subjective appraisal throughout the study.

3. Test substance intake

The approximate, mean daily test-substance intake in mg/kg bw/d over the entire study period is shown in the following table:

Table 5.5-23: Mean test substance intake

	Males			Females		
Dose level [ppm]	**20**	**50**	**200**	**20**	**50**	**250**
BAS 750 F (mg/kg bw/d)	3.5	9.1	36	4.9	12.6	61.5

E. TEST SUBSTANCE CONCENTRATION IN BLOOD PLASMA

Results of the BAS 750 F plasma concentration measurements are presented Figure 5.5-3 and in Table 5.5-24.

Figure 5.5-3: BAS 750 F plasma concentration in satellite group mice

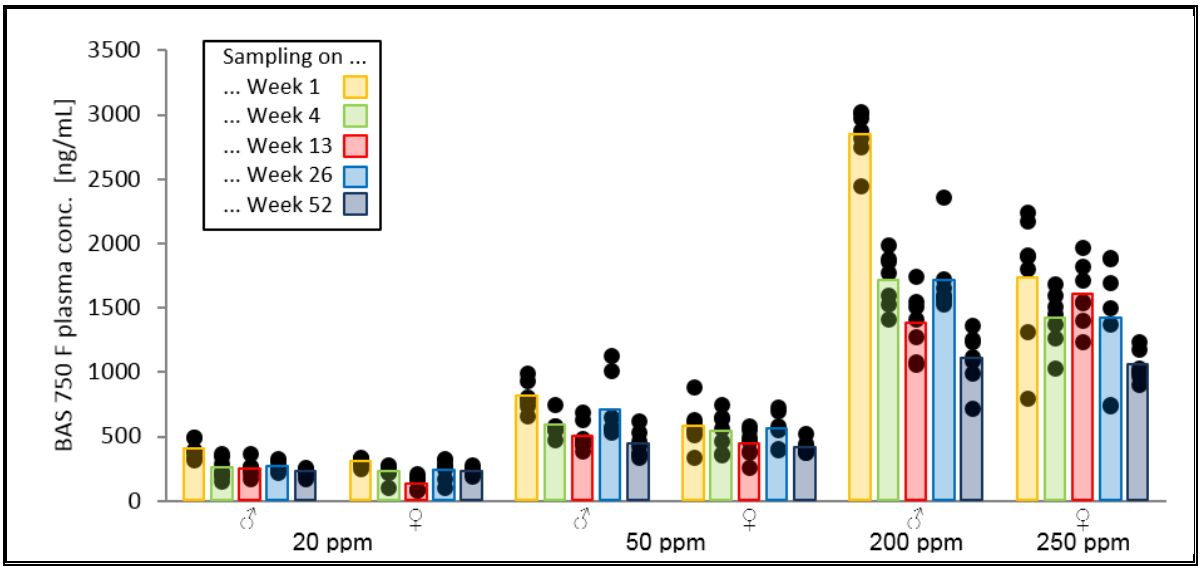


Table 5.5-24: BAS 750 F plasma concentration

		Males (N=7)	Females (N=7)
BAS 750 F Diet concentration [ppm]	**Sampling on Study Week**	**Mean ± SD [#] [ng/mL]**	**Mean ± SD [#] [ng/mL]**
0	1	n.d.	n.d.
	4	n.d.	n.d.
	13	n.d.	n.d.
	26	n.d.	n.d.
	52	n.d.	n.d.
20	1	404 ± 69	304 ± 32
	4	258 ± 79	223 ± 57
	13	255 ± 61	146 ± 54
	26	272 ± 45	246 ± 80
	52	228 ± 31	236 ± 36
50	1	815 ± 114	582 ± 165
	4	582 ± 84	542 ± 150
	13	508 ± 112	445 ± 110
	26	716 ± 247	563 ± 129
	52	448 ± 103	423 ± 52
200 / 250	1	2844 ± 202	1734 ± 513
	4	1720 ± 211	1418 ± 219
	13	1377 ± 251	1606 ± 250
	26	1717 ± 291	1406 ± 488
	52	1111 ± 213	1049 ± 118

[#] Values may not calculate exactly due to rounding of mean and standard deviation values

BAS 750 F was generally not detected at quantifiable amounts in control samples. A roughly dose-dependent (slightly underproportional) increase of the mean plasma concentrations were found in both sexes: In male mice, increasing the dietary concentration of BAS 750 F from 20 to 50 ppm (2.5-fold) corresponded to about 2.2-fold increases in the mean plasma concentration. A 10-fold increase of BAS 750 F in the diet (from 20 to 200 ppm) caused about a 5-to-7-fold increase in the plasma concentration of male mice. Very similar observations were made in female mice. Male mice tended to exhibit slightly higher plasma concentrations than female mice for a given dose group, even at the high-dose level although females were fed a higher concentrated diet (250 ppm) than males (200 ppm). In male mice the plasma concentration was consistently higher at the first sampling time point (Day 7) compared to later time points, especially at the high-dose level, which may partly be due to increased elimination efficiency in the course of further treatment.

F. HEMATOLOGY

No treatment-related effects were observed by comparison of blood smears from control and high-dose groups sampled either after 12 months or at the end of the treatment period. The variations in haematology parameters after 12 months, or at end of treatment, were considered unrelated to treatment due to the minimal magnitude of the change, or absence of biological relevance.

Table 5.5-25: Hematology

Sex		MALES				FEMALES			
Sampling time point		12 months		18 months		12 months		18 months	
Dose (ppm)		0	200	0	200	0	250	0	250
Hematology parameter									
Neutrophils	(%WBC)	13.4	14.6	28.7	**33.2**$^{+}$	15.5	14.0	32.8	34.8
Lymphocytes	(%WBC)	82.2	81.7	66.3	61.9	78.2	80.9	62.2	59.7
Monocytes	(%WBC)	2.7	2.6	4.5	4.4	3.9	3.6	4.2	4.9
Eosinophils	(%WBC)	1.7	1.1	0.5	0.6	2.4	**1.5**$^{++}$	0.9	0.7
Basophils	(%WBC)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Anisocytosis	(Score 0/3)	0	0	0	0	0	0	0	**0**$^{+}$
Polychromasia	(Score 0/3)	1	1	0	1	1	1	1	1
Poikilocytosis	(Score 0/3)	0	**1**$^{++}$	1	1	1	1	1	1
Hypochromia	(Score 0/3)	0	0	0	0	0	0	1	0
Hyperchromia	(Score 0/3)	0	0	0	0	0	0	0	0
Microcytosis	(Score 0/3)	0	0	0	0	0	0	0	0
Macrocytosis	(Score 0/3)	0	0	1	**0**$^{++}$	0	0	1	1
Erythroblasts	(‰ RBC)	0	0	0	0	0	0	0	0
Normoblasts	(‰ RBC)	0	0	0	0	0	0	0	0
Basophilic punct.	(‰ RBC)	0	0	0	0	0	0	0	0
Howell-Jolly bodies	(‰ RBC)	3	4	2	2	2	**1**$^{++}$	1	1

Statistical analysis: Steel-test +: $p \leq 0.05$; ++: $p \leq 0.01$

G. NECROSCOPY AND PATHOLOGY

1. Macroscopic examination

There were no treatment-related changes. The range of gross findings recorded in this study was concluded to be within the normal range of background alterations that may be seen in untreated animals of this age and strain.

2. Organ weights

Organ weights with statistically significant differences to the control groups are presented in Table 5.5-26 below.

Table 5.5-26: Mean absolute and relative organ weights of mice administered BAS 750 F for 18 months (percent change compared to control)

Sex	Males			Females		
Dose level [ppm]	**20**	**50**	**200**	**20**	**50**	**250**
Animals examined	48	50	50	46	41	42
TERMINAL BODY WT	-5%	-4%	**-7%***	-3%	-7%	**-13%****
LIVER						
Absolute	+6%	**+14%****	**+33%****	+4%	+22% [+7%]&	**+41%****
Relative to body weight	**+12%****	**+18%****	**+42%****	+5%	**+31%*** [+13%]&	**+57%****
KIDNEYS						
Absolute	**-5%***	**-8%****	**-13%****	0%	+32% [-1%]@	-5%
Relative to body weight	+1%	-4%	**-7%***	+2%	+40% [+6%]@	+8%
ADRENAL GLAND						
Absolute	-7%	+10%	**+27%****	-15%	-6%	+8%
Relative to body weight	+1%	+16%	**+39%****	-14%	+1%	**+22%***
HEART						
Absolute	+3%	+1%	-1%	-1%	-1%	**-7%***
Relative to body weight	**+10%****	+6%	+6%	+1%	+6%	+5%
UTERUS						
Absolute				+8%	+7%	+11%
Relative to body weight				+13%	+19%	**+32%***

Statistical analysis: Dunnett's-test, * : p≤0.05; ** : p≤0.01; & The high absolute and relative mean liver weight in 50 ppm females is caused by female 622 having a massive infiltration of a histiocytic sarcoma. When this female is removed from calculation, the difference to the control group is only minor (see values in brackets)

@ The high absolute and relative mean kidney weights in 50 ppm females are caused by female #609 having a very large cyst. When this female is removed from calculation, no differences with the control group is observed (see values in brackets).

Some mean organ weight differences were statistically significant when compared to the control group but were considered to be the results of a test item-related effect on final body weight.

The dose-dependent significantly increased mean liver weights in treated males (mid- and high-dose) and females (high-dose) were considered to be test item-related and correlated with the microscopic findings of increased incidences and severities of fatty change (diffuse and macrovesicular).

The mean kidney weights (absolute and relative to body weight) of the males were decreased at 50 and 200 ppm (only at 200 ppm both absolute weight and kidney weight relative to body weight were statistically significant). Decreased kidney weights at 50 and 200 ppm were considered to be test item related and may be correlated with the decreased incidence of tubular vacuolation in the kidneys of males in these dose groups (see histopathology).

Absolute and relative mean adrenal gland weights were significantly increased in the males treated at 200 ppm whereas in the females at 250 ppm, only the relative mean adrenal gland weight was increased. In males, there were no macroscopic and microscopic findings in this treatment group that could explain this adrenal gland weight increase. In the females the increased mean adrenal weight relative to body weight might be correlated to the increased incidence of eosinophilic cytoplasmic change in the cortical cells together with a minimal to slight size increase of individual eosinophilic cells. There were no other test-item-related organ weight changes.

The mean relative heart weight was significantly increased in males of the low-dose group. In the absence of a dose-response relationship, this finding was not considered to be treatment-related.

The mean relative uterus weight was significantly increased at 250 ppm. Since there was no significant change in absolute uterus weights, the increased relative uterus weight was considered to be secondary to the body weight reduction in the high-dose group females and thus an unspecific finding.

3. Histopathology

3.1. Neoplastic findings

In general, the incidence of neoplastic lesions was slightly higher in females than in males but the neoplastic lesions were equally distributed over the controls and treatment groups without any statistical differences.

In the thyroid glands, follicular cell adenomas were observed in few males and females, randomly distributed in almost all dose groups. In the liver, hepatocellular adenomas and carcinomas were observed in males and females of the control group and in the males of the 20 and 50 ppm treatment groups. One Ito cell tumor was present in the female control group. Incidences of the neoplastic findings in the thyroid glands and the liver are summarized in the table below.

Table 5.5-27: Incidence of selected neoplastic findings in mice administered BAS 750 F for 18 months

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
No. of animals	50	50	50	50	50	50	50	50
THYROID exam	50	50	50	50	50	50	50	50
Adenoma, follicular cell				2	1		1	3
LIVER exam	50	50	50	50	50	50	50	50
Adenoma, hepatocellular	1	2	3					
Carcinoma, hepatocellular	1	1	1		1			
Ito cell tumor					1			

HISTORICAL CONTROL DATA (Test facility, four 18-month studies in C57BL/6JRj mice, started: 2013 - 2014)

♂ Thyroid, follicular cell adenoma:	Mean: 1.5% (3/198);	min.: 0% (0/50);	max. 2% (1/50)
♀ Thyroid, follicular cell adenoma	Mean: 2.6% (5/196);	min.: 0% (0/50);	max: 6% (3/47)

HISTORICAL CONTROL DATA (BASF, five 18-month studies in C57BL/6JRj mice, started: 1998 - 2007)

♂ Thyroid, follicular cell adenoma:	Mean: 1.2% (3/250);	min.: 0% (0/50);	max. 6% (3/50)
♀ Thyroid, follicular cell adenoma	Mean: 3.6% (9/250);	min.: 0% (0/50);	max: 8% (4/50)

The incidence of the adenomas in the thyroid glands were slightly higher in the 200/250 ppm treated animals. However, the incidence was not statistically significantly changed compared to the control group and was comparable to the background incidences in this strain of mice. This indicates that the follicular cell adenomas were incidental and not related to treatment.

The neoplastic lesions in the liver were clearly not treatment-related as the 200/250 ppm treatment groups were not affected.

All neoplastic findings (including in thyroid gland and liver) occurred either individually or were biologically equally distributed over the control group and treatment groups. They were considered to be spontaneous in nature and not related to treatment.

3.2. Non-neoplastic findings

Test item-related non-neoplastic microscopic findings after treatment with BAS 750 F were noted in the liver and thyroid glands of both male and female treated animals, in the kidneys of treated males and in the adrenal glands of treated females. These findings are summarized in the table below.

Liver

The majority of male and female mice from both control and treatment groups showed a diffuse fatty change of hepatocytes in livers, ranging from minimal to marked severity. This diffuse fatty change was characterized by a microvesicular cytoplasmic change of hepatocytes with a more or less diffuse pattern. Although there was no difference in total incidence of this change compared to the control animals, the severity of this background finding was slightly increased in males treated at 50 and 200 ppm and in females at 250 ppm.

Table 5.5-28: Incidence of selected non-neoplastic liver findings in mice administered BAS 750 F for 18 months

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
No. of animals	50	50	50	50	50	50	50	50
LIVER exam	50	50	50	50	50	50	50	50
Fatty change, diffuse (microvesic.)	48	46	47	48	40	44	38	43
Minimal (gr. 1)	7	5	2	1	10	11	5	3
Slight (gr. 2)	30	15	3	3	29	32	21	6
Moderate (gr. 3)	10	24	33	35	1	1	11	8
Marked (gr. 4)	1	2	9	9			1	26
Mean severity grade	*<2.0>*	*<2.3>*	*<2.9>*	*<3.0>*	*<1.4>*	*<1.6>*	*<1.7>*	*<2.9>*
Fatty change, macrovesicular	23	16	**35***	**46****	21	16	22	**39****
Minimal (gr. 1)	20	14	8	13	14	13	7	3
Slight (gr. 2)	3	2	14	16	7	3	12	2
Moderate (gr. 3)			11	13			3	11
Marked (gr. 4)			2	4				23
Mean severity grade	*<0.5>*	*<0.4>*	*<1.5>*	*<2.0>*	*<0.6>*	*<0.4>*	*<0.8>*	*<2.6>*
Eosinophilic inclusions, centrilob.				**38****				
Single cell necrosis, increased							1	**10***
Minimal							1	10

Statistical analysis: Fisher's Exact Test (1-sided); * : p≤0.05; ** : p≤0.01

A statistically significant test-item related and dose-dependent (in males) increased incidence and/or severity of macrovesicular fatty change was observed in the 50 and 200 ppm treated males and the 250 ppm treated females. This finding was characterized by the presence of large vesicles within the hepatocytes. The distribution of this macrovesicular fatty change was mainly midzonal.

The livers of the two animals per group per sex that were stained with Oil-Red-O to confirm that the vacuoles in the liver represents fat (fatty change), were all positively stained.

In addition to the findings of hepatocellular fatty change (diffuse and macrovesicular), there was a test-item related presence of eosinophilic cytoplasmic inclusions in hepatocytes (centrilobular distribution) in the majority of the males treated at 200 ppm (only) and a test-item related increased incidence of hepatocellular single cell necrosis (minimal severity) in 20% of the 250 ppm treated females (only).

Thyroid

There was a test-item related increased incidence and multiplicity in follicular cell hyperplasia in the thyroid glands of males treated at 200 ppm and possibly (as the finding was not statistically significantly different from control females) also in females treated at 250 ppm. The most important criteria for the diagnosis follicular cell hyperplasia were piling up of the epithelium into the lumen with presence of (a) stromal component(s). The follicular cell hyperplasia incidences observed in the male and female high-dose groups, but also in the female control group (38%), were outside of the 1-year historical control range of the test facility (females, 6-28%). The incidence of follicular cell hyperplasia in control males (42%) was also very close to the upper limit (45%) of the 1-year HC range for males.

Table 5.5-29: Incidence of selected non-neoplastic thyroid gland findings

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
No. of animals	50	50	50	50	50	50	50	50
THYROID exam	50	50	50	50	50	50	50	50
Hyperplasia, follicular cell, (m)focal	21	16	17	**37****	19	14	**8***	26

Statistical analysis: *: $p \leq 0.05$, **: $p \leq 0.01$ (Fisher's Exact test, 1-sided)

HISTORICAL CONTROL DATA (Four 18-month studies in C57BL/6JRj mice, started: Jul-2013 to Mar-2014)

♂ Thyroid, follicular cell hyperplasia: Mean: 31% (52/148); min.: 18% (9/50); max. 45% (22/49)
♀ Thyroid, follicular cell hyperplasia Mean: 18% (31/146); min.: 6% (3/50); max: 28% (13/47)

Kidney

A statistically significant test-item related and dose-dependent decreased incidence of tubular vacuolation was observed in the 50 and 200 ppm treated males. This vacuolation is a common finding in untreated C57BL/6JRj mice. The decreased tubular vacuolation at 200 ppm seemed to be correlated with the treatment-related decreased body weights in this treatment group.

Table 5.5-30: Incidence of selected non-neoplastic kidney findings

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
No. of animals	50	50	50	50	50	50	50	50
KIDNEY exam	50	50	50	50	50	5	10	50
Tubular vacuolation	42	42	**34***	**6****	0	0	0	0

Statistical analysis: Fisher's Exact Test (1-sided); * : $p \leq 0.05$; ** : $p \leq 0.01$

Adrenal gland

A statistically significant test-item related increased incidence of eosinophilic cytoplasmic change was observed in the adrenal glands of 250 ppm treated females. This adrenal gland finding was characterized by diffuse eosinophilic appearance of the cortical cell cytoplasm of all three zones together with a minimal to slight diffuse size increase of the individual cells. The incidence of cortical hypertrophy without cytoplasmic changes in treated animals was low and comparable with the females of the control.

Table 5.5-31: Incidence of selected non-neoplastic adrenal gland findings

	Males				Females			
Dose level [ppm]	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
No. of animals	50	50	50	50	50	50	50	50
ADRENAL GLAND exam	50			50	50	50	50	50
Cytoplasmic change, eosinophilic	0			0	2	6	3	**20****
Hypertrophy, cortex, diffuse	1			0	6	6	**0***	4
Minimal					3	3		2
Slight	1				3	3		2

Statistical analysis: Fisher's Exact Test (1-sided); * : p≤0.05; ** : p≤0.01

III. DISCUSSION

Treatment up to 200 ppm in males and 250 ppm in females neither increased the incidence of mortalities nor increased the incidence of neoplastic findings. This demonstrates that BAS 750 F did not induce or accelerate life threatening changes and did not have a carcinogenic potential up to the highest doses administered in this study (200 ppm in males and 250 ppm in females).

In the liver an increased incidence and/or severity in fatty change was observed in male mice at 50 and 200 ppm and in females at 200 ppm, which was associated with increased liver weights. An Oil-Red-O staining performed on representative samples confirmed the presence of fat in the hepatocytes. A diffuse, more microvesicular change was noted at the same incidence in control and treated animals but was more severe at 50 and 200 ppm in males and at 250 ppm in females and a macrovesicular fatty change was increased in both incidence and severity at 50 and 200 ppm in males and at 250 ppm in females. Only at the highest dose (200 ppm in males and 250 ppm in females), additional liver changes indicative of degeneration (eosinophilic cytoplasmic inclusions/increased single cell necrosis) were observed. Mild to moderate lipidosis alone would not be anticipated to impair liver function (Cattley R.C. and Popp J.A. (2002): "Chapter 31 – Liver", in: "Handbook of Toxicologic Pathology" (Haschek W.M, Rousseaux C.G. and Wallig M.A., eds.), 2nd edition, San Diego, Academic Press). In the absence of degenerative changes in the liver at lower doses, only the changes in the liver at the highest dose group were considered to be adverse.

Non-neoplastic findings in the thyroid glands consisted of an increased incidence and multiplicity of follicular cell hyperplasia in males of the highest dose group. In the females of the highest dose group, the increase in follicular cell hyperplasia at the high-dose level was not statistically significant, a dose-response relationship was not present, and the variability of this finding was quite high (rel. incidence at the mid dose was decreased by ~50% [19/50 to 8/50], then increased by ~50% [19/50 to 26/50] at high dose), therefore a test item relationship is not supported.

In 4 recent studies from the test facility with C57BL 6J/Rj mice that were run shortly before or in parallel with the BAS 750 F study and conducted within a 1-year timeframe, the incidences of follicular cell hyperplasia ranged between 18–45% in males and 6–28% in females. By comparison, the incidences of thyroid follicular cell hyperplasia in the BAS 750 F study were above these ranges in high-dose group males (74%, $p<0.01$) and in both female control (38%) and high-dose (52%, not statistically significant) groups. On this basis, the increased occurrence of follicular cell hyperplasia was considered treatment-related in high-dose group males, while a relationship to treatment of the slightly increased incidence in high-dose group females (from 19/50 in controls to 26/50 at 250 ppm) could not be supported. In the present study, the increased incidence of follicular cell hyperplasia was not related to an increased incidence of thyroid gland tumors. The occurrence of thyroid hyperplasia in high-dose group animals is considered to reflect a slight exacerbation in the incidence or severity of age-related thyroid changes that occur also in untreated controls, and is therefore assessed as non-adverse.

The non-neoplastic change in the kidneys of 50 and 200 ppm treated males (a dose-dependent decrease in spontaneous tubular vacuolation) was associated with a decreased mean kidney weight. The observed reduction of vacuolation in kidney tubule cells of male mice of the mid- and high-dose group represents a very subtle change. Although associated with slight kidney weight decreases, there were no indications of any degenerative process in the male kidney. Therefore, the observed change is not considered to reflect an adverse effect, but rather likely an expression of increased (energy-consuming) excretion activity.

The eosinophilic cytoplasmic change (together with a minimal to slight diffuse size increase) in the adrenal gland cortex of females at 250 ppm was considered to be non-adverse as no signs of degenerative processes were additionally observed. For example in rats, mild long-term chronic stress can lead to histological and ultrastructural changes in the adrenal gland cortex including decreased neutral lipid vacuoles and cellular hypertrophy (Everds N.E. et al. (2013): “Interpreting stress responses during routine toxicity studies: a review of the biology, impact and assessment”, Toxicologic Pathol. 41, pp. 560-614). These changes are very comparable to the morphologic features of the adrenal glands noted in the 250 ppm treated female mice of the present study which makes mild chronic stress a plausible explanation for these findings.

Adverse findings in the mouse 18-month carcinogenicity study with BAS 750 F were limited to the high-dose groups as follows:

Males (200 ppm)

- overall decreased mean body weight gain (up to -15%) observed from week 11/12 onwards
- increased relative liver weight (+42%)
- increased incidence and severity of fatty change of hepatocytes in the liver in combination with (pre)degenerative changes (eosinophilic cytoplasmic inclusions)

Females (250 ppm)

- overall decreased mean body weight gain (up to -33%) observed from week 7 onwards
- increased relative liver weight (+57%)
- increased incidence and severity of fatty change of hepatocytes in the liver in combination with (pre)degenerative changes (increased single cell necrosis)

Other findings were considered not to be adverse based on the nature of the changes and/or their amplitude. No adverse findings were noted up to 50 ppm for both sexes, corresponding with 9.1 mg/kg bw/d for males and 12.6 mg/kg bw/d for females.

IV. CONCLUSION

Under the conditions of this 18-month carcinogenicity study in C57BL/6JRj mice, the NOAEL for systemic toxicity was 50 ppm, corresponding to 9.1 mg/kg bw/d in males and 12.6 mg/kg bw/d in females, based on reductions of body weight gain, and increased liver weights in combination with histopathological evidence of liver cell degeneration in both sexes at the high dose level of 200 ppm in males (36 mg/kg bw/d) and 250 ppm in females (61.5 mg/kg bw/d). BAS 750 F was not carcinogenic in mice up to the highest dose level tested.

CA 5.6. Reproductive Toxicity

BAS 750F was investigated in a 2-generation reproduction toxicity study in rats and in prenatal developmental toxicity studies in rats and rabbits.

In the rat 2-gen study, BAS 750 F was administered via the diet to groups of 25 male and 25 female Wistar rats and the dose levels were adjusted to achieve mean daily intakes of 0, 25, 75 or 200 mg/kg bw/d. The NOAEL for parental toxicity was 25 mg/kg bw/d, based on clinical chemistry changes and increased liver weights in F_0 males at 75 mg/kg bw/d. At 200 mg/kg bw, adverse clinical chemistry changes were also observed in females and liver weight increases were associated with hypertrophy in males. Reductions in feed intake and body weight were seen in parental animals of both generations given 200 mg/kg bw/d. Body weight gains were reduced during major parts of the study in males (up to -33% in F_0 and up to -58% in F_1 males); in parental females reductions in body weight gain were most severe during gestation (up to -24% in F_0 and up to -32% in F_1 females) resulting in reduced body weight in F_1 females during early lactation (up to 17% below controls). BAS 750 F treatment did not adversely affect fertility of male rats or reproduction and delivery of female rats. All observations were within the normal range of biological variation and the corresponding values were within the historical control range of the test facility. Slight reductions in the number of live F_2 pups at birth and of live F_1 and F_2 pups on postnatal day 4 were noted at the high-dose level, but the resulting pup viability indices were not statistically significantly changed compared to control values. Moreover the findings were significantly influenced by one F_1 dam with only stillborn pups (the dam showing reduced general condition and piloerection during late pregnancy and very low feed intake after parturition) and by one total litter loss each in the F_0 and F_1 generation during early lactation. These affected F_0 and F_1 dams showed markedly reduced feed intake during lactation with associated signs of insufficient maternal care, leading to a slight increase in pup mortality and decreased body weights in surviving pups. Pup weight gains of the high-dose group were reduced to a comparable degree as the body weight changes of the parent animals and caused secondary effects on pup organ weights. There were no treatment-related histopathological changes of reproductive organs in parental animals or effects on sexual development of pups. The NOAEL for fertility and reproductive performance for the parental rats is 200 mg/kg bw/d, the highest tested dose. The NOAEL for developmental toxicity in the F_1 and F_2 progeny is 75 mg/ kg bw/d, due to the pup mortality and decrease in the pre-weaning pup body weights/pup weight gains observed at the 200 mg/kg bw/d dose. These effects are considered to be secondary to the decreased food consumption by the parental females during the last part of gestation and the early part of lactation. Developmental toxicity did not occur in the absence of parental toxicity. Therefore no signs of selective developmental toxicity were noted in the 2-generation reproduction toxicity study.

Oral gavage treatment of pregnant Wistar rats with BAS 750 F did not lead to significant or biologically relevant fetal toxicity or teratogenicity when administered from GD 6-19 at doses of up to 400 mg/kg bw/d. At this dose level, maternal toxicity was evident by statistically significant reductions in feed intake, body weight gain, carcass weight and corrected body weight gain. Hence, the NOAEL for maternal toxicity is 150 mg/kg bw/d and the NOAEL for prenatal developmental toxicity is 400 mg/kg bw/day, the highest dose tested. There were no toxicologically relevant fetal findings evident. BAS 750 F is not teratogenic in rats.

BAS 750 F was administered daily to inseminated New Zealand White rabbits by stomach tube during gestation days 6-28 post insemination (p.i.) at dose levels of 0, 5, 10 and 25 mg/kg bw/d.

The high dose of 25 mg/kg bw was selected on the basis of range-finding studies, where high toxicity including mortality was observed at 50 mg/kg bw/d and higher dose levels. In the definitive prenatal developmental toxicity study, no maternal or fetal toxicity was observed up to 25 mg/kg bw/d (the highest tested dose), and no treatment-related external, visceral or skeletal malformations or variations were noted. The maternal and developmental NOAEL in this rabbit prenatal developmental toxicity study was 25 mg/kg bw/d. BAS 750 F is not teratogenic up to the highest dose tested in rabbits.

Table 5.6-1: Summary of reproductive toxicity studies with BAS 750 F

Study **Species,** **Dose levels** **(batch / purity)**	**NOAEL (mg/kg bw/d)**	**LOAEL (mg/kg bw/d)**	**Critical effects**	**Reference** **Author(s), year** **BASF DocID**
Rat 2-generation study				[redacted] 2015c 2014/1170754
Rat (Wistar, Crl:WI(Han) 2-gen study Dietary administration with dose adjustment 0-25-75-200 mg/kg bw/d (COD-001740; 98.8%)	**Parental toxicity**			
	25	**75**	F_0 gen <u>≥ 75 mg/kg bw/d</u>: ↑ ALP (♂+♀), CHOL (♂), liver wt (♂+♀) <u>200 mg/kg bw/d:</u> ↓ fc, bw and bwg (♂+♀) ↑ Liver cell hypertrophy (♂) 1 dam with total litter loss by PND 2, one pup showing signs of insufficient nursing	
			F_1 gen <u>200 mg/kg bw/d:</u> ↓ fc, bw and bwg (♂+♀) ↑ ALP (♂+♀), urea + INP (♂), TRIG (♀) ↑ liver wt (♂+♀), liver cell hypertrophy (♂) 1 dam with total litter loss, pups showing signs of insufficient nursing 1 dam with only stillborn pups, dam showing poor general state and piloerection	
	Fertility			
	200	**–**	F_0-gen & F_1 gen No treatment-related adverse effect	
	Offspring toxicity			
	75	**200**	F_1 pups (200 mg/kg bw/d) ↓ live pups (PND 4) due to 1 total litter loss ↓ pup wt and wt gain during lactation (& secondary organ wt effects)	
			F_2 pups (200 mg/kg bw/d) ↓ live pups (PND 0 and PND 4) due to 1 dam with only stillborn pups and 1 dam with total litter loss ↓ pup wt and bwg during lactation (& secondary organ wt effects	

Table 5.6-1: Summary of reproductive toxicity studies with BAS 750 F

Study Species, Dose levels (batch / purity)	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Critical effects	Reference Author(s), year BASF DocID
Rat oral prenatal developmental toxicity				[redacted] 2015a 2014/1170755
Rat (Wistar, Crl:WI(Han) 0-50-150-400 mg/kg bw/d, gavage GD 6-19 vehicle: 1% CMC (L84-176; 97.7%)	**Maternal toxicity**			
	150	**400**	↓ fc, bw and bwg	
	Developmental toxicity			
	400	---	No treatment-related, adverse effects	
Rabbit oral prenatal developmental toxicity				[redacted] 2015b 2014/1170757
Rabbit (NZW) 0-5-15-25 mg/kg bw/d gavage GD 6-28 vehicle: 1% CMC (COD-001662; 95.5%)	**Maternal toxicity**			
	25	---	No treatment-related, adverse effects*	
	Developmental toxicity			
	25	---	No treatment-related, adverse effects	

* Severe toxicity requiring sacrifice of moribund rabbits was found in dose-range finding studies at a dose level of 50 mg/kg bw/d (see chapter 5.6.2/2 for details)

Comparison with CLP criteria:

For the purpose of classification for reproductive toxicity according to the criteria of the CLP (Regulation 1272/2008/EC), substances that are toxic to reproduction are allocated to one of two categories. Within each category, effects on sexual function and fertility, and on development, are considered separately. In addition, effects on lactation are allocated to a separate hazard category.

Category 1: Known or presumed human reproductive toxicant
Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).

Category 1A: Known human reproductive toxicant
The classification of a substance in Category 1A is largely based on evidence from humans.

Category 1B: Presumed human reproductive toxicant
The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.

Category 2: Suspected human reproductive toxicant
Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

Fertility
In the rat 2-generation reproduction toxicity there were no treatment-related significant effects that could be linked to impaired sexual function or fertility, such as increased time-to-mating, increased pregnancy duration, dystocia, effects on sperm parameters, adverse effects on reproductive organs, changes in the differential ovarian follicle count (DOFC), and decreases in the number of implantation sites or of litter size. All parameters were similar to concurrent controls or within the historical control range of the test facility.

In the absence of effects on sexual function or fertility in the rat 2-generation study, no classification for fertility is proposed or is considered necessary.

Developmental toxicity
In the rat 2-generation reproduction toxicity study, dietary exposure of Wistar rats at the top dose level corresponding to 200 mg/kg bw/d caused parental toxicity including reductions in feed intake, body weight and body weight gain in both male and female rats. Marked reductions in feed intake and body weight were noted in single F_0 and F_1 dams of the high-dose group, which lost their litter completely within the first days after birth mostly via cannibalization. Signs of insufficient nursing were noted in some of the pups from affected litters that were still available for examination. The single cases of total litter loss in F_0 and F_1 dams resulted in slight (statistically non-significant) decreases of the pup viability indices at 200 mg/kg bw/d and consequently the average litter size in this dose group was significantly below control on PND 4. Reductions in body weight were also noted in surviving pups at birth and body weight development during lactation, however the extent of these body weight changes between parental animals and pups was comparable. The body weight decreases caused secondary reductions of pup organ weights (brain, thymus, spleen), while organ weights relative to body weight were either unchanged or increased compared to control values. The time-points of vaginal opening or preputial separation as indicators of sexual development were within normal ranges. At necropsy, an increased incidence of dilated renal pelvis was noted in F_2 pups of the high-dose group (200 mg/kg bw/d). The finding was considered by the study authors to be treatment-related but secondary to the general delay in development of the high-dose pups (decreased body weight gain up to 19%), largely reversible and therefore non-adverse in this study. In addition, a treatment-related increase in renal pelvic dilation was *not* observed at any dose in F_1 pups. Dilated renal pelves can be a common finding in developmental toxicity studies in rodents (Haschek and Rousseaux 1998), and was observed in both controls and test groups at an incidence of up to 7.6% of fetuses (across 21% of litters) in the developmental toxicity study in rats. The incidence of this frequently occurring variation was not statistically significantly increased in the treated groups and was well within the historical control range in that study up to the highest tested dose level of 400 mg/kg bw/d; therefore, it was not considered a specific developmental effect.

Overall, developmental toxicity in the offspring due to reductions of body weights and body weight gains are considered to be secondary to the decreased food consumption by the parental females during the last part of gestation and the early part of lactation. Thus, the pup mortality and observed developmental toxicity in the rat 2-gen study is considered to be a secondary, non-specific consequence of maternal toxicity. No signs of selective developmental toxicity were noted.

In the prenatal developmental toxicity studies in Wistar rats, BAS 750 F treatment did not cause developmental toxicity or teratogenicity at dose levels that were maternally toxic. Likewise, no evidence for developmental toxicity or teratogenicity was found in the study with NZW rabbits.

Overall a classification of BAS 750 F for developmental toxicity is not required by comparison with CLP criteria; therefore no classification is proposed for this endpoint.

CA 5.6.1. Generational studies

Report:	CA 5.6.1/1 [redacted] 2015 c BAS 750 F - Two-generation reproduction toxicity study in Wistar rats - Administration via the diet 2014/1170754
Guidelines:	(EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, EPA 870.3800, OECD 416, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (batch COD-001740; purity: 98.8%) was administered to groups of 25 male and 25 female healthy young Wistar rats (F_0 parental generation) as a homogeneous addition to the food in different concentrations, which were adjusted to obtain target dose levels of 0, 25, 75 and 200 mg/kg mg/kg bw/d. At least 75 days after the beginning of treatment, F_0 animals were mated to produce a litter (F_1 generation). Mating pairs were from the same dose group and F_1 animals selected for breeding were continued in the same dose group as their parents. Groups of 25 males and 25 females, selected from F_1 pups to become F_1 parental generation, were offered diets containing target dosages of 0, 25, 75 and 200 mg/kg bw/d of the test substance post weaning, and the breeding program was repeated to produce a F_2 litter. The study was terminated with the terminal sacrifice of the F_2 weanlings and F_1 parental animals. Test diets containing BAS 750 F were offered continuously throughout the study.

The parents' and the pups' state of health was checked each day, and parental animals were examined for their mating and reproductive performances. Food consumption of the F_0 and F_1 parents was determined regularly once weekly during premating, gestation and lactation periods. In general, body weights of F_0 and F_1 parents were determined once weekly. However, during gestation and lactation F_0/F_1 females were weighed on gestation days (GD) 0, 7, 14 and 20 and on postnatal days (PND) 1, 4, 7, 14 and 21. Estrous cycle data were evaluated for F_0 and F_1 generation females over a three week period prior to mating until evidence of mating occurred. Moreover, the estrous stage of each female was determined on the day of scheduled sacrifice. The F_1 and F_2 pups were sexed on the day of birth (PND 0) and were weighed on the first day after birth (PND 1) as well as on PND 4, 7, 14 and 21. Their viability was recorded. At necropsy, all pups were examined macroscopically (including weight determinations of brain, spleen and thymus in one pup/sex/litter). All surviving pups were examined for the presence or absence of nipple/areola anlagen on PND 12 and re-examined towards the end (PND 20) of the lactation period. Anogenital distance (defined as the distance from the anus [center of the anal opening] to the base of the genital tubercle) measurements were conducted in a blind randomized fashion, using a measuring ocular on all live male and female pups on PND 1. Blood samples for clinical pathological investigations were withdrawn from 12 selected F_0 and F_1 parental animals per sex and group shortly before scheduled sacrifice.

Date of sexual maturation, i.e. day of vaginal opening (females) or balanopreputial separation (males), of all F_1 pups selected to become F_1 parental generation was recorded. Various sperm parameters (motility, sperm head count, morphology) were assessed in the F_0 and F_1 generation males at scheduled sacrifice or after appropriate staining. All F_0 and F_1 parental animals were assessed by gross pathology (including weight determinations of several organs) and subjected to an extensive histopathological examination, special attention being paid to the organs of the reproductive system. A quantitative assessment of primordial and growing follicles in the ovaries was performed for all control and high-dose F_1 parental females.

In **F_0 parental animals** administered 200 mg/kg bw/d, food intake was decreased in males during premating (up to 11% below control), mating days 9 - 14 (about 10% below control) and post-mating (up to 10% below control). In females food consumption was decreased throughout the study, most severely during gestation (up to 15% below control) and lactation (about 12% below control). Body weights were decreased in males from premating day 13 onwards until the end of the study (up to 11% below control), decreased body weight change during major parts of the study (up to 33% below control). In females, body weights were decreased throughout the study, most severely during gestation (up to 9% below control) and early lactation (up to 13% below control), decreased body weight change during major parts of the gestation period (up to 24% below control). Clinical pathology revealed increased alkaline phosphatase (ALP) activities in both sexes, and increased cholesterol values in males. Liver weights were increased in males (relative to bw) and females (both absolute and relative). Minimal grade centrilobular hypertrophy were found in 15/25 males. One dam suffered a complete litter loss by PND 2, one of the pups showing signs of insufficient nursing (no milk in stomach at necropsy). As a result of the complete loss of one litter, the mean number of live **F_1 pups** on day 4 was slightly decreased. F_1 pup body weights were decreased (about 10% below control) as was body weight gain (up to 15% below control) during lactation, causing secondary effects on pup organ weights. Otherwise, there were no treatment-related effects in pups.

In **F_1 parental animals** administered 200 mg/kg bw/d, food intake was decreased in males during premating (up to 9% below control) and post-mating (up to 10% below control). In females food consumption was decreased throughout the study, most severely during gestation (up to 20% below control) and lactation (about 20% below control). Body weights were decreased in males from premating day 1 onwards until the end of the study (up to 12% below control), decreased body weight change during major parts of the study (up to 58% below control). In females, body weights were decreased throughout the study, most severely during gestation (up to 16% below control) and early lactation (up to 17% below control), and decreased body weight gain was seen during major parts of the gestation period (up to 32% below control). Clinical pathology revealed increased alkaline phosphatase (ALP) activities in both sexes, increased urea and inorganic phosphate in males and increased triglyceride levels in females. Relative liver weights were increased in both sexes. Minimal grade centrilobular hypertrophy was found in 15/25 males. One dam, which showed piloerection and a distinctly poor general state around birth was noted with only stillborn pups. One dam suffered a complete litter loss by PND 3, pups showing signs of insufficient nursing (severely reduced nutritional condition, no milk in stomach of pups at necropsy). Although the viability index did not show statistically significant differences between treatment and control groups, the number of dead **F_2 pups** during PND 1-4 was higher at the high-dose level (15 vs. 1 in control), consequently, the mean number of live F_2 pups was significantly below controls on PND 4. Eleven of the 15 dead pups came from the dam that suffered total litter loss.

The lactation index indicating pup survival between PND 4-21 was not impacted by treatment. F_2 pup body weights were decreased (about 14% below control) and so was body weight gain (up to 19% below control) during lactation, causing secondary effects on pup organ weights.

In **F_0 parental animals** administered 75 mg/kg bw/d, there were no effects on food intake or on body weight development. ALP was increased in both sexes, cholesterol increased in males, and mean liver weights were increased in males (relative) and females (absolute and relative), however without any histopathological correlate; collectively, only the changes in males were considered to be adverse. In **F_1 parental animals** administered 75 mg/kg bw/d, there were no effects on food intake or on body weight development. Significant clinical chemistry changes were confined to one parameter in each sex (ALP increase in males and triglyceride increase in females), which were therefore not considered to be adverse. Mean relative liver weights were slightly increased in both sexes without associated histopathological change; collectively changes in F1 parental animals were not considered to be adverse. There were no adverse effects noted in F_1 or F_2 pups at the dose level of 75 mg/kg bw/d.

Thus, under the conditions of the present 2-generation reproduction toxicity study the **NOAEL for general, systemic toxicity is 25 mg/kg bw/d** for the F_0 and F_1 parental rats, based on decreased food consumption and body weight/body weight gain observed at 200 mg/kg bw/d, as well as clinical-chemical and pathological evidence of liver toxicity at 75 and 200 mg/kg bw/d, in all F_0 and F_1 parental animals.

The **NOAEL for fertility and reproductive performance** for the parental rats is **200 mg/kg bw/d**, the highest tested dose.

The **NOAEL for developmental toxicity** in the F_1 and F_2 progeny is **75 mg/ kg bw/d,** due to the decrease in the pre-weaning pup body weights/pup weight gains observed at the 200 mg/ kg bw/d dose. These effects are considered to be secondary to the decreased food consumption by the parental females during the last part of gestation and the early part of lactation.

Developmental toxicity did not occur in the absence of parental toxicity. Therefore no signs of selective developmental toxicity were noted in this 2-generation reproduction toxicity study.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description:	solid / white
Batch #:	COD-001740
Purity / content:	98.8%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: Rodent diet

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Source:	Charles River Laboratories, Research Models and Services GmbH, Germany
Sex:	male and female
Age (day 0):	34 ± 1 days
Weight at dosing:	♂: 97.5 - 123.0 g; ♀: 90.3 - 112.2 g
Acclimatization period:	about 6 days
Diet:	Kliba maintenance diet rat/mouse "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water from water bottles, ad libitum
Housing:	Single housing in Polycarbonate cages type III (800 cm² floor area, supplied by Tecniplast, Hohenpeissenberg, Germany and Becker & Co, Castrop-Rauxel, Germany) with dust-free wooden bedding, with the following exceptions • During overnight matings, male and female mating partners were housed together • Pregnant animals and their litters were housed together until PND 21 (end of lactation) Enrichment: wooden gnawing blocks (Typ NGM E-022; supplied by Abedd® Lab. and Vet. Service GmbH, Vienna, Austria) Pregnant females were provided with nesting material (cellulose wadding) toward the end of gestation.

Environmental conditions:

Temperature:	20 – 24 °C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 3-Apr-2013 to 22-Jul-2015 [in life phase: 9-Apr-2013 (start of administration of F_0 parental animals) to 23-Dec-2013 (sacrifice of F_1 females)]

2. Animal assignment and treatment

BAS 750 F was administered in the diet to groups of 25 male and 25 female rats at nominal dose levels of 0, 25 (low dose), 75 (mid dose), and 200 mg/kg bw/d (high dose). The animals used as F_0 parental animals were derived from different litters according to a written statement from the breeder. By this, sibling mating was avoided. The animals were randomly assigned to the test groups by means of computer generated randomization list based on body weights.

After the acclimatization period F_0 parental animals continuously received the test-substance throughout the entire study. About 16 hours prior to sacrifice food was withdrawn.

At least 75 days after the beginning of treatment, male and female rats of the same dose groups were mated overnight (details see below).

Females were allowed to deliver and rear their pups (F_1 generation pups) until day PND 4 (standardization; see below) or day 21 after parturition. After weaning of F_1 pups the F_0 generation parental animals were sacrificed.

After weaning, 25 male and 25 female F_1 pups of each treatment group were randomly selected as F_1 generation parental animals. It was attempted to take each litter into account. If fewer than 25 litters in these groups were available for selection or if one sex was missing in a litter, more animals were taken from different litters from the relevant test group to obtain the required number of animals.

All selected animals were treated with the test substance at the same dose level as their parents from post-weaning through adulthood up to about one day before they were sacrificed. At least 74 days after assignment of the F_1 generation parental animals, the males and females were mated overnight. The partners were randomly assigned so that matings of siblings were avoided.

Like F_0 females, F_1 females were allowed to litter and rear their pups (F_2 generation pups) until day 4 (standardization) or 21 after parturition. Shortly after weaning of F_2 pups, the F_1 parental animals were sacrificed.

With the exception of F_1 generation pups, which were chosen as F_1 generation parental animals, all pups were sacrificed by means of CO_2 after standardization or weaning.

Mating procedure: Males and females were mated overnight at a 1 : 1 ratio for a maximum of 2 weeks. Throughout the mating period, each female was paired with a predetermined male from the same dose group. The animals were paired by placing the male in the cage of the female mating partner from about 4.00 pm until 7.00 - 9.00 am of the following morning. Deviations from the specified times were possible on weekends and public holidays, and were reported in the raw data.

A vaginal smear was prepared after each mating and examined for the presence of sperm. If sperm was detected, pairing of the animals was discontinued. The day on which sperm were detected was denoted "gestation day (GD) 0" and the following day "gestation day (GD) 1".

Standardization of litters: On PND 4, the individual litters were standardized in such a way that, where possible, each litter contained 4 male and 4 female pups (always the first 4 pups/sex and litter were taken for further rearing). If individual litters did not have 4 pups/sex it was proceeded in such a way that the most evenly distributed 8 pups per litter were taken for further rearing (e.g., 5 male and 3 female pups). Standardization of litters was not performed in litters with ≤ 8 pups.

3. Test substance preparation and analysis:

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diet preparations were performed at intervals, which guaranteed that the test substance in the diet remained stable throughout the feeding period.

In principle, dietary concentrations were adjusted to the body weight and food consumption data of the preceding week in order to meet as close as possible the nominal dose levels. Exceptions from this rule are indicated in the table below:

Table 5.6.1-1: Dietary adjustment schedule

STUDY PHASE	FOR F_0 AND F_1 MALES	FOR F_0 AND F_1 FEMALES
Premating period	weekly	weekly
Mating period	once, use last premating concentration*	once, use last premating concentration
Gestation period	weekly	once, use last premating concentration
Lactation period	weekly	once, use 50% of last premating concentration
Post-weaning period	weekly	once, use last premating concentration

* Males received the female's diet during the cohabitation period until there was evidence of mating

The dietary concentration of BAS 750 F was calculated using the following equation:

$$\frac{BW_x \times D}{FC_x} = ppm$$

BWx = mean body weight on day x (g); D = dose (mg/kg bw); FCx = mean daily food consumption on day x (g); ppm = dietary BAS 750 F concentration for the week/period following day x.

During the first week of the premating period, F_0 parental animals received dietary BAS 750 F concentrations based on the body weight of randomization and historical food consumption data, i.e. 18 g for males and 15 g for females.

After weaning until the start of the respective premating period (day 0), the F_1 pups selected as F_1 parental animals received dietary BAS 750 F concentrations which were formulated on the basis of historical body weight and food consumption data for rats of similar age.

During the first week of the premating period for F_1 parental animals, dietary BAS 750 F concentrations were formulated on the basis of actual body weight on day 0 and historical food consumption data. Subsequent dietary BAS 750 F levels for each group and sex were adjusted as described above for F_0 parental animals.

Analyses performed prior to the start of the administration period revealed that the test-substance was stable in the diet for at least 32 days.

Samples of test substance / diet mixtures were taken for homogeneity and concentration control analyses to cover all treatment phases of the study for both generations, i.e. for the begin of the pre-mating period, end of the pre-mating period covering also mating and gestation phases (concentration control analyses only), and for the lactation period. For homogeneity analysis three randomly sampled specimen from the top, middle and bottom of the storage containers were sampled and analyzed. Results of these analyses are presented in Table 5.6.1-2 below.

Table 5.6.1-2: Dietary homogeneity and concentration control analyses

Sampling date	For treatment phase	fed to	Nominal dose level [ppm]	Mean conc. ± SD [ppm]	Relative SD [%]	Mean % of nominal dose level
09-Apr-2013	Begin Premating Week 0	F_0 males	**150**	**144 ± 5**	**3.4**	**95.8**
			450	417	–	92.7
			1193	1166	–	97.7
		F_0 females	171	174	–	101.7
			516	474	–	91.8
			1357	**1329 ± 76**	**5.7**	**97.9**
17-Jun-2013	End Premating Week 10	F_0 males	404	402	–	99.6
			1220	1265	–	103.7
			3358	3216	–	104.4
		F_0 females	327	317	–	97.0
			992	987	–	99.5
			2615	2737	–	104.7
10-Jul-2013	Lactation	F_0 females	**163**	**168 ± 3**	**1.6**	**102.8**
			496	483	–	97.4
			1308	**1319 ± 49**	**3.7**	**100.9**
5-Aug-2013	Post-weaning	F_1 males	**76**	**79 ± 2**	**2.0**	**103.9**
			228	251	–	110.0
			608	636	–	104.6
		F_1 females	83	92	–	110.4
			250	275	–	110.0
			667	**668 ± 16**	**2.4**	**100.2**
21-Oct-2013	End Premating Week 10	F_1 males	323	338	–	104.7
			970	1059	–	109.2
			2600	2839	–	109.2
		F_1 females	412	422	–	102.3
			1210	1285	–	106.2
			3125	3352	–	107.3
11-Nov-2013	Lactation	F_1 females	**162**	**163 ± 5**	**3.1**	**100.5**
			485	479	–	98.7
			1300	**1344 ± 13**	**0.9**	**103.4**

Values may not calculate exactly due to rounding

Relative standard deviations of the homogeneity samples in the range of 0.9 to 5.7% indicate the homogenous distribution of BAS 750 F in the diet preparations. The actual test-item concentrations were in the range of 91.8 to 110.4% of the nominal concentrations.

4. Statistics:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameter	Statistical test
Food consumption (parental animals), body weight and body weight change (parental animals and pups; for the pup weights, the litter means were used), gestation days, duration of sexual maturation (days to vaginal opening, days to preputial separation)	Simultaneous comparison of all dose groups with the control group using the DUNNETT test (two-sided) for the hypothesis of equal means
Male and female mating indices, male and female fertility indices, gestation index, females delivering, females with liveborn pups, females with stillborn pups, females with all stillborn pups	Pair-wise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Mating days until day 0 pc, %postimplantation loss	Pair-wise comparison of the dose group with the placebo-control group using the WILCOXON test (one-sided+) with BONFERRONI-HOLM adjustment for the hypothesis of equal medians
Implantation sites, pups delivered, live pups day x, viability index, lactation index	Pair-wise comparison of the dose group with the placebo-control group using the WILCOXON test (one-sided-) with BONFERRONI-HOLM adjustment for the hypothesis of equal medians
Number of cycles and cycle length (days 54 -74), pup organ weights (absolute and relative)	Non-parametric one-way analysis using the KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair-wise com¬parison of the dose groups with the placebo-control group was per¬formed using the WILCOXON-test (two-sided) for the hypothesis of equal medians.

Statistics of clinical pathology

Blood parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians
Sperm analysis parameters	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians; If only control and one dose group are measured, WILCOXON-test (one-sided) without adjustment were used. For the percentage of abnormal sperms (ABNORMAL6_C) values < 6% were set to 6% (cut off 6 %)

Statistics of pathology

Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
DOFC (differential ovarian follicular count)	Pair-wise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians

C. METHODS

1. Observations:

The animals, i.e. parental animals and pups, were examined for mortality twice daily on working days and once daily on weekends and public holidays. If animals were in a moribund state, they were sacrificed and necropsied. Observations for evident signs of toxicity were performed at least once daily.

The parturition and lactation behavior of the dams was generally evaluated in the mornings in combination with the daily clinical inspection of the dams. Only special findings (e.g., disability to deliver) were documented on an individual dam basis. Except on weekends and public holidays, the parturition behavior was additionally checked in the afternoons. The day of parturition was defined as the 24-hour period from about 3 pm of one day until about 3 pm of the following day.

2. Body weight:

Body weight of **parental animals** was determined on the first day of the premating period and weekly thereafter at the same time of the day. The following exceptions are notable for female parental animals:

a. During mating and post-mating, females were not weighed until there was a positive evidence of sperm in vaginal smears
b. During pregnancy, the F_0 and F_1 generation parental females were weighed on the day of positive evidence of sperm (GD 0) and on GD 7, 14, and 20.
c. During lactation, Females with litter were weighed on the day after parturition (PND 1) and on PND 4, 7, 14, and 21. Females without litter were not weighed during the lactation phase.

Pup body weights were determined on the day after birth (PND 1) and on PND 4 (before standardization), 7, 14, and 21.

3. Food consumption and test substance intake:

Food consumption was determined once a week for parental animals and calculated as mean food consumption in grams per animal and day. The following exceptions are notable for female parental animals:

a. During the mating period, no food consumption was determined.
b. During pregnancy, food consumption of females was determined weekly for GD 0-7, 7-14, and 14-20.
c. During lactation, food consumption of the females was determined for PND 1 - 4, 4 - 7, 7 - 14, and 14 - 21.
d. Food consumption was not determined for F_0 and F_1 females without positive evidence of sperm and females without litter.

The mean daily intake of test substance (group means in mg/kg bw/d) was calculated based upon individual values for body weight and food consumption:

$$\text{Substance intake for day } x = \frac{FC_x \times C}{BW_y}$$

FC_x = daily food consumption on day x [g]; C = concentration in ppm; BW_y = body weight on day y (g) (last weighing before day x)

4. Ophtalmoscopy:

Not performed in this study

5. Hematology and clinical chemistry:

Blood was withdrawn from the retro-orbital venous plexus from non-fasted animals following isoflurane anesthesia.

The examinations were carried out a few days before terminal sacrifice of the animals in 12 randomly selected animals per test group and sex of the F_0 and F_1 parental generation. The following hematological and clinical chemistry parameters were determined:

Hematology:					
	Red blood cells		***White blood cells***		***Differential blood cell count***
✓	Erythrocyte count (RBC)	✓	Leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)	✓	Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		***Metabolites and Proteins***		***Enzymes:***
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

6. Estrous cycle determination:

Estrous cycle length was evaluated by daily analysis of vaginal smear for all F_0 and F_1 female parental rats for a minimum of 3 weeks prior to mating and was continued throughout the mating period until the female exhibited evidence of copulation. Moreover, at necropsy a vaginal smear was examined to determine the stage of the estrous cycle for each F_0 and F_1 female with scheduled sacrifice.

7. Male reproduction data

For the males, mating and fertility indices were calculated for F_1 and F_2 litters according to the following equations:

$$\text{Male mating index [\%]} = \frac{\text{number of males with confirmed mating *}}{\text{number of males placed with females}} \times 100$$

* defined by a female with vaginal sperm or with implants in utero

$$\text{Male fertility index [\%]} = \frac{\text{number of males proving their fertility *}}{\text{number of males placed with females}} \times 100$$

* defined by a female with implants in utero

8. Sperm parameters

Immediately after necropsy and organ weight determination the right testis and cauda epididymis were taken from the F_0 and F_1 males of all dose groups. The following parameters were determined:

- sperm motility
- sperm morphology
- sperm head count (cauda epididymis)
- sperm head count (testis)

Sperm motility examinations and the preparations of the specimens for sperm morphology were carried out in a randomized sequence. Sperm morphology and sperm head count (cauda epididymis and testis) were evaluated for the control and highest dose group, only. Sperm motility was investigated by microscopic evaluation. Sperm morphology was evaluated microscopically after vital staining with eosin. Sperm head counts in cauda epididymis and testes were determined microscopically after homogenization using a MAKLER chamber.

9. Female reproduction and delivery data

For the females, mating, fertility and gestation indices were calculated for F_1 and F_2 litters according to the following equations:

$$\text{Female mating index [\%]} = \frac{\text{number of females mated *}}{\text{number of females placed with males}} \times 100$$

* defined as the number of females with vaginal sperm or with implants in utero

$$\text{Female fertility index [\%]} = \frac{\text{number of females pregnant *}}{\text{number of females mated * *}} \times 100$$

* defined as the number of females with implants in utero
** defined as the number of females with vaginal sperm or with implants in utero

$$\text{Female gestation index [\%]} = \frac{\text{number of females with live pups on the day of birth}}{\text{number of females pregnant *}} \times 100$$

* defined as the number of females with implants in utero

The total number of pups delivered and the number of liveborn and stillborn pups were noted, and the live birth index was calculated for F_1 and F_2 litters:

$$\text{Live birth index [\%]} = \frac{\text{number of liveborn pups at birth}}{\text{total number of pups born}} \times 100$$

The implantations were counted and the postimplantation loss (in %) was calculated. To determine the number of implantation sites, the apparently non-pregnant uteri were stained for about 5 minutes in 10% ammonium sulfide solution according to the method of SALEWSKI.

$$\text{Postimplantation loss [\%]} = \frac{\text{number of implantations} - \text{number of pups delivered}}{\text{number of implantations}} \times 100$$

10. Litter data

All F_1 and F_2 pups were examined as soon as possible on the day of birth to determine the total number of pups and the number of liveborn and stillborn members of each litter. Pups, which died before the first examination on the day of birth, were designated as stillborn pups.

The number of live pups/litter was calculated on the day after birth, and on lactation days 4, 7, 14, and 21. Furthermore, viability and lactation indices were calculated as follows:

$$\text{Viability index [\%]} = \frac{\text{number of live pups on day 4 * after birth}}{\text{number of live pups on the day of birth}} \times 100$$

* before standardization of litters (i.e. before culling)

$$\text{Lactation index [\%]} = \frac{\text{number of live pups on day 21 after birth}}{\text{number of live pups on day 4 * after birth}} \times 100$$

* after standardization of litters (i.e. after culling)

On the day of birth (PND 0) the sex of the pups was determined by determination of the anogenital distance (AGD). The AGD is defined as the distance from the center of the anal opening to the base of the genital tubercle. AGD was determined in all live male and female pups on PND 1. These measurements were performed in randomized order, using a measuring ocular. They were conducted by technicians unaware of treatment group in order to minimize bias.

The anogenital index was calculated according to the following formula:

$$\text{Anogenital index} = \frac{\text{anogenital distance [mm]}}{\text{cubic root of pup weight [g]}} \times 100$$

Subsequently the sex of the pups was assessed by the external appearance of the anogenital region and/or the mammary line. The sex of the animals was finally confirmed at necropsy. The sex ratio was calculated at PND 0 and PND 21 after birth using the following equation:

$$\text{Sex ratio [\%]} = \frac{\text{number of live male or female pups on day 0 / 21}}{\text{number of live male and female pups on day 0 / 21}} \times 100$$

Sexual maturation was determined in F_1 pups selected as parental animals. Females were evaluated daily for vaginal opening with examinations initiating on day PND 27. On the day of vaginal opening the body weights of the respective animals were determined. Males were evaluated daily for preputial separation with examinations initiating on day PND 38. On the day of preputial separation the body weights of the respective animals were determined.

11. Sacrifice and pathology:

Parental animals

All F_0 and F_1 parental animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology with special attention given to the reproductive organs. Animals that were sacrificed in a moribund state were necropsied as soon as possible after death and assessed by gross pathology without determination of organ weights.

Pathology:

The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: All animals of control and high dose groups; male animals suspected of impaired fertility and female animals that were non-pregnant of low and mid dose groups; all low and mid dose animals which died intercurrently of were sacrificed moribund).

C	W	H		C	W	H		C	W	H	
✓	✓	✓	adrenals	✓	✓	#	liver[‡]	✓	✓		spleen
✓	✓		brain	✓	✓	#	ovaries[$]	✓	✓	#	testes[§$]
	✓		cauda epidiymis	✓		#	oviducts	✓	✓		thyroid w. parathyroid gland
✓		#	coagulation glands	✓	✓	#	pituitary	✓	✓	#	uterus (& cervix uteri)
✓	✓	#	epididymides[§$]	✓	✓	#	prostate	✓		#	vagina
✓		✓	gross lesions	✓	✓	#	seminal vesicles[a]		✓		body (anesthethized)
✓	✓		kidneys								

[§] left of paired organs sampled and histopathologically examined; [$] fixed in Bouin's solution for animals killed as scheduled, for animals that died intercurrently the mentioned organs were fixed in neutral buffered 4% formaldehyde; [‡] histopathological examination of livers from all males; [a] with coagulation glands

The organs or tissues were fixed in neutral buffered 4% formaldehyde or in BOUIN's solution. The hematoxylin-eosin (HE) stained slides were examined and assessed by light microscopy.

Pups

With the exception of those F_1 generation pups, which were chosen as F_1 generation parental animals, all pups were sacrificed by means of CO_2 after standardization or weaning.

All culled pups, including stillborn pups and those that died during their rearing period, and pups killed after weaning were subjected to a macroscopic (external and visceral) examination.

Brain, spleen and thymus weights were taken from the first male and the first female pup/litter for pups killed at PND 21 (weaning). Relative organ weights were calculated on PND 21 pup weights.

All pups without notable findings or abnormalities were discarded after their macroscopic evaluation. Animals with notable findings or abnormalities were evaluated on a case-by-case basis, depending on the type of finding noted.

12. Differential Ovarian Follicle Count (DOFC):

For the F_1 maternal animals differential ovarian follicle count (DOFC), the ovaries were embedded in a longitudinal orientation in separate paraffin blocks. Each block was sectioned serially until the total ovary was laminated. Sections were prepared with 2 µm thickness and every 50 µm three serial sections were taken, mounted on glass slides and prepared as follows:

a. the first section from each triplicate was stained with hematoxylin and eosin;
b. the second section from each triplicate was subjected to immunohistochemistry staining using an MVH antibody [mouse vasa homolog], a protein expressed in all oocyte stages);
c. the third section from each triplicate was taken as possible negative control for immunohistochemistry (the third section of the 14th serial sections was used as negative control).

The immunohistochemically stained slides were used to count the primordial and growing follicles. Starting with the second series, the designated section of every third series was immunohistochemically evaluated throughout both ovaries of each female. Primordial follicles and growing follicles were counted by light microscope (magnification: 100x), according to the definitions given by Plowchalk et al., 1993.

To prevent multiple counting on serial sections, especially of the growing follicles, only follicles with an oocyte with visible chromatin were counted. The number of each type of follicle was recorded individually for both ovaries of every animal, giving in summary the incidence of each type of the follicles. Finally, the results of all types of follicles were summarized for all animals per group from the control and high-dose F_1 female groups. As primordial follicles continuously develop into growing follicles, the assessment of the follicles was extended to the combined incidence of primordial plus growing follicles.

In general, the 14th section of both ovaries stained with hematoxylin and eosin from all F_1 females of control and high-dose groups was evaluated for histological findings. Whenever in the ovary the diagnosis: “no abnormalities detected” was used that implies all different stages of functional bodies (especially corpora lutea) were present and normal. A correlation between gross lesions and histopathological findings was performed.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

There were no test substance-related mortalities in any of the groups. One female animal of test group 02 (No. 174 - 75 mg/kg bw/d) was sacrificed unscheduled because of a missing vaginal orifice, which resulted in severe uterus dilation.

2. Clinical observations

F_0 parental animals

No clinical signs or changes of general behavior, which may be attributed to the test substance, were detected in any of the male and female F_0 parental animals in any of the groups during pre-mating, mating, gestation and lactation.

One F_0 dam of the high-dose group (No. 200) had a complete litter loss by PND 2. One of the few pups of this litter, which were not cannibalized and were therefore available for necropsy, had no milk in its stomach.

F_1 parental animals

No clinical signs or changes of general behavior, which may be attributed to the test substance, were detected in any of the male and female F_1 parental animals in any of the groups during the premating and mating period.

During gestation of F_2 litters, there were no clinical findings in the F_1 females of the low- and mid-dose groups. Two sperm-positive females of the high-dose group (Nos. 383 and 393) did not deliver F_2 pups. One F_1 high-dose female animal (No. 379) showed piloerection and a distinctly poor general state before and after birth of its litter on GD 23/24. Its litter consisted only of stillborn pups.

During the lactation period, there were no clinical findings in the F_1 females of the low- and mid-dose groups. High-dose female animal No. 397 (200 mg/kg bw/d) did not nourish her pups properly during PND 1 - 3, as became evident by a severely reduced nutritional condition and the absence of milk in the stomach of the investigated pups. Consequently this animal lost its complete offspring within the first three days after birth.

C. BODY WEIGHT AND BODY WEIGHT CHANGE

Body weight development was impaired in high dose parental F_0 and F_1 animals.

F_0 parental animals

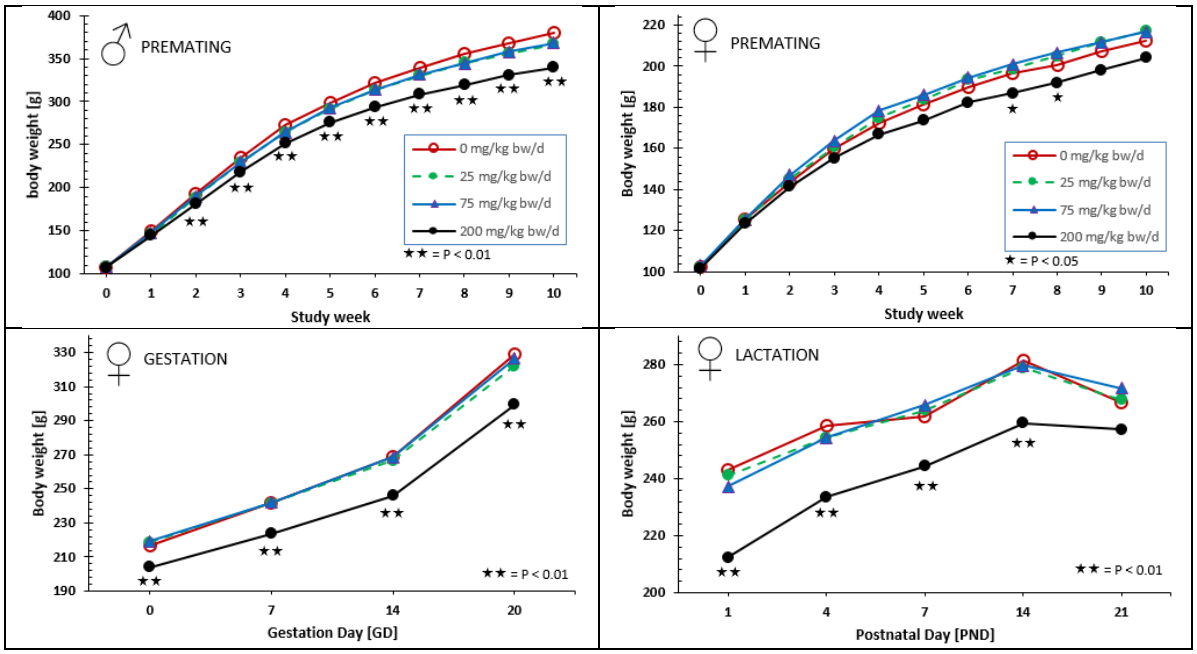
The body weight development of the F_0 animals is shown in Figure 5.6.1-1. Body weights of the high-dose (200 mg/kg bw/d) F_0 males were statistically significantly below the concurrent control values from premating day 13 onwards and remained so until the end of the study (up to 11%). Body weights of the mid- and low-dose parental males were comparable to the concurrent control group throughout the entire study.

Body weights of the high-dose F_0 females (200 mg/kg bw/d) were consistently below concurrent controls throughout the entire study, and gained statistical significance during premating days 48–55 (up to 5%), during the entire gestation period (up to 9%) and during lactation days 1–14 (up to 13%). Body weights of the mid- and low-dose parental females were comparable to the concurrent control group throughout the entire study.

The body weight change of the high-dose parental males was statistically significantly below the concurrent control values during major parts of the premating period (up to 33%). In the other study periods (mating, post-mating) the values were consistently below control, however, the difference did not gain statistical significance. The body weight change of the low- and mid-dose males was comparable to the concurrent control group during the entire study period. This includes a slight decrease in the low-dose group during premating days 0-6, which gained statistical significance but was considered incidental.

The body weight change of the high-dose parental females was statistically significantly below the concurrent control values during major parts of the gestation period (up to 24%). Contrary to this, the F_0 high-dose parental females generally gained more weight during lactation. The body weight change of the low- and mid-dose females was generally comparable to the concurrent control values throughout the entire study period. Increases of body weight gain in the mid-dose females during premating days 6–13 and lactation days 4–7 and (the latter change resulting in a slight increase of the overall body weight gain during PND 1–21) were considered to be spontaneous in nature.

Figure 5.6.1-1: Body weight development of F_0 parental animals



F_1 parental animals

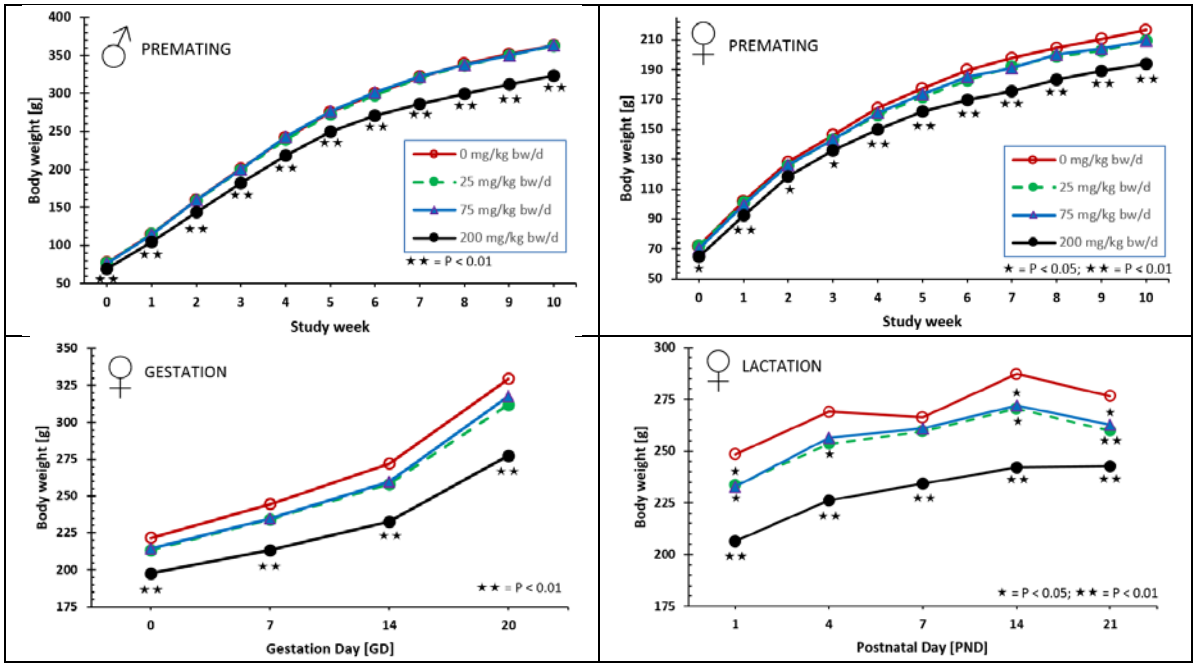
The body weight development of the F_1 animals is shown in Figure 5.6.1-2. Body weights of the high-dose (200 mg/kg bw/d) F_1 males were statistically significantly below the concurrent control values from the beginning of premating onwards and remained so until the end of the study (up to 12%). Body weights of the mid- and low-dose parental males were comparable to the concurrent control group throughout the entire study.

Body weights of the high-dose F_1 females were statistically significantly below the concurrent control values during the entire premating, gestation and lactation periods (up to 11%, 16% and 17%, respectively).The body weights of the low- and mid-dose females were comparable to the concurrent control group during the entire study period. This includes a number of slight statistically significant decreases in these dose groups during several sections of the lactation period. These decreases were neither consistent nor related to the dose, thus they are considered to be incidental findings.

The body weight change of the high-dose parental males was statistically significantly below the concurrent control values during major parts of the premating period (up to 32%) and during post-mating days 0 - 7 (about 58%). The body weight change of the low- and mid-dose males were comparable to the concurrent control group during the entire study period.

The body weight change of the high-dose parental females was below the concurrent control during most parts of premating and during the entire gestation (up to 32%). During premating the difference gained statistical significance on days 20 - 27 (about 23%), days 34 - 41 (about 35%), and when summarized for the whole premating (days 0 - 69, about 11%). Contrary to this, the F_1 high-dose parental females generally gained more weight during lactation. The body weight change of the low- and mid-dose females was comparable to the concurrent control values throughout the entire study. Inconsistent decreases or increases of body weight gain in these animals on several occasions were considered to be spontaneous in nature.

Figure 5.6.1-2: Body weight development of F1 parental animals



D FOOD CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

F_0 parental animals

Food consumption of the high-dose F_0 males (200 mg/kg bw/d) was statistically significantly below the concurrent control during the entire premating period (up to 11%), during mating days 9 - 14 (about 10%) and throughout post-mating period (up to 10%). Food consumption of the male F_0 rats in the mid and low dose groups was comparable to the concurrent control throughout the entire study. This statement includes occasional decreases in the low dose group during premating days 49 - 62 and in the mid-dose group during premating days 14 - 20, which are considered as incidental findings.

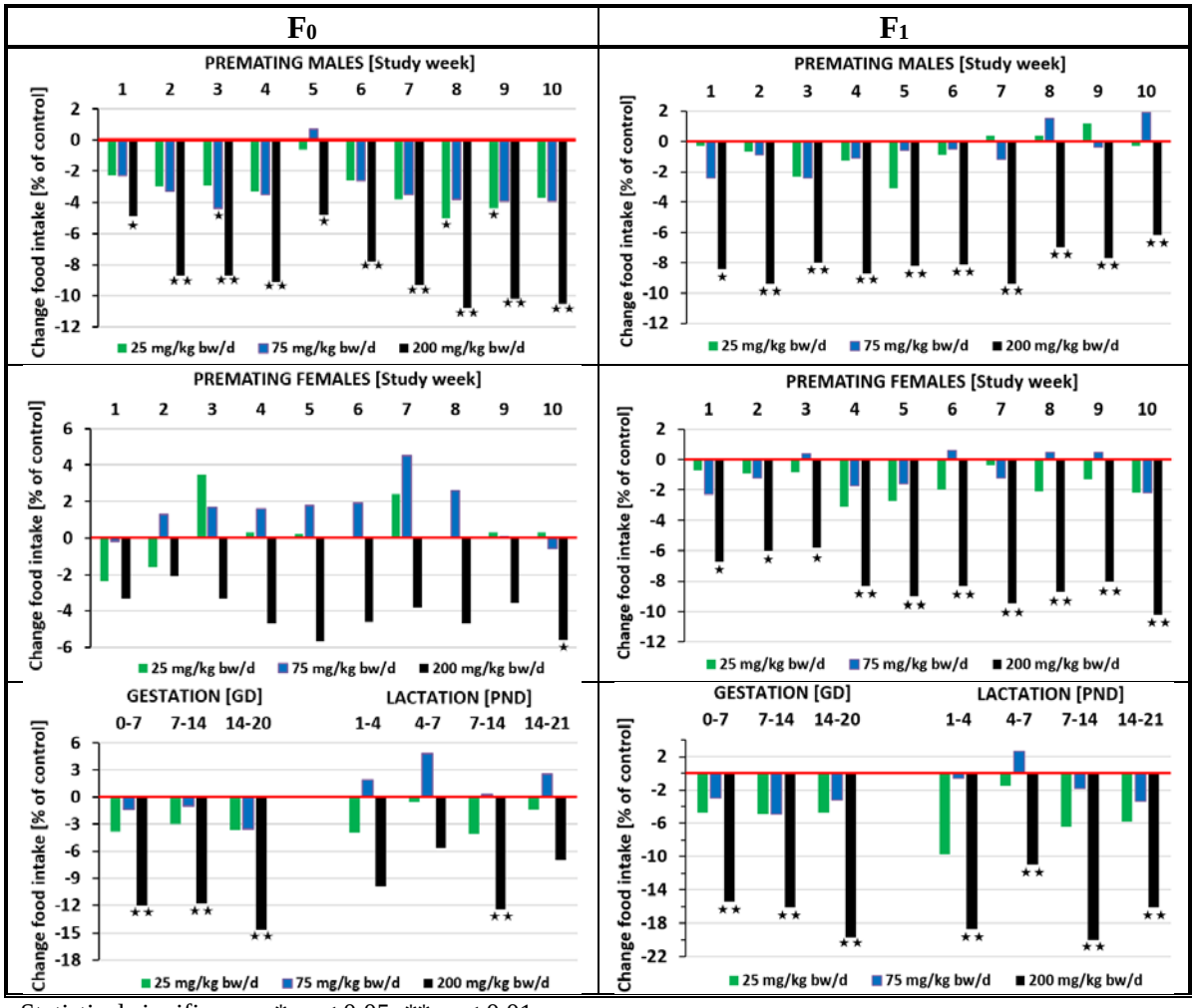
Food consumption of the high-dose F_0 females (200 mg/kg bw/d) was consistently below concurrent control throughout the entire study, however, the difference gained statistical significance below the control values only during premating days 63 - 69 (about 6%), during the entire gestation period (up to 15%) and during lactation days 7 - 14 (about 12%). Food consumption of the female F_0 rats in the mid and low dose groups was comparable to the concurrent control values throughout the entire study.

F_1 parental animals

Food consumption of the high-dose (200 mg/kg bw/d) F_1 males was consistently and statistically significantly below the concurrent control during the entire premating period (up to 9%) and during the entire post-mating period (up to 10%). Food consumption of the low- and mid-dose males was comparable to the concurrent control throughout the entire study.

Food consumption of the high-dose F_1 females was statistically significantly below the concurrent control during the entire premating period (up to 10%), during the entire gestation period (up to 20%) and during the entire lactation period (up to 20%). Food consumption of the female F_1 rats in the mid- and low-dose groups was comparable to the concurrent control values throughout the entire study.

Figure 5.6.1-3: Food consumption of F_0 and F_1 parental animals



Statistical significance: *: $p < 0.05$; **: $p < 0.01$

2. Test substance intake

For all test groups the intake of BAS 750 F correlated well with the desired target doses. The actual test substance intake was calculated on the basis of interpolated mean body weights of each test group. With regard to the target dose levels, the mean values and the range of the actual mean test substance intake per time interval examined are summarized in Table 5.6.1-3.

Table 5.6.1-3:Mean test substance intake in parental animals

	Target dose levels [mg/kg bw/d]		
	25	**75**	**200**
	Mean test substance intakes [mg/kg bw/d]		
F_0 males	24.1	72.2	191.3
F_0 females (premating)	24.3	72.9	194.0
F_0 females (gestation)	25.4	78.0	198.6
F_0 females (lactation)	28.2	89.5	228.0
F_1 males	23.9	71.9	190.5
F_1 females (premating)	24.1	72.2	193.3
F_1 females (gestation)	24.7	74.8	191.9
F_1 females (lactation)	27.9	87.3	220.4

E ESTROUS CYCLE DETERMINATIONS

F_0 parental animals

Estrous cycle data, generated during the last 3 weeks prior to mating for the F_1 litter, revealed regular cycles in the females of all test groups including the control. The mean estrous cycle duration in the different test groups was similar: 4.1 days in control, 4.2 in the low-dose group, 4.1 in the mid-dose group and 4.2 in the high-dose group.

F_1 parental animals

Estrous cycle data, generated during the last 3 weeks prior to mating for the F_2 litter, revealed regular cycles in the females of all test groups including the control. The mean estrous cycle duration in control, low- and mid-dose groups was similar: 4.1 days in control, 4.0 days in the low-dose group and 4.1 days in the mid-dose group.

The mean estrous cycle duration of the high-dose group was statistically significantly above the concurrent control value (4.6* days vs. 4.1 days in control), the number of cycles within the observation period was accordingly lower (3.48** vs. 4.32 in control). However, as the apparent mean cycle prolongation is only half a day and the cycle length value is well within the historical control range [6 studies (2010-2014): 3.99 – 4.8 days], it is not considered to be related to the treatment. In addition, neither time to (successful) pairing nor pairing success indicated any treatment-related effect on estrous cyclicity.

F MATING AND GESTATION DATA

1. Male reproductive performance

F_0 parental animals

Copulation was confirmed for all F_0 parental males, which were paired with females to generate F_1 pups. Thus, the male mating index was 100% in all test groups. Fertility was proven for all F_0 parental males with confirmed copulation. Thus, the male fertility index was 100% in all test groups.

F_1 parental animals

Copulation was confirmed for all F_1 parental males, which were paired with females to generate F_2 pups. Thus, the male mating index was 100% in all test groups. Fertility was proven for most of the F_1 parental males within the scheduled mating interval for F_2 litter. Two high-dose males (Nos. 283 and 293) did not generate F_1 pups. Thus, the male fertility index ranged between 92% and 100%. This reflects the normal range of biological variation inherent in the strain of rats used for this study. All respective values are within the range of the historical control data of the test facility.

Table 5.6.1-4: Reproduction parameters of male rats treated with BAS 750 F

Parental generation	**F_0**				**F_1**			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
- # animals per group	**25**	**25**	**25**	**25**	**25**	**25**	**25**	**25**
- # males placed with females	25	25	24	25	25	25	25	25
- # males mated	25	25	24	25	25	25	25	25
- Male mating index [%]	100	100	100	100	100	100	100	100
- # mated females pregnant	25	25	24	25	25	25	25	23
- Male fertility index [%]	100	100	100	100	100	100	100	92

Statistical analysis: Fisher's Exact test (1-sided -); *: p< 0.05; **: p<0.01

Historical control data [33 studies run 2008-2015 at test facility with Wistar rats (supplier: Charles River)]

- Male fertility index [%]: 80-100

The apparently infertile male rats (Nos. 283 and 293) did not show gross necropsy or histopathological findings or changes in sperm quality that would hint to infertility.

2. Sperm analysis

Sperm analyses, which investigated sperm motility and determined the incidence of sperm head counts in the testis as well as the percentage of abnormal sperm in the testis and cauda epididymis, did not indicate any effects of treatment in F_0 and F_1 males [see Table 5.6.1-5].

Table 5.6.1-5: Sperm parameters of male rats treated with BAS 750 F

Parental generation	**F_0**				**F_1**			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
- # animals per group	25	25	25	25	25	25	25	25
Sperm count [10^6 / g]								
- testis	110	N.A.	N.A.	117	102	N.A.	N.A.	103
- cauda epididymis	740	N.A.	N.A.	824	740	N.A.	N.A.	730
Sperm motility [%]	88	87	88	88	89	87	88	87
Abnormal sperm [%]	6.0	N.A.	N.A.	6.0	6.0	N.A.	N.A.	6.0

Statistical analysis: Wilcoxon with Bonferoni-Holm (1-sided -); *: p< 0.05; **: p<0.01

3. Female reproductive performance [Table 5.6.1-6]

F_0 parental animals

BAS 750 F did not adversely affect reproduction and delivery of the F_0 generation parental females.

The female mating index calculated after the mating period for F_1 litter was 100% in all test groups. The mean duration until sperm was detected (GD 0) varied between 2.4 and 3.0 days without any relation to dosing. All female F_0 rats delivered pups or had implants in utero. The fertility index was 100% in all test groups. The mean duration of gestation was similar in all test groups (i.e. between 22.1 and 22.4* [p≤0.05] days). The statistically significant difference for the high-dose group is not considered to be an effect of the test substance. The average difference is less than half a day and all values are well within the historical control range of the test facility. The gestation index was 100% in all test groups.

Implantation was not affected by the treatment since the mean number of implantation sites was comparable between all test substance-treated groups and the controls, taking normal biological variation into account (12.3 / 11.4 / 12.0 and 11.8 implants/dam in control, low-, mid- and high-dose test groups). Furthermore, there were no indications for test substance-induced intrauterine embryo-/fetolethality since the postimplantation loss did not show any significant differences between the groups.

The mean number of F_1 pups delivered per dam was 11.9, 10.7* [p≤0.05], 11.8 and 11.1 pups/dam in control, low-, mid- and high-dose test groups. As there is no relationship to the dose and the value is well within the historical control range, the slightly lower litter size in the low-dose group is not considered to be related to the treatment.

The rate of liveborn pups was also not affected by the test substance, as indicated by live birth indices of 98.9% (high-dose group), 100% (mid-dose group), 99.6% (low-dose group) and 99.7% (control). Moreover, the number of stillborn pups was comparable between the groups.

Table 5.6.1-6: Reproduction parameters of female rats treated with BAS 750 F

Parental generation	**F_0**				**F_1**			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
- # animals per group	25	25	25	25	25	25	25	25
- # females placed with males	25	25	24	25	25	25	25	25
- # females mated	25	25	24	25	25	25	25	25
- Female mating index [%]	100	100	100	100	100	100	100	100
- # females pregnant	25	25	24	25	25	25	25	23
- Female fertility index [%]	100	100	100	100	100	100	100	92
Pre-coital interval [mean days]	2.8	2.4	3.0	2.8	3.0	3.0	2.5	2.8
Duration of gestation [mean days]	22.1	22.2	22.2	**22.4***	22.2	22.0	22.0	22.2
Implantation sites, total	307	284	288	295	300	285	308	229
- dto per dam	12.3	11.4	12.0	11.8	12.0	11.4	12.3	**10.0***
Postimplantation loss [mean %]	3.9	5.5	1.3	5.2	2.4	5.0	**7.1****	8.9
Females with liveborn	25	25	24	25	25	25	25	21
- with stillborn pups	1	1	0	1	1	3	2	3
- with all stillborn	0	0	0	0	0	0	0	1
- Gestation index [%]	100	100	100	100	100	100	100	91.3
Pups delivered	297	267	284	277	298	269	285	217
- per dam	11.9	**10.7***	11.8	11.1	11.9	10.8	11.4	**9.9****
- liveborn	296	266	284	274	295	263	283	208
- stillborn	1	1	0	3	3	6	2	9
- Live birth index [%]	99.7	99.6	100	98.9	99.0	97.8	99.3	95.9

Statistical analysis: *: p< 0.05; **: p<0.01

Historical control data [33 studies run 2008-2015 at test facility with Wistar rats (supplier: Charles River)]

- Duration of gestation: 21.8 – 22.9 days;
- Postimplantation loss [mean %]: 0.9 – 17.7;
- Gestation index: 87.5 – 100%;
- Pups delivered/dam: 9.2 – 13.4
- Implantation sites/dam: 9.4 – 14.0;
- Live birth index [mean %]: 92.1 – 100

F_1 parental animals

The female mating index calculated after the mating period for F_2 litter was 100% in all test groups. The mean duration until sperm was detected (GD 0) varied between 2.5 and 3.0 days without any relation to dosing. All female F_1 rats delivered pups or had implants in utero except for high-dose female No. 383 (mated with male No. 293) and high-dose female No. 393 (mated with male No. 283), which did not become pregnant. There were no corroborative histopathological findings in the sexual organs of the non-pregnant females and time-to-mating was also unsuspicious.

The fertility index varied between 100% (control, low- and mid-dose group) and 92.0% (high-dose group). These values reflect the normal range of biological variation inherent in the strain of rats used for this study. All respective values are within the range of the historical control data of the test facility. The mean duration of gestation was similar in all test groups (i.e. between 22.0 and 22.2 days). The gestation index was 100% (control, low- and mid-dose groups) and 91.3% (high-dose group). All values are within the historical control range of the test facility

The mean number of implantation sites was slightly but statistically significantly lower in the high-dose group (12.0 / 11.4 / 12.3 and 10.0* ($p \leq 0.05$) implants/dam in control, low-, mid- and high-dose test groups). However, the high-dose mean was within the historical control range of the test facility (9.4 – 13.9) reflecting the normal range of biological variation inherent in the strain of rats used for this study.

As a consequence of the fewer implants, the mean number of F_2 pups delivered per dam was statistically significantly decreased in the high-dose group (11.9 / 10.8 / 11.4 and 9.9** (**:$p \leq 0.01$) pups/dam in test groups 10 - 13). As for the implants, this mean was also within the historical control range of the test facility (9.9 – 12.7) reflecting the normal range of biological variation inherent in the strain of rats used for this study.

There were no indications for test substance-induced intrauterine embryo-/fetolethality since the postimplantation loss did not show biologically relevant differences between the groups (2.4% / 5.0% / 7.1%** ($p \leq 0.01$) and 8.9% loss/dam in control, low-, mid- and high-dose test groups). The apparently higher rates in the treated groups are well covered by the historical control range of the test facility (0.9 – 17.7%), while the control rate was quite low. Also, the high value in high-dose group (without showing a statistically significant difference to control) is owing to female No. 388 which had only one resorbed implant, resulting in a postimplantation loss of 100% for this animal. Thus this animal contributed immoderately to the group mean which would have been 4.76% without this animal.

Apart from the carry-on effect in the high-dose group the rate of liveborn pups (live birth index) was not affected by the test substance, as indicated by live birth indices of 99.0% (control), 97.8% (low-dose group), 99.3% (mid-dose group) and 95.9% (high-dose group). As for the implants and delivered pups, this mean was also within the historical control range of the test facility (92.1 – 100%) reflecting the normal range of biological variation inherent in the strain of rats used for this study.

G PUP DATA

1. Pup survival [Table 5.6.1-7]

F_1 pups

The viability index indicating pup survival during early lactation (PND 0 - 4) varied between 93.5% (high-dose group), 100% (mid-dose group), 99.4% (low-dose group) and 99.1% (control) without showing statistically significant differences between control and treated groups. However, the number of dead pups during PND 1-4 was higher at the high-dose level (18 vs. 3 in control) and consequently the average litter size in this dose group was significantly below control on PND 4. It should be noted, however, that 11 out of the 18 dead pups came from one litter (No. 200), with one of the pups that could be examined showing signs of improper nursing (empty stomach). The remaining 7 dead pups (2 found dead, 5 cannibalized) at the high dose originated from the litters of 6 additional F_0 dams. The significantly lower litter size in the low-dose group is subsequent to the lower litter size at birth and not the result of postnatal pup mortality. The viability indices in the study were within the range of historical controls (89.4–100%)

The lactation index indicating pup survival on PND 4 – 21 was 100% (control, low- and high-dose groups) and 99.0% (mid-dose group).

F_2 pups

The viability index indicating pup survival during lactation (PND 0 – 4) varied between 93.6% (200 mg/kg bw/d), 99.0% (75 mg/kg bw/d), 99.6% (25 mg/kg bw/d) and 99.7% (control) without showing statistically significant differences between control and treated groups. However, the number of dead pups during PND 1 – 4 was higher at the high-dose level (15 vs. 1 in control) and consequently the average litter size in this dose group was significantly below control on PND 4. Eleven out of the 15 dead pups came from one litter (No. 397), and were not properly nourished during PND 1 – 3, as became evident by a severely reduced nutritional condition and the absence of milk in the stomach of the investigated pups. Consequentially this animal lost all offspring within the first three days after birth. The remaining 4 dead pups (cannibalized) originated individually from the litters of 4 F_1 dams.

The lactation index indicating pup survival on PND 4 – 21 was 100% (control and mid-dose group), 99.5 (low-dose group) and 99.4% (high-dose group). There was no evidence for test substance related mortality in any of the treated groups.

2. Sex ratio

The sex distribution and sex ratios of live F_1 or F_2 pups on the day of birth and on PND 21 did not show substantial differences between the control and the test substance-treated groups; slight differences were regarded spontaneous in nature.

Table 5.6.1-7: Pup survival and sex ratio

Parental generation	**F_0**				**F_1**			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
Number of litters	25	25	24	25	25	25	25	22
- with liveborn pups	25	25	24	25	25	25	25	21
- with stillborn pups	1	1		1				1
Pups liveborn	296	266	284	274	295	263	283	208
Pups found dead (day 1-4)	1	2		10			1	2
Pups cannibalized (day 1-4)	2			8	1	1	2	13
Pups PND 4 (pre-cull)	293	264	284	256	294	262	280	193
- Viability index [%]	99.1	99.4	100	93.5	99.7	99.6	99.0	93.6
Pups culled day 4	99	73	92	69	95	66	81	38
Pups PND 4 (post-cull)	194	191	192	187	199	196	199	155
Pups found dead (day 5-21)								
Pups cannibalized (day 5-21)			1			1		1
Pups PND 21	194	191	191	187	199	195	199	154
- Lactation index [%]	100	100	99.0	100	100	99.5	100	99.4
Sex ratio [% live males], PND 0	47.7	49.5	51.3	49.2	49.4	44.4	52.8	50.2
Sex ratio [% live males], PND 21	47.6	50.7	47.8	50.2	48.9	48.0	50.8	48.7

Statistical analysis, viability and lactation indices: Wilcoxon with Bonferoni-Holm (1-sided -), sex ratio: Wilcoxon test (2-sided); * $p \leq 0.05$, ** $p \leq 0.01$

3. Pup clinical observations

F_1 pups

For male pup No. 2 (from control dam No. 107) opacity on both eyes was recorded on PND 20 - 21.

F_2 pups

For six pups from high-dose group dam No. 397 (male pup No. 1, and female pups Nos. 4-8), a reduced nutritional condition and absence of milk in the stomach was recorded during PND 1 – 2. All other pups were without relevant clinical signs of toxicity.

4. Nipple / areola anlagen

The apparent number and percentage of male F_1 or F_2 pups having areolae was not influenced by the test substance when examined on PND 12. Upon the re-check of all animals for nipples/areolae on PND 20, one day prior to weaning, no areolae were detected at all in any of the male F_1 pups regardless of the test group.

Table 5.6.1-8: Areola nipple development in male F_1 and F_2 pups

Pup generation	F_1 pups				F_2 pups			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
- Day 12 [no. examined]	25	25	24	24	25	25	25	25
- Day 12 [Mean ± SD]	59±38	57±32	66±36	61±34	68±40	48±41	60±40	84±30
- Day 20 [no. examined]	25	25	24	24	25	25	25	20
- Day 20 [Mean ± SD]	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Statistical analysis: Wilcoxon with Bonferoni-Holm (1-sided +); * $p < 0.05$; ** $p < 0.01$

5. Anogenital distance / Anogenital index [Table 5.6.1-9]

F_1 pups

Anogenital distance and index of all male F_1 pups of all test groups were comparable to the concurrent control group. The apparently higher distance in the low-dose group is considered to be an incidental finding.

Anogenital distance and anogenital index of all female F_1 pups of all test groups were comparable to the concurrent control group. The slightly lower index in the low-dose group and the slightly higher index in the high-dose group are considered spontaneous findings.

F_2 pups

Anogenital distance and index of all male pups of all test groups were comparable to the concurrent control group. The apparently higher index in the high-dose group is considered to be a consequence of the lower body weight.

Anogenital distance of female pups of all treated groups was slightly but statistically significantly below the concurrent control value on PND 1 (about 2% [low dose], about 3% [mid-dose] and about 4% [high-dose]). However, as the anogenital index was unchanged and quite close to the control in all treated F_2 female pups, these minimal differences were considered incidental and not related to the treatment.

Table 5.6.1-9: Anogenital distance and anogenital index

Pup generation	F_1 pups				F_2 pups			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
MALES (No. examined)	1.43	1.32	148	127	145	115	148	103
- Anogenital distance [mm] (day 1)	3.02	3.08*	3.01	2.97	3.05	3.04	3.05	3.05
[Δ% control]		2.0	-0.3	-1.7		-0.3	±0.0	±0.0
- Anogenital index [---] (day 1)	1.61	1.62	1.62	1.63	1.62	1.62	1.63	1.66**
[Δ% control]		0.6	0.6	1.2		±0	+0.6	+2.5
FEMALES (No. examined)	150	132	136	132	149	147	132	96
- Anogenital distance [mm] (day 1)	1.50	1.47	1.50	1.49	1.58	1.55*	1.53**	1.52**
[Δ% control]		-2.0	±0.0	-0.7		-1.9	-3.2	-3.8
- Anogenital index [---] (day 1)	0.81	0.79*	0.82	0.84**	0.85	0.84	0.84*	0.84
[Δ% control]		-2.5	1.2	3.7		-1.2	-1.2	-1.2

Statistical analysis: * $p < 0.05$; ** $p < 0.01$ (Dunnett-test, 2-sided)

6. Pup weight

F_1 pups

In the high-dose group (200 mg/kg bw/d) lower F_1 pup body weights were noted on postnatal day 1 (about 9% below control). Pup body weights remained lower until weaning (about 10% below control at PND 21). Accordingly, mean body weight change was below the concurrent control in the high-dose F_1 pups (up to 15%) throughout lactation. No test compound-related influence on F_1 pup body weights/body weight change was noted in the mid- and low-dose groups (75 and 25 mg/kg bw/d).

F_2 pups

At 200 mg/kg bw/d lower F_2 pup body weights were noted on postnatal day 1 (about 7% below control). Pup body weights remained lower until weaning (about 14% below control at PND 21). High-dose pup weights were also below the lower limit of the historical control range during the entire lactation period. Accordingly, mean body weight change was below the concurrent control in the high-dose F_2 pups (up to 19%) throughout lactation.

Towards the end of lactation a number of statistically significant changes of pup body weights were noted in the low- and mid-dose groups (25 and 75 mg/kg bw/d): on PND 14 and 21 in the low-dose group as well as on PND 21 in the mid-dose group. Accordingly, mean body weight change was below the concurrent control in these animals from the second lactation week onwards (low-dose) or during the third lactation week (mid-dose). As there was no dose-response and as all the pup body weights are well covered by the historical control range of the test facility this apparent effect was considered to be incidental and of no toxicological relevance.

Table 5.6.1-10: Pup body weights

Pup generation		F_1 pups				F_2 pups			
Dose level [mg/kg bw/d]		0	25	75	200	0	25	75	200
Male pup wt [g]									
- day 1	[HCR: 6.5 – 7.4]	6.7	7.0	6.6	6.1*	6.7	6.7	6.5	6.2*
- day 4	[HCR: 10.2 – 11.2]	10.1	10.6	10.1	9.2	10.4	10.3	10.1	9.7
- day 7	[HCR: 15.9 – 17.5]	16.6	16.9	16.7	14.7**	16.9	16.6	16.2	15.2**
- day 14	[HCR: 32.2 – 35.2]	34.0	33.5	34.1	30.2**	34.3	32.8	33.0	29.6**
- day 21	[HCR: 49.9 – 55.6]	53.7	53.1	53.8	48.4**	54.4	51.2**	51.3**	46.9**
Male pup wt gain [g]									
- day 1 to 21		47.0	46.2	47.2	42.2**	47.7	44.5**	44.8**	40.6**
- day 1 to 21	[Δ% control]		-2	±0	-10		-7	-6	-15
Female pup wt [g]									
- day 1	[HCR: 6.2 – 7.1]	6.4	6.6	6.3	5.8*	6.4	6.3	6.2	6.0*
- day 4	[HCR: 9.7 – 11.0]	9.8	10.2	9.7	8.7*	10.1	9.9	9.7	9.4*
- day 7	[HCR: 15.6 – 17.0]	16.1	16.3	16.1	14.1**	16.3	15.9	15.7	14.7**
- day 14	[HCR: 31.2 – 34.4]	33.2	32.5	33.2	29.3**	33.3	31.7*	32.3	28.5**
- day 21	[HCR: 48.3 – 53.3]	52.9	52.2	52.8	47.5**	52.3	48.9**	49.7**	44.9**
Female pup wt gain [g]									
- day 1 to 21		45.7	44.3	45.6	40.7**	45.9	42.6**	43.4*	38.8**
- day 1 to 21	[Δ% control]		-3	±0	-11		-7	-5	-15

Statistical analysis: * $p < 0.05$; ** $p < 0.01$ (Dunnett-test, 2-sided)

[HCR]: Historical control range from 17 studies (1-gen or 2-gen) with Wistar rats (supplier: Charles River) run at test facility between 2011-2015

7. Pup organ weights [Table 5.6.1-11]

F_1 pups

Mean absolute brain, spleen and thymus weights of male and female F_1 pups from the high-dose group were decreased. Relative brain weights of high-dose male and female F1 pups were increased, there were no significant differences in the relative thymus or spleen weights between control and test groups. The observed organ weight changes were considered secondary to the lower pup body weights in the high-dose group.

In addition, absolute and relative brain weight of low-dose F_1 females was slightly increased, which was considered an incidental finding.

F_2 pups

In the high-dose group, brain, thymus and spleen weights were significantly decreased. The relative brain weights of F_2 pups were significantly increased at all dose levels (slightly at the low- and mid-dose level). Relative spleen and thymus weights were not significantly changed at any dose level. These organ weight changes were considered to be secondary to the lower pup body weights in these groups. The decreased absolute spleen weights in the low-dose group are regarded as an incidental finding.

Table 5.6.1-11: Organ weights of F_1 and F_2 pups

Generation		F_1 (males & females combined)				F_2 (males & females combined)			
	Dose [mg/kg]	Absolute weight [g]	Δ%	Relative weight [% of bw]	Δ%	Absolute weight [mg]	Δ%	Relative weight [% of bw]	Δ%
Brain	0	1.526		2.853		1.527		2.855	
	25	1.549[a]	(+1.5)	3.012*	(+3.3)	1.510	(-1.1)	3.016*	(+5.6)
	75	1.531	(+0.3)	2.927	(+0.9)	1.507	(-1.3)	2.991**	(+4.8)
	200	1.481**	(-2.9)	3.128**	(+9.1)	1.473**	(-3.5)	3.229**	(+13.1)
Thymus	0	0.255		0.480		0.255		0.476	
	25	0.250	(-2.0)	0.477	(-0.7)	0.236	(-7.6)	0.469	(-1.6)
	75	0.252	(-1.1)	0.476	(-0.8)	0.248	(-2.7)	0.492	(+3.4)
	200	0.223**	(-12.6)	0.472	(-1.7)	0.214**	(-16.2)	0.464	(-2.5)
Spleen	0	0.254		0.477		0.249		0.464	
	25	0.246	(-3.1)	0.470	(-1.6)	0.219**	(-12.0)	0.436	(-6.1)
	75	0.258	(-1.5)	0.487	(+2.0)	0.242	(-3.1)	0.477	(+2.8)
	200	0.222**	(-12.7)	0.467	(-2.1)	0.206**	(-17.6)	0.442	(-4.7)

Statistical evaluation: Kruskal-Wallis and Wilcoxon-test (2-sided); * $p \leq 0.05$, ** $p \leq 0.01$
[a] mean absolute brain weights of females were significantly increased (+1.9%)
Values may not calculate exactly due to rounding of figures

8. Pup necropsy findings [Table 5.6.1-12]

F_1 pups

A few F_1 pups showed spontaneous findings at gross necropsy, such as partly cannibalized, dilated ureter, opacity of both eyes, diaphragmatic hernia, small testis, dilated renal pelvis, post mortem autolysis, abnormal liver lobation, and incisors sloped. These findings occurred without any relation to dosing and/or can be found in the historical control data at comparable or even higher incidences. Thus, all these findings were not considered to be associated with the test substance.

From the litter of dam no. 200 with total litter loss of 11 pups, one pup was found with empty stomach at necropsy; nothing abnormal was detected in five pups. The remaining five pups from this litter were cannibalized before they could be clinically examined or necropsied.

F_2 pups

A few F_2 pups showed spontaneous findings at gross necropsy, such as post mortem autolysis, dilated ureter, dark discolored liver, diaphragmatic hernia and hydronephrosis. These findings occurred without any relation to dosing and/or can be found in the historical control data at comparable or even higher incidences. Thus, all these findings were not considered to be associated with the test substance.

Dilated renal pelves were found in 23 high-dose F_2 pups (6 males, 17 females) compared to 3 affected female pups in the control. The finding were considered by the study authors to be treatment-related but secondary to the general delay in development of the high-dose pups (decreased body weight gain up to 19%), largely reversible and therefore non-adverse in this study. In addition, a treatment-related increase in renal pelvic dilation was *not* observed at any dose in F_1 pups. Dilated renal pelves can be a common finding in developmental toxicity studies in rodents (Haschek and Rousseaux 1998), and was observed in both controls and test groups at an incidence of up to 7.6% of fetuses (across 21% of litters) in the developmental toxicity study in rats.

The incidence of this frequently occurring variation was not statistically significantly increased in the treated groups when tested up to 400 mg/kg bw/d and was well within the historical control range in that study; therefore, it was not considered a specific developmental or endocrine-related effect in either study.

Findings of empty stomach in the high-dose pups were preceded by clinical signs of (these and/or further) pups not properly nursed and reduced general nutritional condition in the pups, suggesting poor nutrition is the primary cause of the delayed pup development in the high-dose group in general.

Table 5.6.1-12: Incidence of gross necropsy observations in F_1 and F_2 pups

	Male pups				Female pups			
Dose [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
F_1 pups								
Animals examined	119	107	121	109	127	107	110	117
… normal (NAD)	114	105	114	101	125	101	106	106
… with signs	5	2	7	8	2	6	4	11
... post-mortem autolysis						1		
… partly cannibalized			1					
… not assessed (= missing/cannibalized)				4 (4)	1	2 (2)		6 (3)
Diaphragm, hernia				2 (1)				
Eye, opacity	1							
Incisors, sloped								1
Liver, abnormal lobation							1	
Renal pelvis, dilated	3 (3)	2 (2)	5 (4)	2 (2)	1	3 (3)	3 (3)	3 (3)
Stomach, empty								1
Testis, small	1		1					
Ureter, dilated		1	1				1	1
F_2 pups								
Animals examined	145	120	148	112	152	149	132	105
… normal (NAD)	145	117	145	101	146	139	126	75
… with signs		3	3	11	6	10	6	30
... post-mortem autolysis			2 (2)				1	2 (1)
… not assessed(= missing/cannibalized)				4 (3)	1	2 (2)	2 (2)	10 (3)
Diaphragm, hernia					2 (2)		1	1
Liver, dark discolored		2 (1)				6 (1)		
Renal pelvis, dilated		1	1	6 (5)	3 (3)	2 (2)	1	17 (9)
Renal pelvis, hydronephrosis							1	1
Stomach, empty				1				1
Ureter, dilated				1	1		1	1

( ) values in brackets give litter incidence

9. Sexual maturation [Table 5.6.1-13]

Vaginal opening

Each female F_1 pup, which was selected to become a parental F_1 female, was evaluated for commencement of sexual maturity. The first day when vaginal opening was observed was PND 28, the last was PND 36. The mean number of days to reach the criterion in the control and 25, 75 and 200 mg/kg bw/d test groups amounted to 30.0; 30.9* ($p \leq 0.05$); 30.2 and 31.8** ($p \leq 0.01$) days. The mean body weight on the day, when vaginal opening was recorded, amounted to 91.6 g, 93.5 g, 90.8 g, and 91.9 g in control and low-, mid- and high-dose test groups.

Although a statistically significant difference in the mean time point of vaginal opening was obtained in the low- and high-dose groups when compared to the concurrent control group, the data of these dose groups (30.9 and 31.8 days) were well comparable to the overall historical control mean value (29.5 - 31.9 days), while the mean value of the concurrent control group (30.0 days) was found to be at the low end of the historical control data range. Therefore, any apparent delay of vaginal opening seen in this study is not considered to be the result of a specific mechanism of toxicity.

Preputial separation

Each male F_1 pup, which was selected to become a F_1 parental male, was evaluated for commencement of sexual maturity. The first day when preputial separation was observed was PND 42, the last was PND 46. The mean number of days to reach the criterion in the control and 25, 75 and 200 mg/kg bw/d test groups was 43.1, 42.7, 43.2, and 43.5 days. The mean body weight on the day, when preputial separation was recorded, amounted to 182.3 g, 177.3 g, 183.6 g, and 167.9 g** ($p \leq 0.01$) in control and low-, mid- and high-dose test groups. The slightly lower body weight of the high-dose offspring had no impact on the puberty age of the animals.

Table 5.6.1-13: Sexual maturation of F_1 pups

Parental generation	**Females / Vaginal opening**				**Males / Preputial separation**			
Dose level [mg/kg bw/d]	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
# animals examined	25	25	25	25	25	25	25	25
- Days to criterion	30.0	30.9*	30.2	31.8**	43.1	42.7	43.2	43.5
- Body weight at criterion [g]	91.6	93.5	90.8	91.9	182.3	177.3	183.6	167.9**

Statistical analysis: * $p < 0.05$; ** $p < 0.01$ (Dunnett-test, 2-sided)

Historical control data: 17 studies (2010-2015) from the test facility with Wistar rats (supplier: Charles River)
Day of vaginal opening: 29.5 – 31.9 days (body wt at criterion: 83.1 – 100.7 g)
Day of preputial separation: 40.5 – 45.2 days (body wt at criterion: 168.1 – 195.3 g)

H CLINICAL CHEMISTRY DATA [Table 5.6.1-14]

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related in adults:

F_0 generation

- ↑ Alkaline phosphatase at ≥ 75 mg/kg bw/d in both sexes (adverse)
- ↑ Cholesterol at ≥ 75 mg/kg bw/d in males (adverse)
- ↓ Total bilirubin at ≥ 75 mg/kg bw/d in males (adaptive change in the absence of anemia signs, considered to reflect increased phase II metabolism in the liver resulting in accelerated biliary excretion of bilirubin)

F_1 generation

- ↑ Alkaline phosphatase in males at ≥ 75 mg/kg bw/d and in females at 200 mg/kg bw/d (adverse)
- ↑ Urea in males at 200 mg/kg bw/d (adverse)
- ↓ Total bilirubin in males at ≥ 75 mg/kg bw/d and in females at 200 mg/kg bw/d (adaptive change)
- ↑ Triglyceride concentration in females at ≥ 75 mg/kg bw/d (adverse)

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

F_0 generation

- ↓ absolute neutrophil count in females at 25 mg/kg bw/d (not dose-dependent)
- ↑ GGT in females at 200 mg/kg bw/d (within HCR)
- ↓ ALT in females at ≥ 25 mg/kg bw/d (not dose-dependent)
- ↑ Urea in females at 25 and 75 mg/kg bw/d (not dose dependent)

F_1 generation

- ↓ Triglyceride concentration in males at ≥ 25 mg/kg bw/d (not dose-dependent and within historical control range)
- ↓ Glucose concentration in males at 25 and 200 mg/kg bw/d (not dose-dependent and within historical control range)
- ↑ Inorganic phosphate in males at ≥ 75 mg/kg bw/d (not dose-dependent and within historical control range)

Table 5.6.1-14: Clinical-chemistry findings in rat 2-gen study

		Males				Females			
Dose level [mg/kg bw/d]		**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
F_0 generation									
ALP[µkat/l]	F_0-gen	1.23	1.45	**2.03****	**2.26****	0.77	0.74	**0.96***	**1.15****
	F_1-gen	1.24	1.55	**1.95****	**2.36****	0.89	0.86	0.84	**1.26****
ALT	F_0-gen	0.90	0.84	0.82	0.80	0.65	0.55*	0.51*	0.55*
	F_1-gen	0.87	0.91	0.79	0.94	0.63	0.54	0.52	0.60
GGT[nkat/l]	F_0-gen	0	0	2	4	5	5	6	14*
	F_1-gen	0	0	0	0	0	0	0	2
						Historical control: 0 – 10			
INP [mmol/L]	F_0-gen	1.64	1.69	1.77	1.74	1.52	1.37	1.32	1.77
	F_1-gen	1.56	1.70	1.69*	**1.81****	1.25	1.21	1.25	1.16
		Historical control: 1.49 – 1.79							
Glucose [mmol/l]	F_0-gen	6.52	6.12	5.95	5.89	6.31	5.65	5.98	5.67
	F_1-gen	6.64	6.14*	6.32	5.97**	5.08	5.26	4.75	4.61
		Historical control: 5.50 – 7.37							
Triglycerides [mmol/l]	F_0-gen	0.95	0.81	0.76	0.80	0.99	0.88	1.12	1.47
	F_1-gen	1.19	0.87*	0.89*	0.81*	0.72	0.94	**0.98***	**1.09****
		Historical control: 0.61 – 1.29							
Cholesterol [mmol/l]	F_0-gen	1.87	2.11	**2.30***	**2.56****	2.05	2.10	1.95	2.21
	F_1-gen	2.13	2.35	2.21	2.45	1.99	2.11	1.86	2.10
Urea [mmol/l]	F_0-gen	7.22	7.30	7.81	7.70	9.81	8.53*	8.62*	10.09
	F_1-gen	6.02	6.62	6.40	**7.91****	7.10	7.62	7.00	7.33
Total bilirubin [µmol/l]	F_0-gen	2.11	2.02	1.58**	1.58**	3.04	3.16	3.25	3.38
	F_1-gen	1.52	1.37	1.21**	1.11**	1.69	1.55	1.44	1.24*

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis + Wilcoxon test (2-sided)

Historical control data: 46-48 3-month studies, 18-wk Wistar rats (16-h fasting), sampling between Sep-2009 and Jun-2014

I PARENTAL TERMINAL INVESTIGATIONS

1. Organ weights [Table 5.6.1-15 and Table 5.6.1-16]

F_0 generation

The terminal body weight was significantly decreased in male and female animals at 200 mg/kg bw/d, resulting in males in decreased absolute adrenal, brain, kidney, spleen, and testes weights, in decreased weights of cauda epididymis and epididymides, as well as in increased relative weights of brain, epididymides and the seminal vesicle. In females of the high-dose group, the absolute weights of ovaries and the pituitary gland were decreased and the relative weight of the brain was increased due to the decreased terminal body weight.

The mean liver weights were dose-relatedly increased in males (relative) and females (absolute and relative) at 75 and 200 mg/kg bw/d. The mean relative liver weight in males of the mid-dose group was within the range of historical control data, whereas it was slightly above the historical control range at the high-dose level. In females, the mean relative liver weights in both treatment groups were above the historical control range. The increased liver weights were considered to be treatment-related.

The increased relative weight of the epididymides in males administered 75 mg/kg bw/d was related to the slightly but not significantly decreased terminal body weight in this test group (-3%).

Because there was no dose-response relationship, the increased relative weight of the seminal vesicle in males of the low-dose group was regarded to be incidental.

F_1 generation

The terminal body weight was significantly decreased in male and female animals at 200 mg/kg bw/d, resulting in males in decreased absolute adrenal, brain, and testes weights, in decreased weights of cauda epididymis and epididymides, as well as in increased relative weights of brain, and the kidneys. In females administered 200 mg/kg bw/d, the absolute weights of the adrenal glands, brain, kidneys, and the pituitary gland were decreased and the relative weight of the brain was increased due to the decreased terminal body weight. The mean relative liver weights were dose-relatedly increased in males and females at and above 75 mg/kg bw/d. The mean relative liver weights in males and females of these test groups were above the historical control range and were considered to be treatment-related.

The increased relative weights of the brain in females of the low- and mid-dose groups were related to the slightly but not significantly decreased terminal body weight (each -4%) in these test groups.

The following weight changes were regarded to be incidental:

- the decreased absolute and relative weights of the cauda epididymis and of the testes at 25 mg/kg bw/d (no dose-response relationship)
- the decreased absolute weight of the epididymides at 25 mg/kg bw/d (no dose-response relationship),
- increased absolute and relative weights of the pituitary gland in males at 25 mg/kg bw/d and increased relative pituitary gland weights in males at 200 mg/kg bw/d (no dose-response relationship).
- increased absolute and relative kidney weights in males given 75 mg/kg bw/d (absolute kidney weight did not show a dose-response relationship).

Table 5.6.1-15: Terminal body weight and organ weights - males

Generation		Dose [mg/kg]	F_0 Males Absolute weight	Δ%&	F_0 Males Relative weight [% of bw]	Δ%&	F_1 Males Absolute weight [mg]	Δ%&	F_1 Males Relative weight [% of bw]	Δ%&
Terminal weight	[g]	0	397.676		0.016		383.764			
		25	384.532	(-3)	0.016		384.600	(±0)		
		75	385.384	(-3)	0.016		386.144	(+1)		
		200	**354.112****	(-11)	0.016		**339.328****	**(-12)**		
Adrenal gland	[mg]	0	63.28		0.016		66.88		0.017	
		25	60.68	(-4)	0.016	(-1)	63.60	(-5)	0.017	(-5)
		75	60.12	(-5)	0.016	(-2)	61.88	(-3)	0.016	(-8)
		200	**55.60****	**(-12)**	0.016	(-1)	**58.24****	**(-13)**	0.017	(-1)
Brain	[g]	0	2.0555		0.519		2.106		0.552	
		25	2.053	(±0)	0.536	(+3)	2.131	(+1)	0.557	(+1)
		75	2.064	(±0)	0.538	(+4)	2.109	(±0)	0.548	(-2)
		200	**1.998***	**(-3)**	**0.568****	**(+9)**	**2.018****	**(-4)**	**0.598****	**(+8)**
Cauda epididymis	[g]	0	0.428		0.108		0.436		0.114	
		25	0.424	(-1)	0.111	(+2)	**0.408***	**(-6)**	**0.107***	**(-6)**
		75	0.435	(+2)	0.113	(+4)	0.431	(-1)	0.112	(-2)
		200	**0.397***	**(-7)**	0.112	(+4)	**0.397****	**(-9)**	0.118	(+3)
Epididymides	[g]	0	1.186		0.299		1.193		0.312	
		25	1.158	(-2)	0.303	(+1)	**1.136***	**(-5)**	0.297	(-5)
		75	1.200	(+1)	**0.312***	**(+4)**	1.187	(±0)	0.308	(-1)
		200	**1.115****	**(-6)**	**0.316***	**(+6)**	**1.102****	**(-8)**	0.326	(+4)
Kidneys	[g]	0	2.501		0.630		2.415		0.631	
		25	2.445	(-2)	0.636	(+1)	2.514	(+4)	0.654	(+4)
		75	2.491	(±0)	0.647	(+3)	**2.556****	**(+6)**	**0.663***	**(+5)**
		200	**2.329****	**(-7)**	0.659	(+5)	2.323	(-4)	**0.686****	**(+9)**
Liver	[g]	0	9.170		2.303		9.178		2.387	
		25	9.012	(-2)	2.342	(+2)	9.478	(+3)	2.460	(+3)
		75	9.324	(+2)	**2.417****	**(+5)**	9.737	(+6)	**2.521****	**(+6)**
		200	9.094	(-1)	**2.567****	**(+11)**	9.132	(-1)	**2.689****	**(+13)**
Pituitary gland	[mg]	0	11.24		0.003		9.16		0.002	
		25	11.88	(+6)	0.003	(+9)	**10.36***	**(+13)**	**0.003***	**(+14)**
		75	11.76	(+5)	0.003	(+7)	10.04	(+10)	0.002	(+9)
		200	10.96	(-2)	0.003	(+9)	9.16	(±0)	**0.003***	**(+14)**
Prostate	[g]	0	1.094		0.275		1.018		0.267	
		25	1.070	(-2)	0.280	(+2)	1.038	(+2)	0.271	(+2)
		75	0.980	(-10)	0.255	(-7)	1.017	(±0)	0.264	(-1)
		200	0.991	(-9)	0.279	(+1)	0.941	(-8)	0.278	(+4)
Seminal vesicle	[g]	0	1.314		0.331		1.299		0.340	
		25	1.395	(+6)	**0.364***	**(+10)**	1.350	(+4)	0.353	(+4)
		75	1.368	(+4)	0.355	(+7)	1.346	(+4)	0.350	(+3)
		200	1.321	(+1)	**0.371****	**(+12)**	1.242	(-4)	0.367	(+8)
Spleen	[g]	0	0.573		0.144		0.619		0.161	
		25	0.551	(-4)	0.144	(±0)	0.604	(-2)	0.157	(-2)
		75	0.565	(-1)	0.147	(+2)	0.612	(-1)	0.159	(-1)
		200	**0.508****	**(-11)**	0.144	(±0)	0.559	(-10)	0.164	(+2)
Testes	[g]	0	3.791		0.955		3.932		1.029	
		25	3.724	(-2)	0.972	(+2)	**3.712***	**(-6)**	**0.970***	**(-6)**
		75	3.836	(+1)	0.999	(+5)	3.951	(±0)	1.025	(±0)
		200	**3.589****	**(-5)**	1.019	(+7)	**3.651****	**(-7)**	1.080	(+5)
Thyroid glands	[mg]	0	25.60		0.006		25.48		0.007	
		25	25.08	(-2)	0.007	(+2)	26.12	(+3)	0.007	(+2)
		75	26.12	(+2)	0.007	(+5)	24.44	(-4)	0.006	(-5)
		200	24.04	(-6)	0.007	(+6)	23.92	(-6)	0.007	(+7)

Statistical analysis: * $p \leq 0.05$, ** $p \leq 0.01$ [Kruskal-Wallis and Wilcoxon-test (two-sided)]

& Values may not calculate exactly due to rounding of figures. The values given are based on the unrounded means

Table 5.6.1-16: Terminal body weight and organ weights - females

Generation		Dose [mg/kg]	F_0 Females Absolute weight	Δ%&	Relative weight [% of bw]	Δ%&	F_1 Females Absolute weight [mg]	Δ%&	Relative weight [% of bw]	Δ%&
Terminal weight	[g]	0	277.456				228.616			
		25	224.664	(-1)			219.321	(-4)		
		75	220.546	(-3)			219.104	(-4)		
		200	**213.160****	**(-6)**			**202.472****	**(-11)**		
Adrenal gland	[mg]	0	82.640		0.036		81.240		0.036	
		25	78.800	(-5)	0.035	(-3)	76.625	(-6)	0.035	(-2)
		75	78.333	(-5)	0.036	(-1)	79.800	(-2)	0.037	(+3)
		200	77.800	(-6)	0.037	(+1)	**71.840****	**(-12)**	0.035	(±0)
Brain	[g]	0	1.918		0.845		1.906		0.840	
		25	1.928	(+1)	0.861	(+2)	**1.955***	**(+3)**	**0.894****	**(+7)**
		75	1.908	(-1)	0.877	(+4)	1.934	(+1)	**0.886***	**(+6)**
		200	1.887	(-2)	**0.888****	**(+5)**	**1.850****	**(-3)**	**0.917****	**(+9)**
Kidneys	[g]	0	1.808		0.795		1.812		0.794	
		25	1.785	(-1)	0.796	(±0)	1.768	(-2)	0.806	(+2)
		75	1.790	(-1)	0.822	(+3)	1.800	(-1)	0.822	(+3)
		200	1.750	(-3)	0.823	(+4)	**1.618****	**(-11)**	0.798	(+1)
Liver	[g]	0	7.285		3.198		7.596		3.317	
		25	7.183	(-1)	3.197	(±0)	7.253	(-5)	3.303	(±0)
		75	**7.843***	**(+8)**	**3.595****	**(+12)**	7.686	(+1)	**3.509***	**(+6)**
		200	**8.033***	**(+10)**	**3.772****	**(+18)**	7.393	(-3)	**3.633***	**(+10)**
Ovaries	[mg]	0	104.480		0.046		105.560		0.047	
		25	99.920	(-4)	0.045	(-3)	103.792	(-2)	0.047	(+2)
		75	107.083	(+2)	0.049	(+7)	107.840	(+2)	0.049	(+6)
		200	**94.960****	**(-9)**	0.045	(-3)	100.480	(-5)	0.050	(+5)
Pituitary gland	[mg]	0	12.880		0.006		11.360		0.005	
		25	12.480	(-3)	0.006	(-2)	11.333	(±0)	0.005	(+5)
		75	12.417	(-4)	0.006	(+1)	11.440	(+1)	0.005	(+5)
		200	**11.040****	**(-14)**	0.005	(-9)	**9.840***	**(-13)**	0.005	(-2)
Spleen	[g]	0	0.502		0.220		0.493		0.215	
		25	0.492	(-2)	0.220	(±0)	0.472	(-4)	0.215	(±0)
		75	0.495	(-1)	0.227	(+3)	0.499	(+1)	0.228	(+6)
		200	0.497	(-1)	0.233	(+6)	0.462	(-6)	0.228	(+6)
Thyroid glands	[mg]	0	17.80		0.008		19.16		0.008	
		25	19.48	(+9)	0.009	(+11)	19.25	(±0)	0.009	(+5)
		75	18.25	(+3)	0.008	(+7)	19.52	(+2)	0.009	(+7)
		200	18.32	(+3)	0.009	(+10)	18.64	(-3)	0.009	(+10)
Uterus	[g]	0	0.685		0.301		0.622		0.273	
		25	0.665	(-3)	0.299	(-1)	0.538	(-14)	0.246	(-10)
		75	0.608	(-11)	0.279	(-7)	0.658	(+6)	0.302	(+10)
		200	0.681	(-1)	0.317	(+6)	0.565	(-9)	0.281	(+3)

Statistical analysis: * $p \leq 0.05$, ** $p \leq 0.01$ [Kruskal-Wallis and Wilcoxon-test (two-sided)]

& Values may not calculate exactly due to rounding of figures. The values given are based on the unrounded means

2. Gross pathology

F_0 generation
All findings occurred individually. They were considered to be incidental or spontaneous in origin and without any relation to treatment. All females examined were pregnant.

One female of the mid-dose group (No. 174) was sacrificed in a moribund condition because of a missing vaginal orifice, resulting in severe uterus dilation.

F_1 generation
All findings occurred individually. They were considered to be incidental or spontaneous in origin and without any relation to treatment. The female animals which were not pregnant (Nos. 383 and 393), as well as the male mating partners (Nos. 283 and 293) did not show relevant gross lesions.

3. Histopathology

F_0 generation
In the liver, a minimal centrilobular hypertrophy was observed in 15 (out of 25) F_0 males of the high-dose group that was considered to be treatment-related. All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental and without any relation to treatment.

F_1 generation
In the liver, a minimal centrilobular hypertrophy was observed in 15 (out of 25) F_1 males of the high-dose group that was considered to be treatment-related. All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental and without any relation to treatment.

4. Differential ovarian follicle count (DOFC)

The results of the differential ovarian follicle count (DOFC) – comprising the numbers of primordial and growing follicles, as well as the combined incidence of primordial plus growing follicles – did not reveal biologically or statistically significant differences between controls and animals of the high-dose group (see Table 5.6.1-17).

Table 5.6.1-17: Differential ovarian follicle count (DOFC)

Number of animals	Dose [mg/kg bw/d]	Absolute values			Relative values		
		Primordial	Growing	Primordial + growing	Primordial	Growing	Primordial + growing
25	0	7641	500	8141	305.64	20.00	325.64
25	200	7422	458	7880	296.88	18.32	315.20

Statistical analysis: * $p \leq 0.05$, ** $p \leq 0.01$, Wilcoxon-test (1-sided)

III. DISCUSSION

BAS 750 F was administered to groups of 25 male and 25 female healthy young Wistar rats (F_0 parental generation) as a homogeneous addition to the food in different concentrations, which were adjusted to obtain target dose levels of 0, 25, 75 and 200 mg/kg bw/d. For all test groups the intake of BAS 750 F correlated well with the desired target doses.

The high-dose of the test substance (200 mg/kg bw/d) produced clinical signs of adverse systemic effects in the F_0 and F_1 male, as well as in the F_0 and F_1 female Wistar rats.

In both sexes and generations **food consumption and body weights** were consistently reduced throughout the study, the difference to the control was statistically significant during major parts of all study sections. Particularly in the F_0 and F_1 females, most significant decreases of body weight were noted towards the end of gestation and during the first days of lactation.

Regarding **clinical pathology** in male and female F_0 and F_1 rats of the mid- and high-dose group alkaline phosphatase (ALP) activities were slightly increased in the plasma. Substrates synthesized in the liver like cholesterol (in F_0 males) or triglycerides (in F_1 females) as well as urea as indicator of an increased protein metabolism (in F_1 males of the high-dose group) were increased. This was most probably due to a dysregulation of the liver cell metabolism.

Regarding pathology, target organ was the liver. In the parental animals of both the F_0 and F_1 generation, the mean relative liver weights were dose-relatedly increased in males and females (additionally in F_0 absolute liver weight increases) at 75 and 200 mg/kg bw/d. At the high-dose level in F_0 and F_1 males, the increased liver weight correlated with a minimal centrilobular hypertrophy that was observed in 15 (out of 25) animals. These findings were considered to be treatment-related and in combination with clinical chemistry regarded to be adverse.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental and without any relation to treatment.

There were no indications from clinical examinations as well as gross and histopathology, that BAS 750 F adversely affected the **fertility or reproductive performance** of the F_0 parental animals up to and including the administered high-dose of 200 mg/kg bw/d. Estrous cycle data, sperm quality of males, mating behavior, conception, gestation, parturition, lactation and weaning as well as sexual organ weights and gross and histopathological findings of these organs were largely comparable between the rats of control and the treatment groups or ranged within the historical control data of the test facility.

Mating of the F_1 parental animals resulted in statistically significant fewer implantation sites in the high-dose group (200 mg/kg bw/d), and correspondingly fewer pups delivered per dam (total and liveborn). However, these values were within the historical control range of the test facility, thus reflecting the normal range of biological variation inherent in the strain of rats used for this study. It should be also noted that no morphologic correlate was observed which gave evidence for any effect of the test compound in the ovaries (in particular the number and appearance of primordial and growing follicles), oviducts or uteri. Also, morphology of the male reproductive organs and sperm quality were unaffected. Thus a relationship to the treatment is not assumed.

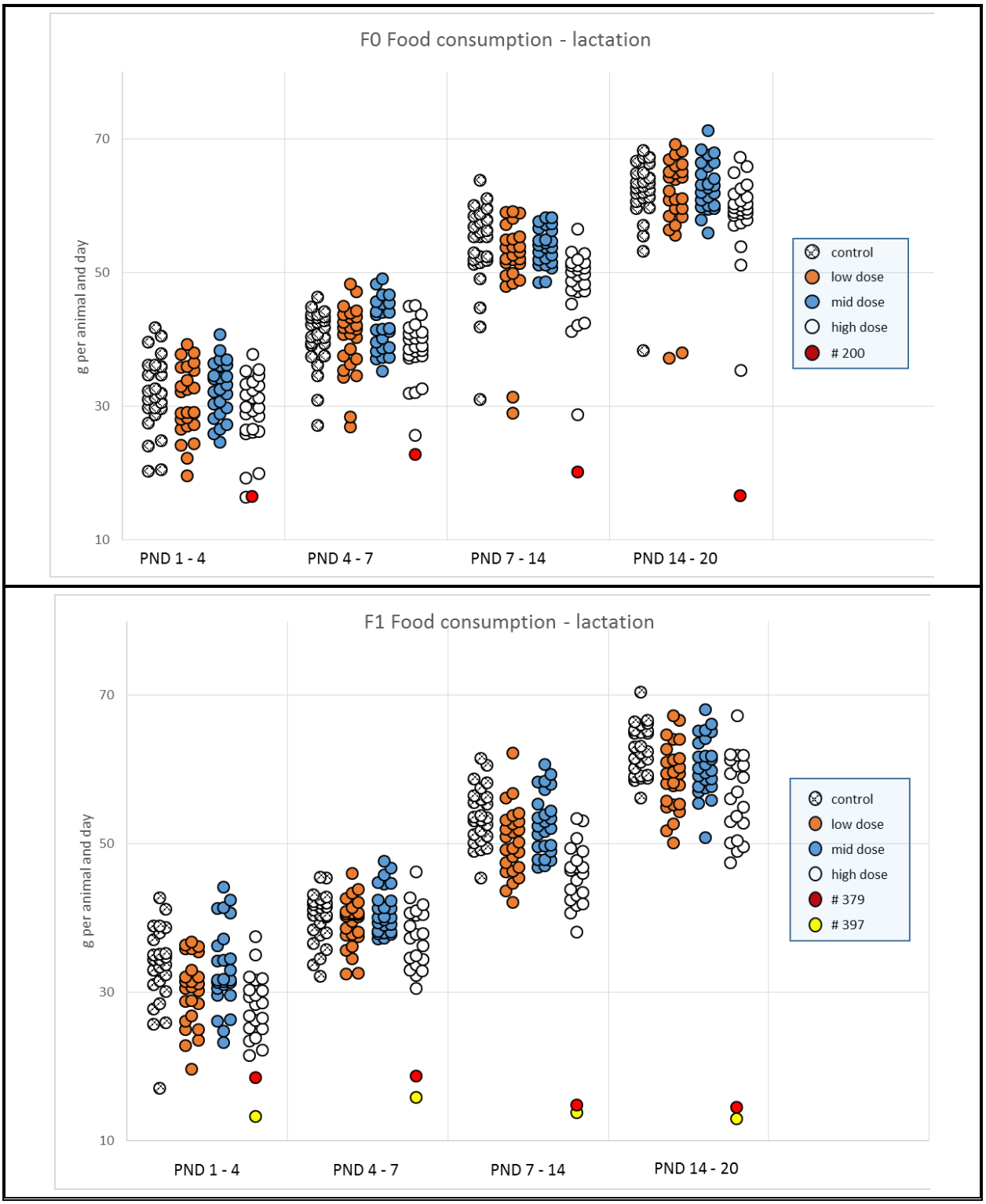
There was one high-dose F_1 female (No. 379) which had a completely stillborn litter. This high-dose female showed distinct signs of impaired well-being such as piloerection and a distinctly poor general state before its litter was born on GD 23/24. Litters with only stillborn pups occur at rare intervals also in control animals. In the last two years, 3 such completely stillborn control litters were noted at the test facility. Neither in the entire F_0 generation nor in the other dams of the F_1 generation high-dose group findings indicative of a general delay of parturition or any parturition complications were observed, nor were there any indications of prenatal developmental toxicity in late gestation. Taking all this together, an association of this finding to the treatment seems rather unlikely; if not incidental, it might have been the result of the impaired well-being of this rat before parturition but not an independent impact of the test compound on reproductive performance.

For all liveborn male and female pups of the F_0 and F_1 parents, no test substance-induced signs of **developmental toxicity** were noted at dose levels as high as 75 mg/kg bw/d. Postnatal survival, pup body weight gain as well as post-weaning development of the offspring of this test group until puberty remained unaffected by the test substance. Furthermore, clinical and/or gross necropsy examinations of the F_1 and F_2 pups revealed no adverse findings.

The F_1 and F_2 offspring in the high-dose group (200 mg/kg bw/d) had reduced body weights and/or also gained less weight than the control offspring prior to weaning. Decreases in body weight and body weight gain which were evident in the high-dose pups directly after birth, became larger as the lactation phase progressed. Decreased spleen and thymus, as well as increased brain organ weights were also observed at this dose, but were considered secondary to the changes in body weight parameters, rather than independent findings.

One high-dose F_0 female and one high-dose F_1 female had a complete litter loss during early lactation. In the deceased litters, the described body weight effects were also accompanied either by clinical signs of pups not properly nursed, reduced general nutritional condition in the pups, or by no stomach contents at autopsy, suggesting poor nutrition is the primary cause of the decreased body and organ weight parameters in general. These effects may have led to the death/cannibalization of those two high-dose litters, which adversely affected postnatal survival at the high dose (200 mg/kg bw/d) in general. This is, in turn, probably secondary to the decreased food consumption by the parental females during the last part of gestation and the early part of lactation. The quantity and quality of mammary secretions is highly dependent on maternal nutrition, and so is pup growth during the first few weeks of life. Since Figure 5.6.1-4 provides proof that the most severely affected dams neglected their offspring completely it seems safe to assume that this lack of maternal care would also have happened in the rest of the high-dose group, although at a lesser extent. Therefore, it is highly likely that insufficient maternal care at least contributed to (if not caused) the general delay in offspring development.

Figure 5.6.1-4: Individual food consumption of F_0 and F_1 dams during lactation



Individual food consumption of F_0 and F_1 dams during lactation. In order to assess whether the dams which had litter losses (either stillborn or dead within 3 days after birth) were affected most severely by maternal toxicity, the individual food consumption of all dams was plotted against the time course of lactation. Animals affected by litter loss (F_0 dam No. 200, and F_1 dams No. 379 and No. 397) were specifically color-coded.

Postnatal survival after PND 4 of the offspring of all test groups until weaning remained unaffected by the test substance. Furthermore, clinical and/or gross necropsy examinations of the weaned F_1 pups revealed no adverse findings. In particular, **anogenital distance/index** and **presence of nipples/areolas**, both sensitive markers of potential endocrine-mediated imbalances, revealed no test substance-related effects.

A statistically significant delay in **vaginal opening** of more than one day beyond the concurrent control was observed in the female F_1 offspring of the high-dose group (200 mg/kg bw/d). However, the time-point of vaginal opening in the concurrent control group was found to be at the lower end of the historical control range, while time of vaginal opening in all treated groups was well covered by the historical controls. Thus, any apparent delay is not considered a result of a specific mechanism of toxicity.

No effect was observed in the sexual maturation of the male offspring. No delay in **preputial separation** was noted in the F_1 animals of the high-dose group (200 mg/kg bw/d), despite a slightly slowed general development. However, the small degree of general developmental delay (high-dose body weight 8% below the control weight at the time) was probably insufficient to exercise a pivotal influence on timing of puberty. It should also be noted that the F_1 males of all groups including the control had a rather late commencement of puberty if compared to the historical control data of the test facility. As this is consistent for all groups, no influence on the above-made judgement is assumed.

IV. CONCLUSION

Thus, under the conditions of the present 2-generation reproduction toxicity study the **NOAEL for general, systemic toxicity** is 25 mg/kg bw/d for the F_0 and F_1 parental rats, based on decreased food consumption and body weight/body weight gain observed at 200 mg/kg bw/d, as well as clinical-chemical and pathological evidence of liver toxicity at 75 and 200 mg/kg bw/d, in all F_0 and F_1 parental animals. At a dose of 200 mg/kg bw/d also borderline effects related to impaired maternal care were noted in 3 individual litters.

The **NOAEL for fertility and reproductive performance** for the parental rats is 200 mg/kg bw/d, the highest tested dose.

The **NOAEL for developmental toxicity** in the F_1 and F_2 progeny is 75 mg/ kg bw/d, due to pup mortality and the decrease in the pre-weaning pup body weights/pup weight gains observed at the 200 mg/ kg bw/d dose. These effects are considered secondary to the decreased food consumption by the parental females during the last part of gestation and the early part of lactation.

Developmental toxicity did not occur in the absence of parental toxicity. Therefore, no signs of selective developmental toxicity were noted in this 2-generation reproduction toxicity study.

CA 5.6.2. Developmental toxicity studies

Report: CA 5.6.2/1
[redacted] 2015 a
BAS 750 F - Prenatal developmental toxicity study in Wistar rats - Oral administration (gavage)
2014/1170755

Guidelines: (EC) No 440/2008 of 30 May 2008 - Part B No. L 142, OECD 414, EPA 870.3700

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch L84-176, Purity 97.7%) in 1% aqueous Carboxymethylcellulose suspension was administered once daily by stomach tube to groups of 25 time-mated Crl:WI (Han) Wistar rats during gestation days 6-19 at dose levels of 0, 50, 150 and 400 mg/kg bw/d.

The high dose of 400 mg/kg bw/d elicited clear signs of maternal toxicity during treatment as indicated by reductions in feed intake (8% below control), reduced body weight gain (17% below control), net corrected body weight gain (34% below control) and net body (carcass) weight (7% below control). In addition, mean placental weights were slightly increased at this dose level (13% above control).

No treatment-related effects on cesarean section parameters were observed at any dose. No treatment-related external, visceral or skeletal malformations were noted. BAS 750 F was not teratogenic and did not induce fetal toxicity up to the highest dose tested.

Thus, based on the results of this study the developmental NOAEL was at 400 mg/kg bw/d. Based on the adverse effects on food intake and body weight gain, the maternal NOAEL was considered to be 150 mg/kg bw/d.

(DocID 2014/1170755)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	L84-176
Purity / content:	97.7%
Stability of test compound:	The stability was guaranteed for the duration of the study.
2. Vehicle:	1% Carboxymethylcellulose suspension in drinking water (1% CMC)
3. Test animals:	
Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Sex:	female
Age (GD 6):	ca. 11-13 weeks
Weight at dosing (GD 6):	198.8 ± 12.43 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimation period:	from GD 0 (day of supply) until day GD 6 (day of first administration)
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cage type III, floor area ca. 800 cm^2; dust-free wooden bedding; wooden gnawing blocks as enrichment ((Typ NGM E-022), Abedd®, Lab. and Vet. Service GmbH, Vienna, Austria)
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 18-Apr-2012 to 10-Mar-2015 [in life phase: 24-Apr-2012 (treatment) to 09-May-2012 (sacrifice on GD20 of last cohort)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 25 "time-mated", presumably pregnant rats by gavage at dose levels of 0, 50 (low dose), 150 (mid dose) and 400 mg/kg bw/day (high dose) during days 6 to 19 of gestation. The animals were paired by the breeder and supplied on Day 0 post coitum (= detection of vaginal plug / sperm). The animals were assigned at random to the treatment groups. The test substance was administered as a suspension in the vehicle 1% aqueous CMC at a dose volume of 10 ml/kg bw. A control group was dosed with the vehicle in parallel to the test groups. At terminal sacrifice on GD 20, 24 to 25 females per group had implantation sites.

3. Test substance preparation and analysis

Prior to study initiation the aqueous test substance preparations under refrigerator conditions were shown to be stable over a 7-day period. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration and homogeneity analyses were performed at the beginning of the study. The homogeneity of the dose suspensions was verified by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (50 and 400 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Table 5.6.2-1: Analysis of preparations for homogeneity and test-item content

Vehicle	Date of sampling	Dose level [mg/kg bw/d]	Nominal concentration [g/100 mL]	Sample [#]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± RSD
1% CMC	24.04.2012 [Homogeneity and concentration control analyses]	50	0.5	3	0.505	101.1	102.9 ± 2.3
				4	0.510	102.0	
				5	0.528	105.6	
		150	1.5	6	1.614	107.6	
		400	4.0	7	3.938	98.4	97.9 ± 1.4
				8	3.850	96.3	
				9	3.956	98.9	

Relative standard deviations of maximum 2.3% indicated the homogenous distribution of BAS 750 F in the dosing suspensions. The actual nominal test-item concentrations were in the range of 96.3 to 105.6% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption[a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

[a)] For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed

C. METHODS

1. Observations

The animals were examined for mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on gestation days (GD) 0, 1, 3, 6, 8, 10, 13, 15, 17, 19, and 20. The body weight change of the animals was calculated from these results. In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on Day 20 p.c. minus weight of the unopened uterus minus body weight on Day 6 p.c.).

Food consumption was recorded for GD 0-1, 1-3, 3-6, 6-8, 8-10, 10-13, 13-15, 15-17, 17-19 and 19-20.

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on GD 20 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Sacrifice

On GD 20, the dams were sacrificed in randomized order by cervical dislocation (after isoflurane anesthesia) and the fetuses were removed from the uterus. Dams were subsequently assessed by gross pathology in randomized order to minimize bias.

The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Number of corpora lutea
- Number, position and distribution of implantation sites in the uterus classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to Salewski in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue)
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]:} \quad \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]:} \quad \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]:} \quad \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

4. Examination of fetuses:

At necropsy each fetus was weighed, sexed, and external tissues and all orifices were examined macroscopically. The sex was determined by observing the distance between the anus and the base of the genital tubercle and was later confirmed in all those fetuses fixed in Harrison's fluid by internal examination. If there were discrepancies between the "external" and the "internal" sex of a fetus, the fetus was finally sexed according to the appearance of its gonads.

Furthermore, the viability of the fetuses and the condition of the placentae, the umbilical cords, the fetal membranes, and fluids were examined. Individual placental weights were recorded. Subsequently the fetuses were sacrificed by s.c. injection of pentobarbital.

After these examinations, approximately one half of the fetuses per dam were eviscerated, skinned and placed in ethyl alcohol, the other half was placed in Harrison's fluid for fixation and further evaluation.

Soft tissue examination of the fetuses

The fetuses fixed in Harrison's fluid were examined for any visceral findings according to the method of Barrow and Taylor. After this examination these fetuses were discarded.

Skeletal examination of the fetuses

The skeletons of the fetuses fixed in ethyl alcohol were stained according to a modified method of Kimmel and Trammell. Thereafter, the skeletons of these fetuses were examined under a stereomicroscope. After this examination the stained fetal skeletons were retained individually.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) and its updated version of Makris et al. (2009) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (1999) and Solecki et al. (2001, 2003); for detailed references see study report:

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Please note: Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on day 20 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

- Control female No. 22 (not pregnant)
- Low dose (50 mg/kg) none
- Mid dose (150 mg/kg) female No. 70 (not pregnant)
- High dose (400 mg/kg) female No. 92 (not pregnant)

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

No clinical signs were observed throughout the study.

2. Clinical signs of toxicity

No mortality was observed.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [Table 5.6.2-2]

The mean food consumption of the dams treated with 400 mg/kg bw/d was substantially reduced from GD 8 onwards until scheduled sacrifice on GD 20, attaining statistical significance on GD 8-10 and from GD 17 until term (12-14% below control). The average reduction of food consumption in the high-dose dams during the entire treatment period (GD 6-19) was 8%, in comparison to the concurrent control group. The differences between the control rats and the high-dose group were considered an effect of the test substance. The mean food consumption of the dams from the low- and mid-dose group (50 and 150 mg/kg bw/d) was comparable to the control.

Table 5.6.2-2: Food consumption

Dose [mg/kg bw/d]	Day of Gestation 0–6	6–19	0–20	6–8	8–10	10–13	13–15	15–17	17–19	19–20
0 [g/animal]	16.4	21.5	20.1	18.4	20.4	20.8	21.9	23.4	24.1	22.4
50 [g/animal]	16.2	21.3	19.8	18.8	19.9	21.0	21.8	23.1	23.1	22.0
[% control]	99%	99%	99%	102%	98%	101%	100%	99%	96%	98%
150 [g/animal]	16.5	21.4	20.0	18.8	19.8	21.1	22.4	23.4	23.1	21.4
[% control]	101%	100%	100%	102%	97%	101%	102%	100%	96%	96%
400 [g/animal]	16.0	19.8	18.6	17.9	**17.9****	19.5	20.6	21.9	**21.0****	**19.3****
[% control]	98%	92%	93%	97%	**88%**	94%	94%	94%	**87%**	**86%**

* p < 0.05, ** p < 0.01 (Dunnett test, two-sided)

2. Body weight and body weight gain [**Table 5.6.2-3 and Table 5.6.2-4**]

Significant effects on body weight and body weight change were seen only at the high dose level of 400 mg/kg bw/d. Mean body weights were significantly decreased between GD 15–20.

Table 5.6.2-3: Body weight

Dose	Day of Gestation 0	6	8	10	13	15	18	19	20
0 mg/kg bw/d Mean bw [g]	163.9	198.2	206.9	216.5	233.3	243.8	261.7	282.6	294.9
50 mg/kg bw/d Mean bw [g]	161.0	196.3	203.8	213.1	228.9	238.0	255.0	274.3	285.7
[% control]	98%	99%	99%	98%	98%	98%	97%	97%	97%
150 mg/kg bw/d Mean bw [g]	167.8	202.7	211.2	220.5	234.9	246.6	264.0	283.7	297.0
[% control]	102%	102%	102%	102%	101%	101%	101%	100%	101%
400 mg/kg bw/d Mean bw [g]	164.1	197.5	204.5	212.0	224.3	**232.1***	**248.0***	**266.8***	**278.6***
[% control]	100%	100%	99%	98%	96%	**95%**	**95%**	**94%**	**94%**

* p < 0.05, ** p < 0.01 (Dunnett test, two-sided)

The mean body weight gain of the high-dose rats (400 mg/kg bw/d) was statistically significantly reduced during GD 10-15 (up to 27% below the concurrent control value), which was likely to be a subsequent effect of reduced feed consumption. If calculated for the entire treatment phase (GD 6-19), mean body weight gain was about 17% below controls. If calculated for the whole study period (GD 0-20), mean body weight gain was about 13% below the concurrent control value. This effect was considered test substance-related. The mean body weights and the average body weight gains of the low- and mid-dose rats (50 and 150 mg/kg bw/d) were in general comparable to the controls.

Table 5.6.2-4: Body weight gain

Dose [mg/kg bw/d]	Day of Gestation 0–6	6–19	0–20	6–8	8–10	10–13	13–15	15–17	17–19	19–20
0 [g/animal]	34.9	83.8	131.0	8.2	9.6	16.8	10.4	17.9	20.9	12.3
50 [g/animal]	35.3	77.9	124.7	7.5	9.3	15.8	9.1	16.9	19.3	11.4
[% control]	101%	93%	95%	91%	97%	94%	88%	94%	92%	93%
150 [g/animal]	34.8	81.1	129.2	8.5	9.3	14.4	10.7	18.5	19.7	13.3
[% control]	100%	97%	99%	104%	97%	86%	103%	103%	94%	108%
400 [g/animal]	33.4	69.3**	114.5**	7.0	7.5	**12.3****	**7.8***	15.9	18.8	11.7
[% control]	96%	**83%**	**87%**	85%	78%	**73%**	**75%**	89%	90%	95%

* p < 0.05, ** p < 0.01 (Dunnett test, two-sided)

D. NECROPSY OBSERVATIONS

1. Uterus weight, carcass weight, corrected body weight gain [see Table 5.6.2-5]

The mean weights of the unopened uteri were comparable between groups. The corrected body weight gain (terminal body weight on GD 20 minus weight of the unopened uterus minus body weight on GD 6) was distinctly and statistically significantly lower at 400 mg/kg bw/d (about 34% below the concurrent control value). Furthermore, the carcass weight of the high-dose dams was also statistically significantly lower in comparison to the control group (about 7% below controls).These effects are assessed as test-substance-related signs of maternal toxicity. The corrected body weight gain at dose levels of 50 and 150 mg/kg bw/d revealed no difference of any biological relevance to the corresponding control group. Moreover, mean carcass weights also remained unaffected by the treatment.

Table 5.6.2-5: Uterus weight, carcass weight and corrected (net) body weight gain

Parameter (g)	BAS 750 F Dose level (mg/kg bw/d) 0	50	150	400
Gravid uterus	51.7 ± 10.0	49.3 ± 14.9	54.3 ± 9.3	51.8 ± 12.2
[% control]		95%	105%	100%
Carcass	243.2 ± 15.0	236.4 ± 17.2	242.7 ± 20.1	**226.8** ± 10.5**
[% control]		97%	100%	**93%**
Net weight change from GD 6	44.4 ± 5.6	40.1 ± 9.3	40.1 ± 9.5	**29.3** ± 5.6**
[% control]		90%	90%	**66%**

* p < 0.05, ** p < 0.01 (Dunnett test, two-sided)
Carcass weight = terminal body weight minus uterine weight
Net weight change from GD 6 = carcass weight minus GD 6 body weight

2. Gross necropsy observations

Dilated renal pelvis was diagnosed in two control group dams, and in one mid-dose and four high-dose group animals. One low-dose dam was found with diaphragmatic hernia. These findings noted during Caesarean section were not considered to be treatment-related.

E. CAESAREAN SECTION DATA [Table 5.6.2-6, Table 5.6.2-7]

Twenty-four, 25, 24 and 24 dams were pregnant at 0, 50, 150 and 400 mg/kg bw/d. None of the pregnant dams aborted or gave premature birth. With conception rates of 96-100%, a sufficient number of pregnant females were available according to the test guidelines for the purpose of the study.

Table 5.6.2-6: Pregnancy status

BAS 750 F (mg/kg bw/d)		0	50	150	400
Pregnancy status					
Females mated	N	25	25	25	25
Females pregnant	N	24	25	24	24
Conception rate	%	96	100	96	96
Aborted	N	0	0	0	0
Premature deaths	N	0	0	0	0
Dams with viable fetuses	N	24	24	24	24
Dams with all resorptions	N	0	1	0	0
Mortality	N	0	0	0	0

There were no test substance-related and/or biologically relevant differences between control and test groups in conception rate [Table 5.6.2-6], in the mean number of corpora lutea and implantation sites or in the values calculated for the pre- and the postimplantation losses, the number of resorptions and viable fetuses [see Table 5.6.2-7]. All observed differences were considered to reflect the normal range of fluctuations for animals of this strain and age (see also Table 5.6.2-8 for historical control data).

Table 5.6.2-7: Caesarean section data

BAS 750 F (mg/kg bw/d)		0	50	150	400
Corpora lutea	[N]	10.2 ± 1.50	10.8 ± 2.42	11.1 ± 1.48	11.3 ± 1.52
total number	[N]	245	270	267	272
Implantation sites	[N]	9.7 ± 2.01	9.3 ± 2.65	10.4 ± 1.88	9.9 ± 2.10
total number	[%]	232	232	249	238
Pre-implantation loss	[%]	6.0 ± 11.3	14.7 ± 19.5	7.1 ± 8.7	12.1 ± 17.8
Post-implantation loss	[%]	3.7 ± 6.2	8.2 ± 20.5	7.2 ± 8.0	6.8 ± 9.1
Resorptions	[N]	0.4 ± 0.65	0.4 ± 0.71	0.8 ± 0.88	0.7 ± 0.92
total number	[N]	9	11	19	16
Early resorptions	[N]	0.4 ± 0.65	0.4 ± 0.71	0.8 ± 0.90	0.5 ± 0.59
total number	[N]	9	11	18	11
Late resorptions	[N]	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.20	0.2 ± 0.59
total number	[N]	0	0	1	5
Dead fetuses	[N]	0	0	0	0
Live fetuses	[N]	9.3 ± 1.99	9.2 ± 2.06	9.6 ± 1.74	9.3 ± 2.29
total number	[N]	223	221	230	222
males	[N]	112	109	125	108
females	[%]	111	112	105	114
male / female ratio		50.2 / 49.8	49.3 / 50.7	54.3 / 45.7	48.6 / 51.4
Placental weight	[g]	0.44 ± 0.06	0.44 ± 0.05	0.46 ± 0.05	**0.50** ± **0.07**
males	[g]	0.46 ± 0.07	0.46 ± 0.06	0.47 ± 0.05	**0.51*** ± **0.08**
females	[g]	0.43 ± 0.06	0.42 ± 0.05	0.45 ± 0.06	**0.49** ± **0.06**
Fetal weight	[g]	3.6 ± 0.35	3.7 ± 0.13	3.7 ± 0.22	3.5 ± 0.21
males	[g]	3.7 ± 0.36	3.8 ± 0.17	3.8 ± 0.23	3.6 ± 0.22
females	[g]	3.6 ± 0.39	3.5 ± 0.20	3.6 ± 0.22	**3.4*** ± **0.24**

* $p < 0.05$, ** $p < 0.01$ (Dunnett test, two-sided)

The sex distribution of the fetuses in test groups was comparable to the control fetuses. Any observable differences were without biological relevance.

Mean placenta weights were slightly but statistically significantly increased at 400 mg/kg bw/d (approx. 113% of the control group value). Although well within the historical control range, this change was considered to be potentially treatment-related, but as non-adverse, since it was not associated with impaired fetal development.

The mean fetal weights in all test groups (50, 150 and 400 mg/kg bw/d) were not influenced by the test substance and did not show any biologically relevant differences in comparison to the control group. Female fetal weights from the high-dose group were marginally decreased (3.4* vs. 3.6 g, 94% of control value), However, as the group mean exactly matches the mean of the historical control (3.4 g) and as there is no effect in the corresponding male fetuses, a relationship with the treatment is not assumed.

Table 5.6.2-8: Historical control data

Historical control data	Mean	±	SD	Range (per study) Minimum	Range (per study) Maximum	95% spread 2.5% – 97.5%
Corpora lutea	11.8	±	1.96	10.0	16.0	n.d.
Implantation sites	11.0	±	2.26	9.1	15.3	n.d.
Pre-implantation loss [mean %]	6.9	±	13.09	1.4	17.5	n.d.
Post-implantation loss [mean %]	7.3	±	11.19	3.5	18.1	n.d.
Resorptions [N]	0.8	±	1.00	0.3	1.5	n.d.
Live litter size [N]	10.2	±	2.34	8.3	14.8	n.d.
Placenta weights [g]	0.46			0.33	1.16	0.32 – 0.60
Males	0.47			0.30	1.16	0.33 – 0.61
Females	0.45			0.28	0.99	0.32 – 0.58
Fetal weights [g]	3.5			2.3	5.1	2.2 – 4.1
Males	3.6			2.4	5.4	3.0 – 4.2
Females	3.4			2.2	4.9	2.9 – 4.1

76 studies performed at the test facility between 2009–2014 with Wistar rats (Charles River)
(1518 pregnant dams; 1502 litters with 15402 viable fetuses)

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination [Table 5.6.2-9]

External malformations were recorded for one fetus each in the mid- and high dose groups (150 and 400 mg/kg bw/d). Female fetus No. 73-07 (150 mg/kg bw/d) showed a mandibular micrognathia associated with severely malformed skull bones found during skeletal examination. Furthermore, for female fetus No. 79-09 (400 mg/kg bw/d) multiple external malformations were recorded: cleft palate, microphthalmia (on right eye) and mandible malformed (pointed appearance). This finding was associated with multiple skeletal malformations found during skeletal examination. One fetus with limb hyperflexion (external variation) was found at the low dose of 50 mg/kg bw/d. Finally several fetuses of the control and high-dose group were observed with coagulated blood around the placenta and/or discolored amniotic fluid.

None of these findings were considered to be treatment-related.

Table 5.6.2-9: External malformations, variations and unclassified observations

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		223	221	230	222
Total external malformations					
Fetal incidence	# (%)			1 (0.4)	1 (0.5)
Litter incidence	# (%)			1 (4.2)	1 (4.2)
Affected fetuses / litter	%			0.5 ± 2.27	0.7 ± 3.40
Fetus with multiple external malformations					
Fetal incidence	# (%)				1 (0.5)
Litter incidence	# (%)				1 (4.2)
Affected fetuses / litter	%				0.7 ± 3.40
Mandibular micrognathia					
Fetal incidence	# (%)			1 (0.4)	
Litter incidence	# (%)			1 (4.2)	
Affected fetuses / litter	%			0.5 ± 2.27	
Total external variations					
Fetal incidence	# (%)		1 (0.4)		
Litter incidence	# (%)		1 (4.2)		
Affected fetuses / litter	%		0.5 ± 2.27		
Limb hyperextension					
Fetal incidence	# (%)		1 (0.4)		
Litter incidence	# (%)		1 (4.2)		
Affected fetuses / litter	%		0.5 ± 2.27		
Total fetal external unclassified observations					
Fetal incidence	# (%)	3 (1.3)			4 (1.8)
Litter incidence	# (%)	1 (4.2)			1 (4.2)
Affected fetuses / litter	%	0.4 ± 2.04			1.7 ± 8.16
Blood coagulam around placenta					
Fetal incidence	# (%)	1 (0.4)			4 (1.4)
Litter incidence	# (%)	1 (4.2)			1 (4.2)
Affected fetuses / litter	%	0.4 ± 2.04			1.7 ± 8.16
Amniotic fluid discolored					
Fetal incidence	# (%)	2 (0.9)			3 (1.8)
Litter incidence	# (%)	1 (4.2)			1 (4.2)
Affected fetuses / litter	%	0.8 ± 4.08			1.3 ± 6.12

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

2. Visceral examination [Table 5.6.2-10]

No treatment-related visceral (soft tissue) malformations were noted in this study. The only observed malformation consisted of a small spleen in a control group fetus. Two soft tissue variations, i.e. dilated renal pelvis and dilated ureter, were detected in all test groups including the controls. The incidences of these frequently occurring variations were not statistically significantly increased in the treated groups and well within the historical control range (Table 5.6.2-11). Thus they were not considered biologically relevant. No unclassified visceral findings were observed.

Table 5.6.2-10: Total soft tissue malformations and variations

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		106	106	110	105
Live		106	106	110	105
Dead		0	0	0	0
Total soft tissue malformations					
Fetal incidence	# (%)	1 (0.9)			
Litter incidence	# (%)	1 (4.2)			
Affected fetuses / litter	%	0.8 ± 4.08			
Small spleen					
Fetal incidence	# (%)	1 (0.9)			
Litter incidence	# (%)	1 (4.2)			
Affected fetuses / litter	%	0.8 ± 4.08			
Total soft tissue variations					
Fetal incidence	# (%)	2 (1.9)	1 (0.9)	3 (2.7)	8 (7.6)
Litter incidence	# (%)	2 (8.3)	1 (4.2)	1 (13)	5 (21)
Affected fetuses / litter	%	2.2 ± 7.78	0.8 ± 4.08	2.5 ± 6.76	7.4 ± 16.17
Dilated renal pelvis					
Fetal incidence	# (%)	2 (1.9)	1 (0.9)	3 (2.7)	8 (7.6)
Litter incidence	# (%)	2 (8.3)	1 (4.2)	1 (13)	5 (21)
Affected fetuses / litter	%	2.2 ± 7.78	0.8 ± 4.08	2.5 ± 6.76	7.4 ± 16.17
Dilated ureter					
Fetal incidence	# (%)	1 (0.9)			1 (0.9)
Litter incidence	# (%)	1 (4.2)			1 (4.2)
Affected fetuses / litter	%	2.2 ± 7.78			2.2 ± 7.78

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

Table 5.6.2-11: Historical control data – fetal soft tissue variations

	Fetuses (7096)			Litters (1453)			Affected fetuses / litter	
	No.	%	Range	No.	%	Range	% Mean	Range
Dilated renal pelvis	173	2.4	0.0 – 11.8	159	10.9	0.0 – 57.1	2.5	0.0 – 11.9

76 studies performed at the test facility between 2009–2014 with Wistar rats (Charles River)
(1453 litters with 7096 viable fetuses examined)

3. Skeletal examination [Table 5.6.2-12, Table 5.6.2-13]

Skeletal malformations (see Table 5.6.2-12) were observed in two fetuses of the control group (misshapen cervical vertebra and severely malformed sternum), one fetus of the mid-dose group (severely malformed skull bones) and two fetuses of the high-dose group (one with malpositioned and bipartite sternebra; one fetus with multiple skeletal malformations (findings of this fetus are described in section on external findings, see above). The overall incidence of total skeletal malformations was not changed by treatment with BAS 750 F. The single incidences of these observations and the distribution within control and treatment groups do not indicate a relationship to treatment.

Table 5.6.2-12: Skeletal malformations

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		117	115	120	117
Live		117	115	120	117
Dead		0	0	0	0
Total skeletal malformations					
Fetal incidence	# (%)	2 (1.7)		1 (0.8)	2 (1.7)
Litter incidence	# (%)	2 (8.3)		1 (4.2)	2 (8.3)
Affected fetuses / litter	%	1.7 ± 5.65		0.8 ± 4.08	1.6 ± 5.77
Fetus with multiple skeletal malformations					
Fetal incidence	# (%)				1 (0.9)
Litter incidence	# (%)				1 (4.2)
Affected fetuses / litter	%				1.0 ± 5.10
Severely malformed skull bones					
Fetal incidence	# (%)			1 (0.8)	
Litter incidence	# (%)			1 (4.2)	
Affected fetuses / litter	%			0.8 ± 4.08	
Misshapen cervical vertebra					
Fetal incidence	# (%)	1 (0.9)			
Litter incidence	# (%)	1 (4.2)			
Affected fetuses / litter	%	0.8 ± 4.08			
Malpositioned and bipartite sternebra (unchanged cartilage)					
Fetal incidence	# (%)				1 (0.8)
Litter incidence	# (%)				1 (4.2)
Affected fetuses / litter	%				0.6 ± 2.92
Severely malformed sternum					
Fetal incidence	# (%)	1 (0.8)			
Litter incidence	# (%)	1 (4.2)			
Affected fetuses / litter	%	0.8 ± 4.08			

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

A wide range of **skeletal variations** was observed in fetuses of all dose groups including the control. The overall incidences of skeletal variations were comparable to the historical control data. There were two skeletal findings with statistically significant increases (see Table 5.6.2-13), i.e., supraoccipital hole(s) and misshapen sacral vertebra. Both findings were within the historical control range of the test facility (Table 5.6.2-14).

Table 5.6.2-13: Skeletal variations with statistically significant changes

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		117	115	120	117
Live		117	115	120	117
Dead		0	0	0	0
Total skeletal variations					
Fetal incidence	# (%)	117 (100)	115 (100)	120 (100)	116 (100)
Litter incidence	# (%)	24 (100)	24 (100)	24 (100)	24 (100)
Affected fetuses / litter	%	100 ± 0.00	100 ± 0.00	100 ± 0.00	99 ± 5.10
Supraoccipital hole(s)					
Fetal incidence	# (%)	8 (6.8)	6 (5.2)	21 (18)	17 (15)
Litter incidence	# (%)	6 (25)	5 (21)	**15 (63)****	**13 (54)***
Affected fetuses / litter	%	6.5 ± 12.18	5.1 ± 10.81	**18.4 ± 19.24****	**17.9 ± 23.44***
Misshapen sacral vertebra					
Fetal incidence	# (%)	3 (2.6)	5 (4.3)	7 (5.8)	12 (10)
Litter incidence	# (%)	3 (15)	3 (13)	5 (21)	**9 (38)***
Affected fetuses / litter	%	2.7 ± 7.37	3.9 ± 10.79	5.8 ± 12.55	**10.9 ± 18.06***

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* : $p \leq 0.05$; ** : $p \leq 0.01$

Table 5.6.2-14: Historical control data – fetal skeletal variations

	Fetuses (7684)			Litters (1431)			Affected fetuses / litter	
	No.	%	Range	No.	%	Range	% Mean	Range
Supraoccipital holes	954	12.4	0.0 – 52.3	521	36.4	0.0 – 100.0	12.2	0.0 – 50.8
Misshapen sacral vertebra	250	3.3	0.0 – 10.2	212	14.8	0 0 – 41.7	3.4	0.0 – 10.9

76 studies performed at BASF SE (2009–2014) with Wistar rats (Charles River)
(1431 litters with 7684 viable fetuses examined)

Further information regarding the observation "misshapen sacral vertebra" is provided in the next section.

Additionally, some isolated cartilage findings without impact on the respective bony structures, which were designated as **fetal skeletal unclassified cartilage observations**, occurred in all test groups. The observed unclassified cartilage findings were related to the skull, the sternum and ribs. The incidence of bipartite processus xiphoideus and, as a consequence, the incidence of total unclassified cartilage observations were statistically significantly increased in the low-dose group (50 mg/kg bw/d). Since there is no dose-response relationship and other test substance-treated groups were unaffected, an association with the treatment and a toxicological relevance is not assumed.

Table 5.6.2-15: Unclassified cartilage observations statistically significant changed

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		117	115	120	117
Total skeletal variations					
Fetal incidence	# (%)	88 (75)	102 (89)	89 (74)	91 (78)
Litter incidence	# (%)	24 (100)	24 (100)	24 (100)	24 (100)
Affected fetuses / litter	%	75.9	88.8*	74.9	78.1
Bipartite processus xiphoideus					
Fetal incidence	# (%)	87 (74)	101 (89)	89 (74)	88 (75)
Litter incidence	# (%)	24 (100)	24 (100)	24 (100)	24 (100)
Affected fetuses / litter	%	75.1	87.9*	74.9	75.6

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* : $p \leq 0.05$; ** $p < 0.01$

4. Assessment of all fetal external, soft tissue and skeletal observations

There were noted external (Table 5.6.2-9) and skeletal (Table 5.6.2-12) malformations in all groups. When the different findings were combined, the following can be concluded: Two fetuses were multiply malformed. Female fetus No. 73-07 from the mid-dose group (150 mg/kg bw/d) had a mandibular micrognathia (comprising severely malformed skull bones), while the findings in high-dose female fetus No. 79-09 (400 mg/kg bw/d) consisted of cleft palate, microphthalmia and a malformed mandible, which were associated with multiple skeletal malformations. No ontogenetic pattern is recognizable for the individual malformations nor was there any cluster of any of these individual malformations seen in the other offspring of these test groups. Other malformations, such as small spleen, misshapen cervical vertebra, malpositioned and bipartite sternebrae and severely malformed sternum, observed in all groups including controls are common for this rat strain and can be found in the historical control data at a comparable or higher frequency. An association of these findings to the treatment is also not assumed.

An external variation (Table 5.6.2-9), two soft tissue variations (Table 5.6.2-10) and a range of skeletal variations (Table 5.6.2-13 summarizing the variations with statistically significant changes) occurred in all test groups including the controls. None of the incidences showed a relation to dosing. The majority of the skeletal variations are equally distributed among the different test groups, if normal biological variation is taken into account, and can be found in the historical control data at a comparable frequency. However, the incidence of one skeletal variation - misshapen sacral vertebra – was slightly increased above the concurrent control but just within the historical control range of the test facility. The term "misshapen sacral vertebra" refers to a minor change in the direction (from ventral to cranial) of one of the sacral vertebral arches which gives the first sacral vertebra a more "lumbar-like" appearance. Although the term "misshapen" may suggest this finding to be a malformation, it rather represents a minor anatomic variant which is not permanent and which is not detrimental to postnatal survival or health. The frequent occurrence in the control population of this rat strain suggest that it is appropriately classified as variation on the one hand and of low toxicological concern on the other hand. It also does neither form a pattern or syndrome with other minor anomalies which may raise toxicological concern nor does it influence the overall rate of variants in this study. A spontaneous origin is also assumed for the unclassified external and skeletal cartilage observations which were observed in several fetuses of test groups including the control. The distribution and type of these findings do not suggest any relation to treatment.

Overall, the treatment with BAS 750 F did not lead to any significant changes of the total incidences of malformations or of variations (Table 5.6.2-16)

Table 5.6.2-16: Total malformations and variations

BAS 750 F (mg/kg bw/d)		0	50	150	400
Litters evaluated		24	24	24	24
Fetuses evaluated		223	221	230	222
Live		223	221	230	222
Dead		0	0	0	0
Total malformations					
Fetal incidence	# (%)	3 (1.3)	0 (0.0)	1 (0.4)	2 (0.9)
Litter incidence	# (%)	3 (13)	0 (0.0)	1 (4.2)	2 (8.3)
Affected fetuses / litter	%	1.3 ± 3.41	0.0 ± 0.00	0.5 ± 2.27	1.0 ± 3.68
Total variations					
Fetal incidence	# (%)	119 (53)	116 (52)	123 (53)	124 (56)
Litter incidence	# (%)	24 (100)	24 (100)	24 (100)	24 (100)
Affected fetuses / litter	%	53.5 ± 4.49	52.7 ± 3.52	53.5 ± 3.66	55.7 ± 8.13

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

III. DISCUSSION

In a prenatal developmental toxicity study the test compound BAS 750 F was administered to pregnant Wistar rats daily by gavage from implantation to one day prior to the expected day of parturition (GD 6-19) to evaluate its potential maternal and prenatal developmental toxicity. Analyses confirmed the correctness of the prepared concentrations and the stability of the test substance in the vehicle.

Clinical observations revealed no toxicologically relevant signs of toxicity in the animals receiving 50, 150 or 400 mg/kg bw/d BAS 750 F.

The test substance caused a decrease in food consumption at a dose of 400 mg/kg body weight/day. Consequently, the body weights/body weight gain were reduced during major phases of the treatment period in this dose group. A significant treatment-related reduction was also noted for the corrected (net) body weight and body weight gain (about 7% and 34% below the corresponding control group, respectively).

In addition, the mean placental weights were slightly increased (+14%) at a dose of 400 mg/kg body weight/day. However, the mean placental weight recorded in the high-dose group was well within the historical control range. Moreover, the potential morphological changes which are coherent to this weight change did not influence the development of the offspring at all. Therefore, the slight placental weight increase was judged to be non-adverse.

No such effects were noted in the low- and mid-dose animals (50 and 150 mg/kg bw/d). No differences of toxicological relevance between the control and the treated groups (50, 150 or 400 mg/kg bw/d) were determined for any reproductive parameters, such as conception rate, mean number of corpora lutea, mean number of implantations, as well as pre- and postimplantation loss. Similarly, no influence of the test compound on sex distribution of the fetuses was noted at any dose.

The mean weights of test group 3 (400 mg/kg bw/d) female fetuses were marginally reduced. However, as the group mean exactly matches the mean of the historical control (3.4 g) and as there is no effect in the corresponding male fetuses, a relationship with the treatment is not assumed.

Overall, there was no evidence for toxicologically relevant adverse effects of the test substance on fetal morphology at any dose.

IV. CONCLUSION

Under the conditions of the study, oral gavage treatment of pregnant Wistar rats with BAS 750 F did not lead to significant or biologically relevant fetal toxicity or teratogenicity when administered from GD 6–19 at doses of up to 400 mg/kg bw/d, the highest dose tested. At this dose level, maternal toxicity was evident by statistically significant reductions in feed intake, body weight gain, carcass weight and corrected body weight gain. Hence, the NOAEL for maternal toxicity is 150 mg/kg bw/d and the NOAEL for prenatal developmental toxicity is 400 mg/kg bw/day, the highest dose tested. There were no toxicologically relevant fetal findings evident.

The test substance is not teratogenic in rats.

Report:	CA 5.6.2/2 [redacted] 2015 b BAS 750 F - Prenatal developmental toxicity study in New Zealand white rabbits - Oral administration (gavage) 2014/1170757
Guidelines:	OECD 414, EPA 870.3700, (EC) No 440/2008 of 30 May 2008 - Part B No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

BAS 750 F (Batch COD-001662, Purity 95.5%) was administered daily to inseminated (presumably pregnant) New Zealand White rabbits by stomach tube during gestation days 6-28 post insemination (p.i.) at daily dose levels of 0, 5, 10 and 25 mg/kg bw/d. The high dose of 25 mg/kg bw was selected on the basis of range-finding studies (data supplied within the report), where 2 out of 3 non-pregnant rabbits dosed with 50 mg/kg bw/d had to be sacrificed moribund, showing signs of reduced nutritional condition, no or reduced feces and lateral position. Food consumption was reduced to almost zero levels within 2-5 days into the study. At higher dose levels, the animals either died or had to be sacrificed moribund, showing similar signs but earlier and more severe.

In the definitive prenatal developmental toxicity study, no maternal toxicity was observed at dose levels of ≤ 25 mg/kg bw/d.

No treatment-related effects on cesarean section parameters were observed at any dose. No treatment-related external, visceral or skeletal malformations or variations were noted.

The maternal and developmental NOAEL in this rabbit prenatal developmental toxicity study is 25 mg/kg bw/d. BAS 750 F is not teratogenic up to the highest dose tested.

(DocID 2014/1170757)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / beige
Batch #:	COD-001662
Purity / content:	95.5%
Stability of test compound:	The stability was guaranteed for the duration of the study.
2. Vehicle:	1% Carboxymethylcellulose suspension in drinking water (1% CMC)
3. Test animals:	
Species:	Rabbit
Strain:	New Zealand White / Crl:KBL(NZW)
Sex:	female
Age (GD 0):	ca. 10-15 weeks (sexually mature)
Weight (GD 0):	3368 ± 217.8 (2747 – 3819 g) – actually pregnant animals
Source:	Charles River Laboratories, Research Models and Services, Germany GmbH
Acclimation period:	At least 8 days (from day of supply until day GD 6 (day of first administration)
Diet:	Pelleted Kliba maintenance diet for rabbits/guinea pigs "GLP" (Provimi Kliba SA, Kaiseraugst, Switzerland), ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Type 4X03B700CP cages with a floor area of about 4200 cm² and 45 cm cage height (Tecniplast Deutschland GmbH, Germany); underneath the cages waste trays were loaded with absorbent material (dust-free wooden bedding). For enrichment, cages were equipped with wooden gnawing blocks, type KNH E-041 (Abedd®, Lab. and Vet. Service GmbH, Vienna, Austria)
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 16-Aug-2012 to 30-Apr-2015 [in life phase: 26-Aug-2012 (start of treatment, first cohort) to 07-Nov-2012 (sacrifice on GD29 of last cohort)]

2. Animal assignment and treatment

BAS 750 F was administered to groups of 30-33 inseminated (presumably pregnant) New Zealand White (NZW) rabbits by gavage at dose levels of 0, 5 (low dose), 15 (mid dose) and 25 mg/kg bw/day (high dose) during days 6 to 28 of gestation. The test substance was administered as a suspension in the vehicle 1% aqueous CMC at a dose volume of 10 ml/kg bw. A control group was dosed with the vehicle in parallel to the test groups. At terminal sacrifice on GD 29, 20 to 24 females per group had implantation sites.

Artificial insemination details:

The artificial insemination of the animals was carried out as follows: 0.2 mL Receptal®, a synthetic hormone which stimulates release of LH and FSH from the anterior pituitary lobe, was injected intramuscularly to the female rabbits about 1 hour before insemination. The ejaculate samples used for the artificial insemination were obtained from male NZW rabbits of the same breed as the females. Each female was inseminated with the sperm of a defined male donor as documented in the raw data. The male donors were kept under conditions (air conditioning, diet, water) comparable to those of the females participating in this study. The day of insemination was designated as gestational (GD) 0 and the following day as GD 1.

Dose rationale:

Two dose range-finding studies preceded this definitive prenatal developmental toxicity study. Initially dose levels of 50, 150 and 400 mg/kg bw/d were tested in a range-finding study in three female *non-pregnant* New Zealand White rabbits per group. At the lowest tested dose of 50 mg/kg bw/d, 2 out of 3 does were sacrificed in a moribund condition, showing signs of reduced nutritional condition, no or reduced feces, and lateral position. At dose levels of 150 and 400 mg/kg bw/d all animals in the study either died or were sacrificed in moribund condition, showing similar signs but earlier and more severe. In all dose groups, food consumption was reduced to almost zero levels within 2-5 days into the study. As a consequence, the animals constantly lost weight until their pre-terminal death or sacrifice. For these reasons, the dose level of 50 mg/kg bw/d was considered to be potentially lethal to the does in the dose range-finding and definitive OECD 414 study.

Subsequently in a maternal toxicity dose range-finding study, groups of 5 *pregnant* NZW rabbits were administered the test substance by oral gavage from GD 6 through GD 28. The dose levels were 0, 5, 10 and 20 mg/kg bw/d. In this study with pregnant rabbits, no consistent adverse effects in the does were produced.

The selected high dose for the present study represented half of the lethal dose determined in non-pregnant rabbits. This approved procedure of decreasing a lethal dose by a factor of two to become the high dose in a subsequent regulatory study meets the principles of guidelines OECD 414 (adopted 2001) and OPPTS 870.3700 (US EPA), as well as ECHA practical guide 10 (“how to avoid unnecessary testing on animals”; chapter 4 “animal welfare”; ECHA-10-B-17-EN, 2010) which is in in compliance with EU Directive 86/609/EEC on animal protection.

3. Test substance preparation and analysis

The stability of the test material in the chosen vehicle 1% aqueous carboxymethyl cellulose (1% CMC) was investigated with a comparable batch (L84-176) – it could be demonstrated that the aqueous test substance preparations are stable for at least 7 days under refrigerator conditions. Details are found in the study report. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration and homogeneity analyses were performed at the beginning of the study. The homogeneity of the dose suspensions was verified by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (5 and 25 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Table 5.6.2-17: Analysis of preparations for homogeneity and test-item content

Vehicle	Date of sampling	Dose level [mg/kg bw/d]	Nominal concentration [mg/100 mL]	Sample [#]	Analytical concentration [mg/100 mL]	% of nominal concentration	Mean ± RSD
1% CMC	24.08.2012 [Homogeneity and concentration control analyses]	5	50	3	45.838	91.7	91.5 ± 1.3
				4	45.109	90.2	
				5	46.304	92.6	
		15	150	6	134.403	89.6	-
		25	250	7	219.633	87.9	89.6 ± 1.7
				8	226.186	90.5	
				9	226.312	90.5	
1% CMC	06.09.2012 [Concentration control analyses]	5	50	12	45.353	90.7	-
		15	150	13	138.283	92.2	-
		25	250	14	225.750	90.3	-
1% CMC	12.10.2012 [Homogeneity and concentration control analyses]	5	50	3	48.040	96.1	92.4 ± 4.6
				4	43.870	87.7	
				5	46.759	93.5	
		15	150	6	145.477	97.0	-
		25	250	7	238.899	95.6	96.1 ± 0.5
				8	241.350	96.5	
				9	240.444	96.2	

Relative standard deviations of maximum 4.6% indicated the homogenous distribution of BAS 750 F in the dosing suspensions. The mean values of BAS 750 F in 1% CMC in drinking water were found to be in the range of 90% - 110% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical pathalogy	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using Kruskal-Wallis test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians
Blood parameters with uni-directional changes	Pairwise comparison of each dose group with the control group using the Wilcoxon-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption[a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of preimplantation loss, proportions of postimplantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight, litter mean placental weight	Simultaneous comparison of all dose groups with the control group using the Dunnett-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using Fisher's Exact test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the Wilcoxon-test (one-sided) for the hypothesis of equal proportions
[a)] For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed	

C. METHODS

1. Observations

The animals were examined for mortality twice daily on working days and once daily on weekends and public holidays (GD 0-29). Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on GD 0, 2, 4, 6, 9, 11, 14, 16, 19, 21, 23, 25, 28, and 29. The body weight change of the animals was calculated from these results. In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 29 minus weight of the unopened uterus minus body weight on GD 6).

Food consumption was recorded daily during GD 0-29.

Only pregnant does were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant does with scheduled sacrifice on GD 29 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Clinical pathology

In the morning of GD 29, blood was taken from the ear veins from fasted not anaesthetized animals. The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The results of clinical pathology examinations were expressed in International System (SI) units. The following examinations were carried out in all surviving animals per test group.

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)		Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
	Chloride		Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (γ-GT)
	Potassium	✓	Creatinine		
	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

4. Sacrifice

On GD 29, the surviving does were sacrificed in randomized order by intravenous injection of pentobarbital (Narcoren®; dose 2 mL/animal) and later, fetuses were removed from the uterus. All prematurely dead or sacrificed females were examined following the same procedures as for females sacrificed on schedule with the exception that no uterine weights were determined. After the does had been sacrificed, they were necropsied and were assessed by gross pathology in randomized order to minimize bias.

The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Number of corpora lutea
- Number, position and distribution of implantation sites in the uterus classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to Salewski in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue)
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

After the weight of the uterus had been determined, all subsequent evaluations of the does and the gestational parameters were conducted by technicians unaware of treatment group in order to minimize bias. For this purpose animal numbers were encoded.

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]:} \quad \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]:} \quad \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]:} \quad \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

5. Examination of fetuses:

All fetal analyses were conducted by technicians unaware of the treatment group, in order to minimize bias.

Examinations of the fetuses after dissection from the uterus

At necropsy each fetus was weighed and examined macroscopically for any external findings. Furthermore, the viability of the fetuses and the condition of the placentae, the umbilical cords, the fetal membranes, and fluids were examined. Individual placental weights were recorded. Subsequently the fetuses were sacrificed by s.c. injection of pentobarbital.

Soft tissue examination of the fetuses

After sacrifice the abdomen and thorax were opened in order to be able to examine the organs in situ before they were removed. The heart and the kidneys were sectioned in order to assess the internal structure. The sex of the fetuses was determined by internal examination of the gonads.

The heads of approximately one half of the fetuses per doe (and the heads of those fetuses, which revealed severe findings (e.g. anophthalmia, microphthalmia or hydrocephalus) already during the external examination) were severed from the trunk. These heads were fixed in Bouin's solution and processed and assessed according to Wilson's method subsequently. About 10 transverse sections were prepared per head.

All fetuses (including those without heads) were skinned and fixed in ethyl alcohol. After fixation for approx. 1-5 days, the intact fetuses were removed from the fixative and a transversal incision was made into the frontal/parietal head bones. The two halves of the calvarium were cautiously bent outward and the brain was thoroughly examined. Subsequently, these fetuses were placed back into the fixative for further fixation.

Skeletal examination of the fetuses

After completion of fixation in ethyl alcohol, the skeletons of the fetuses (including those without heads) were stained according to a modified method of Kimmel and Trammell. The stained skeletons were placed on an illuminated plate, investigated and archived individually.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) and its updated version of Makris et al. (2009) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (1999) and Solecki et al. (2001, 2003); for detailed references see study report:

Malformation	A permanent structural change that is likely to adversely affect the survival or health.
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Please note: Only pregnant does were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant does with scheduled sacrifice on GD 29 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

Control group (0 mg/kg bw/d)	- not pregnant: #16, 102 - died after gavage error: #15
Low-dose group (5 mg/kg bw/d)	- not pregnant: #27, 39, 41, 44, 46, 47, 48, 103, 105 - sacrificed after abortion: #104
Mid-dose group (15 mg/kg bw/d)	- not pregnant: #54, 56, 57, 63, 65, 67, 68, 70, 72, 111 - sacrificed after abortion: #113
High-dose group (25 mg/kg bw/d)	- not pregnant: #79, 79, 82, 93, 95, 98, 118 - died after gavage error: #88

Thus, according to the requirements of the corresponding test guidelines, each test group including the controls contained a sufficient number of females with implantation sites at necropsy (approx. 20, but not fewer than 16 females with implantation sites).

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

One low-dose female (#104) and one mid-dose female (#113) were sacrificed after abortion ahead of schedule. Spontaneous abortions in single does are not uncommon findings in the strain of rabbits used for this study. Thus, these were considered to be spontaneous incidents. Control female #15 and high-dose female #88 were found dead during the treatment period. The gross pathological examination of these animals revealed findings indicative of a gavage error.

2. Clinical symptoms

For low-dose female #33 blood in bedding was recorded on GD 26 and 27. There were no abnormal clinical findings in the other does in the study.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption

The food consumption of the low-, mid- and high-dose rabbits (5, 15 or 25 mg/kg bw/d) was comparable to the concurrent control (0 mg/kg bw/d) throughout the entire study period. This statement includes the statistically significantly decreased food consumption value at 25 mg/kg bw/d on GD 6-7, as well as the increased value at 15 mg/kg bw/d on GD 22-23. If calculated for the treatment period (GD 6-28), the mean food consumption values were quite similar in all groups including the control group.

Table 5.6.2-18: Food consumption

Dose	Day of Gestation 0–6	6–28	0–29	6–7	22-23
0 mg/kg bw/d [g/animal]	182.1	142.9	150.3	177.4	118.2
5 mg/kg bw/d [g/animal]	186.6	142.4	150.4	176.2	141.4
[% control]	102%	100%	100%	99%	120%
15 mg/kg bw/d [g/animal]	181.2	154.7	159.2	175.3	**148.8***
[% control]	100%	108%	106%	99%	**126%**
25 mg/kg bw/d [g/animal]	182.1	136.9	144.8	**153.6***	114.6
[% control]	100%	96%	96%	**87%**	97%

* p < 0.05, ** p < 0.01 (Dunnett test, two-sided)

2. Body weight and body weight change

The mean body weights and the average body weight gain of the low-, mid- and high-dose groups (5, 15 or 25 mg/kg bw/d) were generally comparable to the concurrent control group throughout the entire study period (see Figure 5.6.2-1 and Table 5.6.2-19).

Figure 5.6.2-1: Mean body weight of pregnant animals

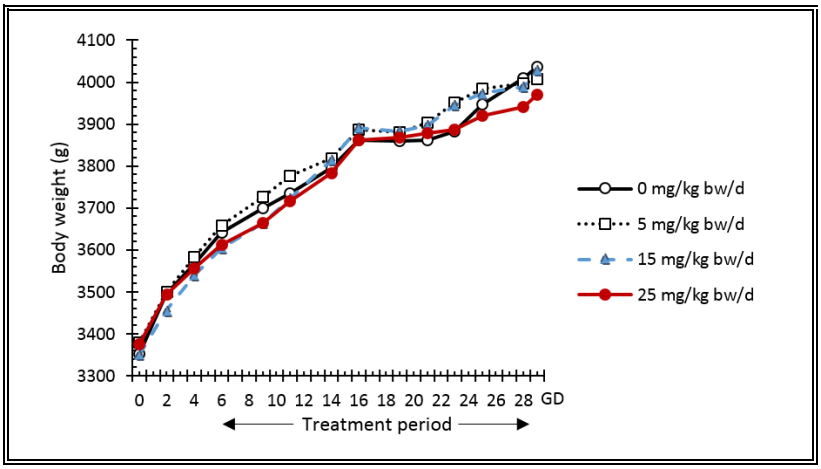


Table 5.6.2-19: Body weight change

Dose	Gestation Day 0–6	6–28	0–29
0 mg/kg bw/d [g/animal]	288.2	367.2	683.5
5 mg/kg bw/d [g/animal]	278.2	32.83	620.0
[% control]	97%	89%	91%
15 mg/kg bw/d [g/animal]	254.5	385.2	683.4
[% control]	88%	105%	100%
25 mg/kg bw/d [g/animal]	239.7	326.3	595.5
[% control]	83%	89%	87%

* $p < 0.05$, ** $p < 0.01$ (Dunnett test, two-sided)

D. TERMINAL EXAMINATIONS OF THE DOES

1 Clinical pathology

No treatment-related, adverse changes among hematological parameters were observed. In clinical chemistry investigations, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were decreased in rabbits of test group 3 (25 mg/kg bw/d). AST activities were already lower in test group 2 (15 mg/kg bw/d). However, the decrease was slight (decrease of mean values in test group 3: ALT 24%; AST 34%) and it was not accompanied by any other changed liver parameters. Therefore, the ALT and AST activity decreases were regarded as treatment-related, but not adverse. Globulins were lower in rabbits of test group 3 (25 mg/kg bw/d) and calcium levels were higher in rabbits of test group 2 (15 mg/kg bw/d) compared to controls. However, calcium values were not altered dose-dependently and the globulins were the only changed parameter in clinical chemistry. Therefore, the globulin decrease was regarded as treatment-related but not adverse.

Table 5.6.2-20: Clinical-pathology

Dose level [ppm]		BAS 750 F (mg/kg bw/d) 0	5	15	25
ALT	[µkat/L]	0.68	0.55	0.55	**0.52****
AST	[µkat/L]	0.61	0.60	**0.43***	**0.40****
ALP	[µkat/L]	0.54	0.55	0.65	0.46
GGT	[nkat/L]	47	48	49	39
Total protein	[g/L]	42.15	42.33	44.48	41.14
Albumin	[g/L]	32.58	32.84	35.30	32.61
Globulin	[g/L]	9.57	9.49	9.18	**8.53****
Glucose	[mmol/L]	6.69	6.50	6.81	6.46
Cholesterol	[mmol/L]	0.31	0.28	0.27	0.27
Triglycerid	[mmol/L]	0.34	0.44	0.41	0.39
Urea	[mmol/L]	5.00	5.37	5.29	5.68
Creatinine	[mmol/L]	103.3	97.2	95.8	98.6
Total bilirubin	[µmol/L]	1.34	1.19	1.16	1.23
Calcium	[mmol/L]	3.06	3.01	**3.22***	3.09
Inorg. phosphate	[mmol/L]	1.43	1.45	1.44	1.42

Statistical evaluation: Kruskal-Wallis + Wilcoxon (2-sided); * $p \leq 0.05$; ** $p \leq 0.01$

2 Uterus weight, carcass weight, corrected body weight change

Mean carcass weights and the corrected body weight gain (terminal body weight on GD 29 minus weight of the unopened uterus minus body weight on GD 6) were comparable among all test groups. The mean gravid uterus weights of the rabbits of test groups 1-3 (5, 15 and 25 mg/kg bw/d) were not influenced by the test substance. The differences between these groups and the control group showed no dose-dependency and were assessed to be without biological relevance.

Table 5.6.2-21: Uterus weight, carcass weight and corrected (net) body weight gain

Parameter (g)	BAS 750 F Dose level (mg/kg bw/d) 0	5	15	25
Gravid uterus	459 ± 142	464 ± 105	365 ± 163	450 ± 114
[% control]		101%	80%	97%
Carcass	3578 ± 308	3544 ± 243	3664 ± 413	3520 ± 342
[% control]		99%	102%	98%
Net weight change from GD 6	-64 ± 134	-123 ± 189	60 ± 247	-94 ± 230

* $p < 0.05$, ** $p < 0.01$ (Dunnett test, two-sided)
Carcass weight = terminal body weight minus uterine weight (values are rounded)
Net weight change from GD 6 = carcass weight minus GD 6 body weight

3. Gross necropsy observations

A number of spontaneous findings were noted in individual females of test groups 0-3 (0, 5, 15 and 25 mg/kg bw/d). These gross findings were:

Control group (0 mg/kg bw/d):	- thoracic cavity filled with blood (indicative of gavage error): No. 15 [found dead on GD 24]
Low-dose group (5 mg/kg bw/d):	- No feces in rectum: No. 29 - Pale liver: No. 107
Mid-dose group (15 mg/kg bw/d):	- multiple erosions in stomach: Nos. 57, 70, 73 - sacrificed after abortion: No. 113
High-dose group (25 mg/kg bw/d):	- multiple erosions in stomach: No. 82, 87 - no feces in rectum: No. 81 - hemometra (right uterus horn): No. 84 - thoracic cavity filled with blood (indicative of gavage error): No. 15 [found dead on GD 24]

E. CAESAREAN SECTION DATA

Female rabbits were placed into the study in five cohorts. The conception rate was 70% in the low- and mid-dose groups (5 and 15 mg/kg bw/d), 77% in the high-dose group (25 mg/kg bw/d) and 93% in the control group (0 mg/kg bw/d). A sufficient number (at least 20 per group) of pregnant females was available for the purpose of the study.

There were no test substance-related and/or biologically relevant differences between test groups 0, 1, 2 and 3 (0, 5, 15 and 25 mg/kg bw/d) in conception rate, in the mean number of corpora lutea or in the values calculated for the pre- and the post-implantation losses and the number of resorptions.

However, in the mid-dose group (15 mg/kg bw/d), the numbers of implantation sites and subsequently of live fetuses (live litter size) was statistically significantly lower than the concurrent control. The mean values were slightly below the historical control range, as was the number of corpora lutea. The latter finding showed – together with the higher percentages of preimplantation losses in this dose group – that the lower litter size observed in the mid-dose group was unrelated to treatment. Further evidence for this assumption is given by the absence of a dose-response relationship and of corroborative findings in the reproductive organs of the affected animals. All other differences observed were considered to reflect the normal range of fluctuations for animals of this strain and age. This includes an incidentally higher post-implantation loss in control and group 2 which were both caused by single does (No. 7 and 51) with completely resorbed litters.

The mean number and weight of live, male and female fetuses as well as the sex ratio of fetuses were not affected by treatment. Finally, no effects on placental weights were observed.

Table 5.6.2-22: Pregnancy status and Caesarean section data

Dose level [mg/kg bw/d]		0		5		15		25	
Pregnancy status									
Females									
- mated	[n]	27		30		33		30	
- pregnant	[n]	25		21		23		23	
conception rate	[%]	93		70		70		77	
- aborted	[n]	0		1		1		0	
- premature birth	[n]	0		0		0		0	
- Does with viable fetuses	[n]	23		20		21		22	
- Does with all resorptions	[n]	1		0		1		0	
- Mortality		1		1		1		1	
- Pregnant terminal sacrifice	[n]	24		**20***		**22***		22	
Caesarean section data[a]									
- Corpora lutea	[n]	9.4	±2.67	8.9	±2.35	8.1	±2.41	9.1	±1.96
total number	[n]	225		179		178		201	
- Implantation sites	[n]	8.9	±2.61	8.4	±2.54	**7.0***	±2.80	8.4	±2.52
total number	[n]	213		169		153		184	
- Pre-implantation loss	[%]	4.8	±10.59	6.3	±9.99	14.5	±21.13	10.4	±16.18
- Post-implantation loss	[%]	12.6	±21.23	4.7	±7.46	12.4	±24.90	3.7	±6.25
- Resorptions	[n]	0.8	±0.78	0.4	±0.60	0.8	±1.97	0.4	±0.66
total number	[n]	19		8		18		8	
- Early resorptions	[%]	10.8	±21.33	2.9	±6.65	11.4	±25.00	2.0	±4.45
number	[n]	0.6	±0.71	0.2	±0.41	0.7	±1.96	0.2	±0.39
total number	[n]	15		4		16		4	
- Late resorptions	[%]	1.8	±5.62	1.8	±4.73	1.0	±3.22	1.6	±4.43
number	[n]	0.2	±0.48	0.2	±0.52	0.1	±0.29	0.2	±0.50
total number	[n]	4		4		2		4	
- Dead fetuses	[n]	0		0		0		0	
- Does with viable fetuses	[n]	23		20		21		22	
- Live fetuses		8.4	±2.29	8.1	±2.42	**6.4***	±2.93	8.0	±2.31
total number	[n]	194		161		135		176	
Mean	[%]	91.2	±10.42	95.3	±7.46	91.8	±15.77	96.3	±6.25
- Total live female fetuses	[n]	4.5	±1.83	4.4	±1.82	**3.0***	±1.75	3.8	±1.71
total number	[n]	104		89		64		84	
Mean	[%]	49.5	±18.05	50.9	±18.24	42.7	±19.99	47.5	±20.13
- Total live male fetuses	[n]	3.9	±1.81	3.6	±1.31	3.4	±1.91	4.2	±1.87
total number	[n]	90		72		71		92	
Mean	[%]	41.7	±14.49	44.4	±16.24	49.1	±21.89	48.8	±18.59
- Percent live females		53.6		55.3		47.4		47.7	
- Percent live males		46.4		44.7		52.6		52.3	
Placental weights	[g]	5.5	±0.81	5.5	±0.89	5.6	±0.93	5.3	±0.75
- male fetuses	[g]	5.5	±0.99	5.6	±0.94	5.6	±0.88	5.2	±0.56
- female fetuses	[g]	5.4	±0.81	5.4	±0.81	5.4	±0.91	5.2	±0.82
Mean fetal weight	[g]	39.4	±6.25	40.6	±5.00	42.6	±5.19	39.8	±6.55
- males	[g]	39.6	±7.32	39.9	±5.29	42.3	±5.03	39.9	±5.50
- females	[g]	38.7	±6.39	40.0	±4.84	41.9	±5.25	39.0	±6.97

[a] Mean ± SD on litter basis; Statistical evaluation: * $p \leq 0.05$; ** $p < 0.01$ (Dunnett-test, two-sided)

Table 5.6.2-23: Historical control data

Historical control data	Mean	±	SD	Range (per study)	
				Minimum	Maximum
Corpora lutea	9.9	±	2.56	9.0	11.0
Implantation sites	8.8	±	2.98	7.1	10.3
Pre-implantation loss [mean %]	11.9	±	18.66	5.4	25.7
Post-implantation loss [mean %]	6.9	±	13.07	2.4	11.3
Resorptions [N]	0.6	±	0.99	0.3	1.1
Live litter size [N]	8.2	±	2.90	6.6	9.7

12 studies performed at BASF SE (Jan 2009– Sep 2013) with New Zealand White rabbits (Charles River)
(303 pregnant dams; 285 litters with 2366 viable fetuses)

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination

External malformations: An omphalocele was recorded for two control fetuses from one litter. This finding was considered to be incidental.

External variations: One control fetus was found with an external variation, i.e. paw hyperflexion. This individual external finding was considered to be incidental.

Table 5.6.2-24: External malformations, variations and unclassified observations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Live					
Dead					
Total external malformations					
Fetal incidence	# (%)	2 (1.0)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	1.0			
Omphalocele					
Fetal incidence	# (%)	2 (1.0)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	1.0			
Total external variations					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.6			
Paw hyperflexion					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.6			
Total fetal external unclassified observations					
Fetal incidence	# (%)				1 (0.6)
Litter incidence	# (%)				1 (4.5)
Affected fetuses / litter	%				0.5
Placentae necrobiotic					
Fetal incidence	# (%)				1 (0.6)
Litter incidence	# (%)				1 (4.5)
Affected fetuses / litter	%				0.5

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

External unclassified observations: One case of necrobiotic placenta was recorded in a litter of the high-dose group (25 mg/kg bw/d). This single finding was not considered biologically relevant. It can be found in the historical control data.

2. Visceral examination

Soft-tissue malformations: Three soft tissue malformations were recorded for single fetuses of test groups 0-3 (0, 5, 15 or 25 mg/kg bw/d). The distribution of the findings across the dose groups does not indicate an association to the treatment. No statistically significant differences between the groups were noted.

Soft-tissue variations: The examinations of the organs revealed a broad variety of soft tissue variations, i.e. dilated cerebral ventricle, cystic dilatation of the brain, malpositioned carotid branch, short innominate and absent lung lobe (Lobus inferior medialis), in fetuses of all test groups including the control (0, 5, 15 and 25 mg/kg bw/d). The incidences of these variations were neither statistically significantly different from control nor dose-dependent and therefore, not considered treatment-related or biologically relevant. All of them can be found in the historical control data at comparable incidences.

Table 5.6.2-25: Soft tissue malformations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total soft tissue malformations					
Fetal incidence	# (%)	1 (0.5)	1 (0.6)	1 (0.7)	1 (0.6)
Litter incidence	# (%)	1 (4.3)	1 (5.0)	1 (4.8)	1 (4.5)
Affected fetuses / litter	%	0.5	0.6	1.0	0.5
Absent subclavian					
Fetal incidence	# (%)	1 (0.5)	1 (0.6)		1 (0.6)[a]
Litter incidence	# (%)	1 (4.3)	1 (5.0)		1 (4.5)
Affected fetuses / litter	%	0.5	0.6		0.5
Diaphragmatic hernia					
Fetal incidence	# (%)			1 (0.7)	
Litter incidence	# (%)			1 (4.8)	
Affected fetuses / litter	%			1.0	
Small thymus					
Fetal incidence	# (%)				1 (0.6)[a]
Litter incidence	# (%)				1 (4.5)
Affected fetuses / litter	%				0.5

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$
[a] fetus #100-06 with additional skeletal malformation

Soft-tissue unclassified observations: Two unclassified soft tissue observations were recorded: a blood coagulum around urinary bladder in two control fetuses and in one fetus each of test group 2 (15 mg/kg bw/d) and test group 3 (25 mg/kg bw/d), furthermore, cyst(s) in one fetus of test group 1 (5 mg/kg bw/d). These findings were neither considered biologically relevant nor treatment-related.

Table 5.6.2-26: Soft tissue variations and unclassified observations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total soft tissue variations					
Fetal incidence	# (%)	4 (2.1)	5 (3.1)	2 (1.5)	3 (1.7)
Litter incidence	# (%)	3 (13)	3 (15)	2 (9.5)	3 (14)
Affected fetuses / litter	%	2.5	3.7	2.4	2.0
Dilated cerebral ventricle					
Fetal incidence	# (%)				1 (0.6)
Litter incidence	# (%)				1 (4.5)
Affected fetuses / litter	%				0.5
Brain: Cystic dilatation					
Fetal incidence	# (%)		1 (0.6)	1 (0.6)	
Litter incidence	# (%)		1 (5.0)	1 (4.8)	
Affected fetuses / litter	%		0.5	0.8	
Malpositioned carotid branch					
Fetal incidence	# (%)	1 (0.5)	2 (1.2)	1 (0.6)	1 (0.6)
Litter incidence	# (%)	1 (4.3)	2 (10)	1 (4.8)	1 (4.5)
Affected fetuses / litter	%	0.5	2.2	0.8	0.6
Short innominate					
Fetal incidence	# (%)				1 (0.6)
Litter incidence	# (%)				1 (4.5)
Affected fetuses / litter	%				0.9
Absent lung lobe (*L. inferior medialis*)					
Fetal incidence	# (%)	3 (1.5)	2 (1.2)		
Litter incidence	# (%)	2 (8.7)	1 (5.0)		
Affected fetuses / litter	%	2.1	1.0		
Total soft tissue unclassified observations					
Fetal incidence	# (%)	2 (1.0)	1 (0.6)	1 (0.7)	1 (0.6)
Litter incidence	# (%)	2 (8.7)	1 (5.0)	1 (4.8)	1 (4.5)
Affected fetuses / litter	%	1.0	0.4	0.5	0.6
Blood coagulum around urinary bladder					
Fetal incidence	# (%)	2 (1.0)		1 (0.7)	1 (0.6)
Litter incidence	# (%)	2 (8.7)		1 (4.8)	1 (4.5)
Affected fetuses / litter	%	1.0		0.5	0.6
Cysts					
Fetal incidence	# (%)		1 (0.6)		
Litter incidence	# (%)		1 (5.0)		
Affected fetuses / litter	%		0.4		

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$

Table 5.6.2-27: Historical control data – fetal soft tissue variations

	Fetuses (2366)			Litters (285)			Affected fetuses / litter	
	No.	%	Range	No.	%	Range	% Mean	Range
Dilated cerebral ventricle	1	0.04	0.0 – 0.7	1	0.4	0.0 – 4.5	0.0	0.0 – 0.5
Brain: Cystic dilatation	2	0.08	0.0 – 0.8	2	0.7	0.0 – 6.3	0.1	0.0 – 0.6
Malpositioned carotid branch	20	0.8	0.0 – 2.4	19	6.7	0.0 – 18.8	0.8	0.0 – 2.0
Short innominate	1	0.04	0.0 – 0.3	1	0.4	0.0 – 2.4	0.0	0.0 – 0.2
Absent lung lobe (L. inferior medialis)	31	1.3	0.0 – 4.5	22	7.7	0.0 – 18.8	1.2	0.0 – 3.5

12 studies performed at BASF SE (Jan 2009– Sep 2013) with New Zealand White rabbits (Charles River)

3. Skeletal examination

Skeletal malformations were recorded for seven control fetuses (0 mg/kg bw/d), two fetuses of test group 1 (5 mg/kg bw/d), one fetus of test group 2 (15 mg/kg bw/d) and three fetuses of test group 3 (25 mg/kg bw/d).

One female control fetus (#3-01 F) with misshapen nasal and severely malformed sternum and one male control fetus (#3-06 M) with severely malformed sternum also each had an omphalocele (external malformation). One female fetus from the high-dose group (#100-06 F) with severely malformed sternum additionally was found with absent subclavian and small thymus (soft tissue malformation).

Table 5.6.2-28: Skeletal malformations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total skeletal malformations					
Fetal incidence	# (%)	7 (3.6)	2 (1.2)	1 (0.7)	3 (1.7)
Litter incidence	# (%)	5 (22)	2 (10)	1 (4.8)	3 (14)
Affected fetuses / litter	%	3.3	1.5	0.5	1.7
Misshapen interparietal					
Fetal incidence	# (%)	2 (1.0)	1 (0.6)	1 (0.7)	1 (0.6)
Litter incidence	# (%)	2 (8.7)	1 (5.0)	1 (4.8)	1 (4.5)
Affected fetuses / litter	%	0.9	0.8	0.5	0.6
Misshapen nasal					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.5			
Small interparietal					
Fetal incidence	# (%)		1 (0.6)		
Litter incidence	# (%)		1 (5.0)		
Affected fetuses / litter	%		0.7		
Lumbar hemivertebra					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.3			
Misshapen lumbar vertebra					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.3			
Severely malformed sternum					
Fetal incidence	# (%)	3 (1.5)			2 (1.1) [b]
Litter incidence	# (%)	2 (8.7)			2 (9.1)
Affected fetuses / litter	%	1.6			1.1
Short rib (cartilage present)					
Fetal incidence	# (%)	1 (0.5)			
Litter incidence	# (%)	1 (4.3)			
Affected fetuses / litter	%	0.5			

Statistics, litter incidence: Fisher's Exact Test (1-sided); affected fetuses/litter: Wilcoxon-Test (1-sided)
* $p < 0.05$, ** $p < 0.01$
[a] control fetuses #3-01 F and #3-06 M with additional external malformation; [b] high-dose fetus #100-06 F with additional soft-tissue malformations

No statistically significant differences in the incidence of skeletal malformations between the test substance-treated groups and the control group were noted and no dose-response relationship was observed.

Skeletal variations: For all control and test groups, skeletal variations of different bone structures were observed, with or without effects on corresponding cartilages. The observed skeletal variations were related to several parts of fetal skeleton and no statistically significant differences between the test substance-treated groups and the control group were noted for any of the observed skeletal variations. Moreover, the overall incidences of skeletal variations were comparable to the historical control data.

Table 5.6.2-29: Skeletal variations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total skeletal variations					
Fetal incidence	# (%)	190 (98)	160 (99)	134 (99)	173 (98)
Litter incidence	# (%)	23 (100)	20 (100)	21 (100)	22 (100)
Affected fetuses / litter	%	97.6	99.4	99.5	98.4

Skeletal unclassified cartilage observations

Some isolated cartilage findings without impact on the respective bony structures, which were designated as unclassified cartilage observations, occurred in all control and test groups. The observed unclassified cartilage findings did not show any relation to dosing and were considered to be spontaneous in nature.

Table 5.6.2-30: Skeletal unclassified cartilage observations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total skeletal unclassified cartilage observations					
Fetal incidence	# (%)	16 (8.2)	160 (99)	134 (99)	12 (6.8)
Litter incidence	# (%)	12 (52)	20 (100)	21 (100)	9 (41)
Affected fetuses / litter	%	7.9	9.1	5.2	7.5

4. Assessment of all fetal external, soft tissue and skeletal observations

External (Table 5.6.2-24), soft tissue (Table 5.6.2-25) and skeletal (Table 5.6.2-28) malformations were noted in eight control, three low-dose, two mid-dose and three high-dose fetuses. The distribution of total malformations about the groups was not related to dose. Some fetuses were multiple-malformed. Female fetus No. 3-01 (control group) had an omphalocele associated with a misshapen nasal and a severely malformed sternum. For male fetus No. 3-06 an omphalocele (comprising a severely malformed sternum) was recorded, while the findings in high-dose female fetus No. 100-06 (25 mg/kg bw/d) consisted of a small thymus, an absent subclavian and a severely malformed sternum. For female fetus No. 2-14 (0 mg/kg bw/d) two malformations affecting the lumbar vertebrae (i.e. lumbar hemivertebra and misshapen lumbar vertebra) were recorded. No ontogenetic pattern is recognizable for the individual malformations nor was there any cluster of any of these individual malformations seen in the other offspring of these test groups.

Other malformations, such as diaphragmatic hernia, misshapen interparietal, small interparietal and short rib were scattered observations in individual fetuses of test groups 0-3. They were not dose-related and all of them can be found in the historical control data at comparable or higher frequency. An association of these findings to the treatment is not assumed. The total incidences of fetal malformations are summarized in Table 5.6.2-31.

Table 5.6.2-31: Total fetal malformations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total fetal malformations					
Fetal incidence	# (%)	8 (4.1)	3 (1.9)	2 (1.5)	3 (1.7)
Litter incidence	# (%)	6 (26)	3 (15)	2 (9.5)	3 (14)
Affected fetuses / litter	%	3.8	2.1	1.4	1.7

A spontaneous origin is assumed for the external variations (Table 5.6.2-24), soft tissue variations, (Table 5.6.2-26) and a broad range of skeletal variations (Table 5.6.2-29) which were observed in fetuses of all test groups including the controls. If all different types of variations are summarized, none of the incidences showed a relation to dosing (Table 5.6.2-32) and can be found in the historical control data at a comparable frequency.

Table 5.6.2-32: Total fetal variations

BAS 750 F (mg/kg bw/d)		0	5	15	25
Litters evaluated		23	20	21	22
Fetuses evaluated		194	161	135	176
Total fetal malformations					
Fetal incidence	# (%)	191 (98)	160 (99)	134 (99)	173 (98)
Litter incidence	# (%)	23 (100)	20 (100)	21 (100)	22 (100)
Affected fetuses / litter	%	98.7	99.4	99.5	98.4

A spontaneous origin is also assumed for unclassified external (Table 5.6.2-24), unclassified soft tissue (Table 5.6.2-26) and unclassified skeletal cartilage observations (Table 5.6.2-30) which were observed in several fetuses of test groups 0, 1, 2 and 3. The distribution and type of these findings do not suggest any relation to treatment.

Finally, fetal examinations revealed that there is no effect of the compound on the respective morphological structures up to a dose of 25 mg/kg bw/d.

III. DISCUSSION

BAS 750 F was administered to pregnant New Zealand White rabbits daily by stomach tube from implantation to one day prior to the expected day of parturition (GD 6-28) at dose levels of 5, 15 and 25 mg/kg bw/day.

Analyses confirmed the correctness of the prepared concentrations, their homogeneous distribution and the stability of the test substance in the vehicle.

Generally, no toxicologically relevant signs of maternal toxicity were observed in any of the control and test groups. Neither clinical examinations nor determination of food consumption and body weights/body weight gain and necropsy revealed any relevant effect on the animals receiving 5, 15 or 25 mg/kg bw/d BAS 750 F. Clinical pathology revealed no treatment-related adverse effects up to a dose of 25 mg/kg bw/d. There were no differences of biological relevance between the control and the substance-treated groups (5, 15 and 25 mg/kg body weight/day) in conception rate, mean number of corpora lutea, total implantations, resorptions and live fetuses, fetal sex ratio or in the values calculated for the pre- and the post-implantation losses.

No test substance-related differences were recorded for placental and fetal body weights, or for fetal sex ratio.

The external, soft tissue and skeletal examinations of the fetuses revealed no differences between the controls and the test substance-treated groups, which might be related to the test substance. Number and type of fetal external, soft tissue and skeletal findings, which were classified as malformations and/or variations, did not show any differences of toxicological relevance between the groups.

The test substance is not teratogenic in rabbits at the tested dose levels.

IV. CONCLUSION

Under the conditions of the study, oral gavage treatment of pregnant NZW rabbits with BAS 750 F did not lead to significant or biologically relevant fetal toxicity or teratogenicity when administered from GD 6–28 at doses of up to 25 mg/kg bw/d, the highest dose tested. No maternal toxicity was noted in this study. Hence, the NOAEL for maternal toxicity and for prenatal developmental toxicity is 25 mg/kg bw/day, the highest dose tested.

The test substance is not teratogenic in rabbits.

CA 5.7 Neurotoxicity Studies

The neurotoxicity of BAS 750 F was specifically examined in an acute oral neurotoxicity test in Wistar rats. As a reminder, neurobehavioual parameters and histopathological examinations of neuronal tissues were also part of the examinations of the 90-day dietary toxicity study in Wistar rats (Buesen et al., 2015a; DocID 2015/1198721), which is summarized in section 5.3.

Table 5.7-1: Summary of neurotoxicity studies with BAS 750 F

Study **Batch / purity** **Intakes (mg/kg bw/d)**	**NOAEL (mg/kg bw)**	**Main adverse effect**	**Reference (BASF DocID)**
Acute neurotoxicity **Wistar rat** Oral gavage; vehicle: 1% CMC Batch: COD-001880 / 98.6% 0 – 200 – **600** – 2000 mg/kg bw	**600**	2000 mg/kg bw ↓ body weight gain (♂+♀) during Day 0-7 ↑ unsteady gait of 5/10 ♂ and 3/10 ♀ on Day 0 ↓ motor activity (♂+♀) on Day 0 ↓ ♂ forelimb grip strength on Day 0 ↑ ♂ landing footsplay on Day 0	[redacted] 2015c (2014/1170759)

In the acute neurotoxicity study, groups of 10 male and 10 female Wistar rats received BAS 750 F by single oral gavage administration at dose levels of 0, 200, 600 or 2000 mg/kg bw. Only at the high-dose level, which also caused transient decreases in body weight gain in both sexes, treatment-related neurobehavioral effects were noted on the day of treatment (Day 0). These consisted of unsteady gait of males and females observed during Open Field examinations and reduced motor activity in both sexes. Male rats were additionally found with reduced forelimb grip strength and increased distance of hindlimbs in the landing footsplay test, also only on the day of treatment. All findings were assessed as being related to an unspecific impairment of the overall condition of the animals (i.e., nonspecific systemic toxicity) following bolus administration of a high dose level rather than being related to a specific neurotoxic mode of action. Brain weight determination, necropsy and neuropathology examinations by light microscopy did not reveal any neuropathological, treatment-related findings at any dose level. The no observed adverse effect level (NOAEL) of this study was 600 mg/kg bw for male and female Wistar rats with regard to both systemic toxicity and neurobehavioural effects. There were no indications of structural damage to the neurons at dose levels up to and including 2000 mg/kg bw, the highest dose level tested and the limit dose.

No indication for clinical (general clinical observation, FOB and motor activity) or histopathological neurotoxicity was observed in dietary repeated-dose studies with BAS 750 F. Importantly, in the 90-day dietary study in Wistar rats, the parameters identified to be treatment-related in the acute neurotoxicity study, were not changed by treatment up to dose levels of 3600 ppm, corresponding to continuous daily intakes of 256 mg/bw in males and 314 mg/kg bw/d in female rats for 3 months, which provides further evidence that findings in the acute neurotoxicity study are nonspecific effects of high-dose bolus treatment and not the expression of specific neurotoxicity.

CA 5.7.1 Neurotoxicity studies in rodents

Report:	CA 5.7.1/1 [redacted] 2015c BAS 750 F - Acute oral neurotoxicity study in Wistar rats - Administration by gavage 2014/1170759
Guidelines:	EPA 870.6200, OECD 424, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.43
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The acute neurotoxicity of BAS 750 F (Batch COD-001880; Purity: 98.6%) was investigated in groups of 10 male and 10 female Wistar rats (Crl:WI(Han) after a single administration by gavage at dose levels of 0, 200, 600, and 2000 mg/kg bw.

Test substance-related findings only occurred at the top dose level. Clinical observations revealed lower mean body weight change values in male (-29%, statistically significantly altered) and in female animals (-20%; not statistically significantly changed) between study days 0 to 7. These effects were assessed to be related to the treatment on study day 0. The effect did not persist and no relevant differences were observed for this parameter on study day 14.

During examinations of the functional observation battery (FOB) on the day of administration (study day 0), slight impairment of coordination, i.e. unsteady gait, was observed during open field observation in 5 male and 3 female animals. With regard to quantitative parameters, grip strength of forelimbs was significantly lower in male animals of test group 3 (-22%). In addition, the landing foot-splay test revealed a statistically significantly increased distance between the hindlimbs for these animals. Decreases in overall motor activity measurement were determined for male and female animals at the top dose level. All findings were assessed as being related to an unspecific impairment of the overall condition of the animals following bolus administration of a high dose level rather than being related to a specific neurotoxic mode of action. No changes of toxicological relevance were observed in male and female animals at dose levels of 600 or 200 mg/kg bw. Regarding neuropathology, brain weight determination, necropsy and neuro-pathology examination by light microscopy did not reveal neuropathological, treatment-related findings at any dose level.

In conclusion, a few parameters demonstrated acute but transient test substance-related findings, attributable to nonspecific high-dose systemic toxicity, following administration of BAS 750 F after single oral gavage dosage in male and female Wistar rats at 2000 mg/kg bw. Regarding neuropathology, brain weight determination, necropsy and neuropathology examination by light microscopy did not reveal any neuropathological, treatment-related findings up to 2000 mg/kg bw (highest dose tested; HDT) in both sexes.

The no observed adverse effect level (NOAEL) of this study was 600 mg/kg bw for male and female Wistar rats with regard to systemic toxicity and neurobehavioural effects. There were no indications of structural damage to the neurons at dose levels up to and including 2000 mg/kg bw, the highest dose level tested. (DocID 2014/1170759)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 750 F (Reg.No. 5834378)
Description:	solid / white
Batch #:	COD-001880
Purity / content:	98.6%
Stability of test compound:	The test substance was stable over the study period (expiry date 30-Nov-2015).
2. Vehicle:	Drinking water containing 1% Carboxymethylcellulose (1% CMC)
3. Test animals:	
Species:	Rat
Strain:	Wistar / Crl:WI (Han)
Sex:	male and female
Age:	34-35 days (Section A) and 32-33 days (Section B) at delivery, 49 days at administration
Weight at dosing (mean):	♂: ca. 201.6 ± 8.3 g; ♀: ca. 152.7 ± 7.9 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimatization period:	at least 8 days
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon Type III cages with wire covers from Ehret, Emmendingen; Germany (floor area about 800 cm²); with dust wooden free bedding enriched with wooden gnawing blocks (TYP NGM E-022; Abedd® Lab. and Vet. Service GmbH, Vienna, Austria).
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 15-Apr-2014 to 04-Jan-2015 [in life phase: 29-Apr-2014 (start of treatment Section A males) to 16-May-2014 (necropsy of Section B females)]

2. Animal assignment and treatment

BAS 750 F was administered once to groups of 10 male and 10 female rats by oral gavage of 0, 200 (low dose), 600 (intermediate dose), and 2000 mg/kg bw (top dose). The application volume was 20 mL/kg bw. Each group per sex was subdivided into 2 subsets (Section A males and Section A females = first 5 animals of each dose group and Section B males and Section B females = second 5 animals of each dose group) in order to balance the groups for FOB and motor activity measurements. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

3. Test substance preparation and analysis

The dosing suspensions were prepared by mixing weighed amounts of test substance with appropriate amounts of the vehicle (1% aqueous CMC) by high speed with an Ultraturrax. During administration of the test substance, preparations were kept homogeneous using a magnetic stirrer. The test-substance preparations were made once before the first administration. Analyses performed prior to the start of the administration period revealed that the test-substance was stable in 1% aqueous CMC for 7 days when stored at room temperature. Homogeneity analyses of suspension preparation were performed for all concentrations. According to the SOP three samples were taken from the top middle and bottom of the beaker at the start and the end of the administration period. For this - as laid down in the SOP - samples were taken from the top, middle and bottom of the storage containers. The homogeneity samples were also used for concentration control analysis.

Table 5.7.1-1: Analysis of preparations for homogeneity and test-item content

BAS 750 F nominal conc. [g/100 mL]	Date of sampling	Starting date of analysis	Sample [#]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± RSD
1.0	28-Apr-2014	08-May-2014	3	0.927	92.7	93.0 ± 0.8
			4	0.924	92.4	
			5	0.938	93.6	
3.0	28-Apr-2014	08-May-2014	6	2.786	92.9	94.3 ± 3.4
			7	2.759	92.0	
			8	2.939	98.0	
10.0	28-Apr-2014	08-May-2014	9	9.177	91.8	94.6 ± 2.7
			10	9.533	95.3	
			11	9.677	96.8	

No test substance could be detected in the vehicle control sample. Relative standard deviations (RSD) in the range of 0.8 to 3.4% indicate the homogenous distribution of BAS 750 F in the diet preparations. The actual nominal test-item concentrations were in the range of 91.8 to 98.0% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	Statistical test
Body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Feces, rearing, grip strength forelimbs and hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

Statistics for pathology	
Parameter	Statistical test
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Clinical Observations

All animals were checked daily for any clinically abnormal signs. Abnormalities and changes were documented for each animal. A check for moribund and dead animals was made twice daily on working days and once daily on Saturdays, Sundays and public holidays. If animals were in a moribund state, they were sacrificed.

2. Body weight

The body weight was determined before the first neurofunctional test in order to randomize the animals. During the conduct of the study, the body weight was determined on the days when functional observational batteries were carried out (study days –7, 0, 7 and 14). The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Individual food consumption was checked daily by visual inspection. No food consumption data were recorded.

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any overt changes in volume. No water consumption data were recorded.

5. Ophthalmoscopy

Not performed in this study.

6. Functional observation battery (FOB)

FOBs were performed in all animals prior to administration (day -7) and on study days 0, 7 and 14. The FOBs were performed starting at about 10:00 a.m. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The examinations were carried out by trained technicians being not aware of the group allocation of the animals. The findings were ranked according to the degree of severity, if applicable.

Home cage observations:

During the home cage observation, special attention was paid to posture, tremors, convulsions, abnormal movements, impairment of gait and other findings (if applicable).

Open field observations:

For open field observation, the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm high) and observed for at least 2 minutes. The following parameters were assessed:

1. Behavior on removal from the cage
2. Fur
3. Skin
4. Salivation
5. Nasal discharge
6. Lacrimation
7. Eyes / pupil size
8. Posture
9. Palpebral closure
10. Respiration
11. Tremors
12. Convulsions
13. Abnormal movements / stereotypes
14. Gait abnormalities
15. Activity / arousal level
16. Feces excreted within 2 min (number of fecal pellets / appearance / consistency)
17. Urine excreted within 2 min (amount / appearance)
18. Number of rearings within 2 minutes

Sensimotor tests / reflexes:

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. Approach response (reaction to an object being moved towards the face)
2. Touch response (touch sensitivity)
3. Visual placing response (vision)
4. Pupillary reflex
5. Pinna reflex
6. Auditory startle response
7. Righting response (coordination of movements)
8. Behavior during handling
9. Vocalization
10. Tail pinch (pain perception)
11. Forelimb grip strength
12. Hindlimb grip strength
13. Landing foot-splay test
14. Other findings

7. Motor activity measurement

Motor activity was measured on the same day as the FOB was performed, from 14:00 h onwards. The examinations were performed using the TSE Labmaster System (TSE Systems GmbH, Bad Homburg, Germany). For this purpose, the animals were placed in new clean polycarbonate cages with a small amount of bedding for the duration of the measurement. Eighteen beams were allocated per cage. The number of beam interrupts was counted over 12 intervals for 5 minutes per interval. The sequence in which the animals were placed in the cages was selected at random. On account of the time needed to place the animals in the cages, the starting time was "staggered" for each animal. The measurement period began when the first beam was interrupted and finished exactly 1 hour later. No food or water was offered to the animals during these measurements and the measurement room was darkened after the transfer of the last animal.

8. Neuropathology

The first five surviving animals per sex and test group were selected for neuropathology evaluation. These animals were sacrificed by perfusion fixation under deep Isoflurane anesthesia. Soerensen's phosphate buffer was used as the rinsing solution and fixation was performed with a solution according to Karnovsky. The remaining animals were sacrificed using CO_2 and discharged without further examination. The sacrificed animals were necropsied and the visible organs or organ sections were assessed by gross pathology as accurately as it is possible for perfused animals. The weight of the brain (without olfactory bulb) was determined in all perfused animals after removal of the brain but before any other preparation. For determination of the relative brain weights the terminal body weights were used.

Additionally to organ/tissues listed in paragraphs below, the following organs/tissues were preserved in neutral buffered 4% formaldehyde:

- Brain (remaining material after trimming)
- Spinal cord (parts of cervical and lumbar cord)
- Gross lesions

Various peripheral nerves, parts of the brain and brain-associated organs, parts of the spinal cord and muscles were embedded and histologically examined. The remaining organ material and the animal bodies were stored in neutrally buffered 4% formaldehyde solution.

The following brain cross sections:

- Frontal lobe
- Parietal lobe with diencephalon and hippocampus
- Midbrain with occipital and temporal lobe
- Pons
- Cerebellum
- Medulla oblongata

from all animals were embedded in paraplast and hematoxylin and eosin (HE) staining and histopathological evaluation was performed on sections from control and high-dose group animals.

Paraplast embedding, HE staining and microscopic evaluation were also performed for the following tissues from animals of the control and high dose groups:

- Eyes with retina and optical nerve
- Longitudinal and/or cross sections of the spinal cord (cervical: C3-C6, lumbar: L1-L4)
- Peripheral nervous system (Trigeminus ganglia with nerve and gastrocnemius muscle),

whereas the tissues from the low- and mid-dose groups were stored in neutrally buffered 4% formaldehyde solution.

From the perfused animals of the control and high-dose groups the following nerves of the peripheral nervous system were embedded in plastic (epoxy resin), semi-thin sections prepared and, stained with Azure-II-Methylene blue-basic fuchsin (AMBF) for subsequent assessment by light microscopy:

- Dorsal root ganglion, (3 of C3–C6)
- Dorsal root fiber (C3-C6)
- Ventral root fiber (C3-C6)
- Proximal tibial nerve (at knee)
- Dorsal root ganglion, (3 of L1-L4)
- Dorsal root fiber (L1-L4)
- Ventral root fiber (L1-L4)
- Distal tibial nerve (at lower leg)

whereas the tissues from the low- and mid-dose groups were stored in neutrally buffered solution.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

No animal died or was sacrificed moribund during the entire study period.

2. Clinical signs of toxicity

No test substance-related, adverse findings were observed during clinical examinations.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Test substance-related effects on body weight were not observed in animals of the low- or mid-dose groups (200 and 600 mg/kg bw) compared to the control (see Table 5.7.1-2).

Table 5.7.1-2: Body weight development

Dose level [mg/kg bw]	Males				Females			
	0	**200**	**600**	**2000**	**0**	**200**	**600**	**2000**
Body weight [g]								
- Day -7	156.1	157.9	155.5	154.5	129.6	134.5	135.8	132.2
- Day 0	201.7	201.2	201.5	202.0	148.3	155.3	156.3	150.8
Δ% (compared to control)		(-0.2)	(-0.1)	(+0.1)		(+4.7)	(+5.4)	(+1.7)
- Day 7	243.8	241.1	245.2	**231.8**	166.8	171.7	175.1	**165.5**
Δ% (compared to control)		(-1.1)	(+0.6)	**(-4.9)**		(+3.0)	(+5.0)	**(-0.7)**
- Day 14	273.4	270.7	275.7	267.4	178.0	186.6	188.2	180.5
Δ% (compared to control)		(-1.0)	(+0.9)	(-2.2)		(+4.8)	(+5.7)	(+1.4)
Overall body weight gain (g)								
Day 0-7	42.1	39.9	43.7	**29.8****	18.5	16.5	18.9	**14.7**
Δ% (compared to control)		(-5.3)	(+3.9)	**(-29.1)**		(-10.9)	(+2.1)	**(-20.2)**
Day 0-14	71.6	69.5	74.2	65.4	29.7	31.3	31.9	29.7
Δ% (compared to control)		(-3.1)	(+3.6)	(-8.7)		(+5.3)	(+7.3)	(0.0)

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided)

At 2000 mg/kg bw, mean body weights of treatment groups were not significantly different from controls. However, mean body weight gain values were lower in high-dose male (-29%, significantly altered) and in high-dose female animals (-20%; not significantly altered) between study days 0 to 7, as compared to respective controls. These effects were assessed to be related to treatment on study day 0. The animals recovered until between study days 8 to 14, i.e. body weight change values were no longer altered in a statistically or biologically relevant way at the end of the study period.

D. FOOD AND WATER CONSUMPTION

Food and water consumption were not quantified. Overt effects of treatment were not noticed based on daily visual inspection.

E. FUNCTIONAL OBSERVATION BATTERY

Deviations from "zero values" were obtained in several animals. However, as most findings were equally distributed between treated groups and controls, were without a dose-response relationship or occurred in single animals, these observations were considered to have been incidental. Beside this, the FOB was performed in different examination segments, which have to be assessed individually:

1. Home cage observations

Test substance-related effects were neither observed on the day of administration nor on study days 7 and 14.

2. Open field observations

On the day of administration (study day 0) slight impairment of coordination, i.e. unsteady gait, was observed at 2000 mg/kg bw in 5 male and 3 female animals, as compared to none in male and female control animals. These slight changes with equivocal biological significance were assessed to be related to the bolus dosing of the relatively high, limit dose level. It did not occur on study days 7 and 14. No changes were observed at dose levels of 200 or 600 mg/kg bw on any study day.

3. Sensorimotor tests / reflexes

Test substance-related effects were not observed in treatment group animals on any day of observation.

On study day 7, male animal #28 of test group 3 (2000 mg/kg bw) did not show adaptation of pupils to light in both eyes. The pupils were permanently contracted after holding the animal in a dark chamber. As the finding occurred only once in a single animal and because it did not occur on study days 0 and 14, it was assessed to be incidental and not related to treatment.

4. Quantitative test parameters

On the day of administration (study day 0) test substance-related effects were observed in male animals of test group 3 (2000 mg/kg bw). Grip strength of forelimbs was significantly lower in male animals of test group 3 (-22%). In addition, the landing foot-splay test revealed a significantly increased distance between the hindlimbs. Both findings indicated a lower body tension. In light of the absence of any (histo-)pathological finding even at a dose level of 2000 mg/kg bw the change was assessed to be unrelated to structural neuronal damage but to be related to general toxicity and impaired well-being on the day of treatment.

In male animals of test group 2 (600 mg/kg bw) landing foot-splay test revealed a significantly increased distance between the hindlimbs, but the mean value of 11.6 cm was still within the historical control range (10 acute neurotoxicity studies started between 2010-2013, range of study means in male Wistar rats (Crl:WI(Han)): 7.5 - 12.0 cm). The change was assessed to be incidental. On study day 0, grip strength of forelimbs was significantly higher in male animals of test group 1 (200 mg/kg bw). As the grip strength was not decreased and did not occur in combination with other changes it was assessed to be incidental and not related to treatment. No findings were observed for female animals at any dose level.

Table 5.7.1-3: Grip strength and landing foot-splay mean values in rats administered BAS 750 F once and observed for 14 days

	Males				Females			
Dose level [mg/kg bw]	**0**	**200**	**600**	**2000**	**0**	**200**	**600**	**2000**
Grip strength forelimbs [Newton]								
- Day –7	4.7 ±1.1	4.8 ±1.1	5.2 ±0.6	5.0 ±0.9	4.6 ±1.3	4.6 ±1.1	4.6 ±1.2	5.2 ±0.9
- Day 0	5.1 ±0.8	**6.2* ±0.8**	4.9 ±1.5	**4.0* ±0.8**	6.4 ±0.8	6.5 ±0.9	6.5 ±1.0	6.8 ±0.8
- Day 7	5.5 ±1.4	4.8 ±1.3	4.8 ±1.1	4.9 ±1.4	5.8 ±1.6	6.4 ±1.3	5.9 ±1.9	6.5 ±1.1
- Day 14	4.1 ±1.7	4.6 ±1.2	5.4 ±1.8	4.8 ±1.5	5.8 ±1.3	6.5 ±1.7	6.8 ±2.1	6.5 ±1.6
Grip strength hindlimbs [Newton]								
- Day –7	2.7 ±0.6	2.8 ±0.4	2.8 ±0.4	2.8 ±0.3	3.8 ±0.5	4.1 ±0.5	4.3 ±0.6	4.1 ±0.4
- Day 0	4.0 ±0.4	3.6 ±0.3	3.5 ±0.5	3.5 ±0.4	3.9 ±0.5	3.9 ±0.6	3.8 ±0.6	3.4 ±0.4
- Day 7	4.6 ±0.8	4.3 ±0.7	4.4 ±0.7	4.5 ±1.1	4.1 ±0.7	3.8 ±0.5	4.3 ±0.8	4.1 ±0.4
- Day 14	4.9 ±0.8	4.6 ±0.9	5.0 ±0.8	5.2 ±1.0	6.2 ±0.7	5.8 ±0.8	6.7 ±0.8	5.8 ±0.8
Landing foot-splay [cm]								
- Day –7	8.9 ±0.9	8.4 ±0.9	8.9 ±1.1	8.6 ±0.8	8.8 ±1.2	9.2 ±1.5	8.6 ±1.2	9.9 ±1.4
- Day 0	9.3 ±1.0	10.0 ±1.4	**11.6** ±1.6**	**12.5** ±1.1**	10.0 ±1.0	10.2 ±1.5	10.9 ±2.2	12.0 ±1.7
- Day 7	9.7 ±1.0	10.0 ±0.9	9.8 ±1.2	9.6 ±1.1	9.3 ±1.3	8.6 ±0.9	8.3 ±0.8	8.7 ±1.5
- Day 14	9.1 ±1.1	9.8 ±1.5	9.5 ±0.7	9.7 ±0.9	8.7 ±1.4	9.7 ±1.4	9.0 ±1.5	9.9 ±1.6

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)

5. Motor activity

Study day -7

No significant differences were observed between test and control groups.

Study day 0

With regard to the overall motor activity, the mean values for male and female animals of test group 3 (2000 mg/kg bw) were significantly reduced. Comparing the single intervals of this test group with the control group, significantly decreased values were measured for male animals at intervals 1, 2, 3, and 4 as well as for female animals at intervals 1, 2 and 3. All mentioned findings were assessed as being related to treatment.

Changes with regard to the overall motor activity were also observed for female animals of test group 2 (600 mg/kg bw). Comparing the single intervals of this test group with the control group, a single statistically significantly decreased value was measured at interval 6 only. However, a similar decrease in interval 6 motor activity was not observed at 2000 mg/kg bw. Because of its isolated occurrence and the lack of other associated findings it was not considered to be a treatment-related adverse effect.

No significant deviations were observed in male animals of test groups 1 and 2 (200 and 600 mg/kg bw) when compared to the control group. The same was true for female animals of test group 1 (200 mg/kg bw).

Table 5.7.1-4: Overall mean motor activity in rats administered BAS 750 F once and observed for 14 days [mean number beam interruptions]

	Males				Females			
Dose level [mg/kg bw]	**0**	**200**	**600**	**2000**	**0**	**200**	**600**	**2000**
Body weight [g]								
- Day -7	2858 ±524	3167 ±546	2924 ±753	3418 ±735	4039 ±1001	3350 ±947	3899 ±889	3708 ±1148
- Day 0	3019 ±807	2990 ±505	2454 ±533	**1436** **±446**	4510 ±784	4240 ±1360	**3480** **±523**	**2233** **±639**
- Day 7	2679 ±587	2829 ±380	3225 ±618	3162 ±809	3812 ±938	4551 ±1251	5573 ±1804	4837 ±2016
- Day 14	2933 ±704	2930 ±546	3140 ±597	2891 ±933	4037 ±1005	3959 ±1399	5889 ±3692	4552 ±1923

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxon test (two-sided)

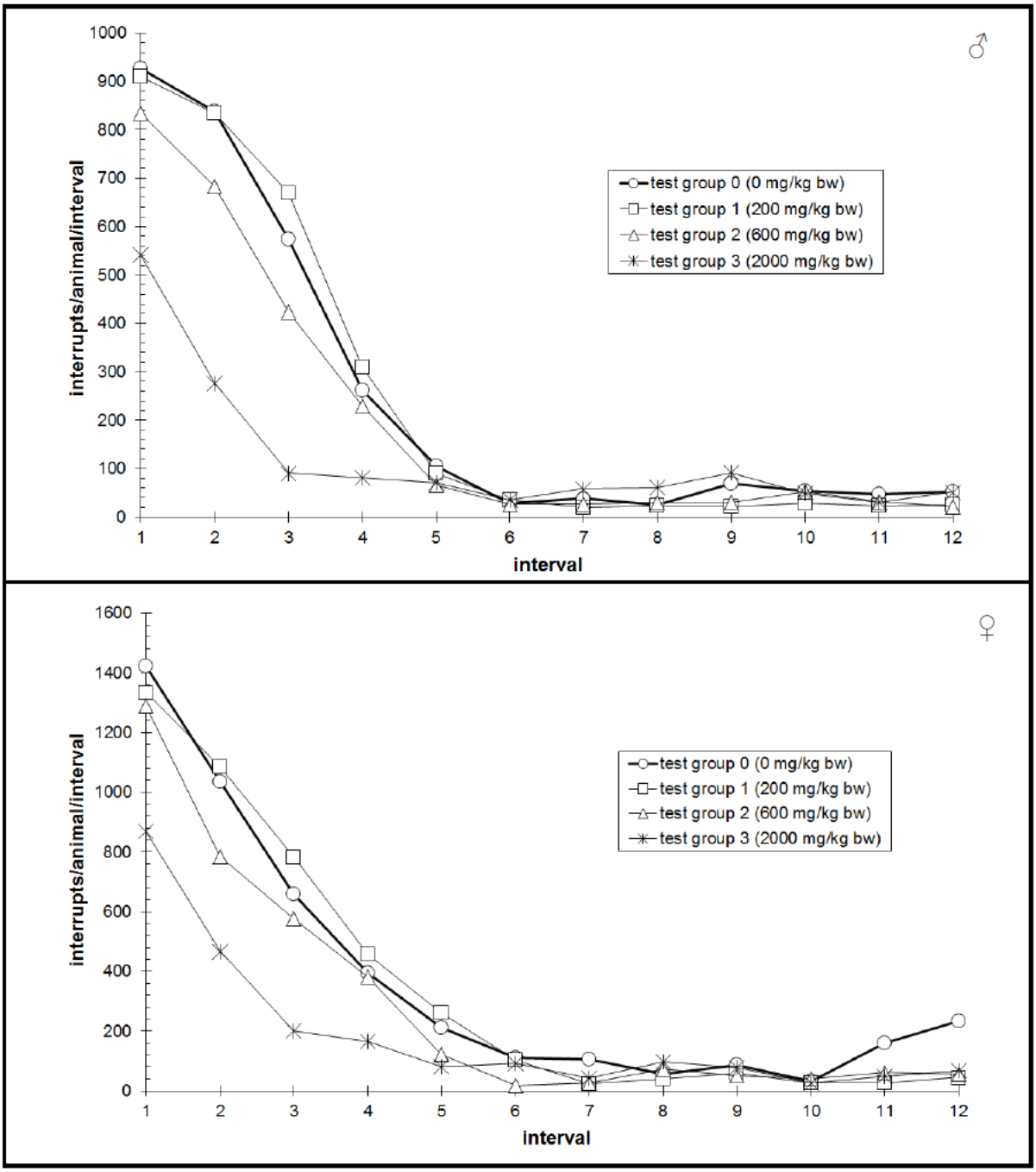
Study day 7

No biologically relevant differences were observed between test and control groups. Comparing the single intervals with the control groups, significantly increased values were measured for female animals of test group 2 and 3 (600 and 2000 mg/kg bw) at interval 3. As these changes indicated a slightly higher activity and did not influence the overall motor activity, they were assessed to be incidental and not related to treatment and underscore the variable nature of motor activity measurements.

Study day 14

No biologically relevant deviations were observed in male and female test group animals when compared to the control group. The significantly reduced single interval 7 for male animals of test group 1 (200 mg/kg bw) was assessed as being incidental and not related to treatment.

Figure 5.7.1-1: Motor activity measurement on Day 0



F. NECROSCOPY AND PATHOLOGY

1. Terminal body weight and brain weight

No significant weight changes were noted.

Table 5.7.1-5: Organ weights

Sex		Males				Females			
Organ weight	Dose [mg/kg bw]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	247.70				167.36			
	200	245.26	(-1)			176.00	(+5)		
	600	255.80	(+3)			172.76	(+3)		
	2000	239.94	(-3)			167.04	(±0)		
Brain (g)	0	1.902		0.768		1.782		1.067	
	200	1.896	(±0)	0.775	(+1)	1.816	(+2)	1.033	(-3)
	600	1.904	(±0)	0.744	(-3)	1.874	(+5)	1.088	(+2)
	2000	1.864	(-2)	0.778	(+1)	1.786	(±0)	1.072	(±0)

no statisical significances were observed ($\alpha = 0.01$) (Kruskal-Wallis and Wilcoxon-test, two sided)
Values may not calculate exactly due to rounding of figures

2. Gross pathology

No abnormalities were observed at gross necropsy.

3. Histopathology

No treatment-related findings were noted. One high-dose male (#17) was found with ocular inflammation and corneal degeneration – this was regarded as incidental as it was unilateral and occurred in one animal only.

G. POSTIVE CONTROLS

No concurrent positive control was employed in this study.

However, in several positive control studies, behavioral and neuropathological sequelae of substances with nervous system effects were evaluated using functional observational batteries (FOB), motor activity measurements and neuropathology (data included in the study report). Clinical signs of peripheral neuropathy (e.g. ataxia, limb weakness), central neuropathy (e.g. tremors) and autonomic signs (e.g. salivation) could be shown. Histopathologically, changes in the peripheral nervous system (e.g. Wallerian-like degeneration) and central nervous system (e.g. neuronal necrosis) were seen. The motor activity device was able to show both increased and decreased activity. The inter-observer reliability of the technicians performing FOBs was proven. Thus, the ability of the methods used to detect signs of neurotoxicity was demonstrated.

Positive control studies employed single or repeated administration of the following neurotoxicants: 3,3'-Iminodipropionitrile, Carbaryl, Nomifensin, Diazepam, Acrylamide, Triethyltin bromide, Methylphenidate hydrochloride, Neostigmine bromide, (R)-(-)-Apomorphine hydrochloride, and Trimethyltinchloride. Study summaries are attached to the report.

III. CONCLUSION

In the acute oral neurotoxicity study in rats, single gavage administration of BAS 750 F at a dose level of 2000 mg/kg bw caused reductions in body weight gain within the first week after treatment in both sexes (-29%* in males and -22%^{ns} in females), followed by recovery during the second week of observation.

A few neurobehavioral effects were noted at the high-dose of 2000 mg/kg bw on the day of administration, which were considered to be test substance related:

- Unsteady gait 5 of 10 males and 3 of 10 females were observed with during the Open Field examinations.
- Reduced motor activity in males and females.
- Grip strength of forelimbs was reduced in males.
- Increased distance between hindlimbs of males in the Landing Foot-splay test.

These parameters were comparable between test and control groups on study days 7 and 14.

Regarding neuropathology, brain weight determination, necropsy and neuropathology examination by light microscopy did not reveal any neuropathological, treatment-related findings up to 2000 mg/kg bw (highest dose tested; HDT) in both sexes.

Therefore, the affected clinical parameters were assessed to be unrelated to structural or functional neuronal damage but to be related to transient general systemic toxicity and impaired well-being, most probably resulting from bolus application of a high dose.

The no observed adverse effect level (NOAEL) of this study was 600 mg/kg bw for male and female Wistar rats with regard to systemic toxicity and neurobehavioural effects. There were no indications of structural damage to the neurons at dose levels up to and including 2000 mg/kg bw, the highest dose level tested.

CA 5.7.2 Delayed polyneuropathy studies

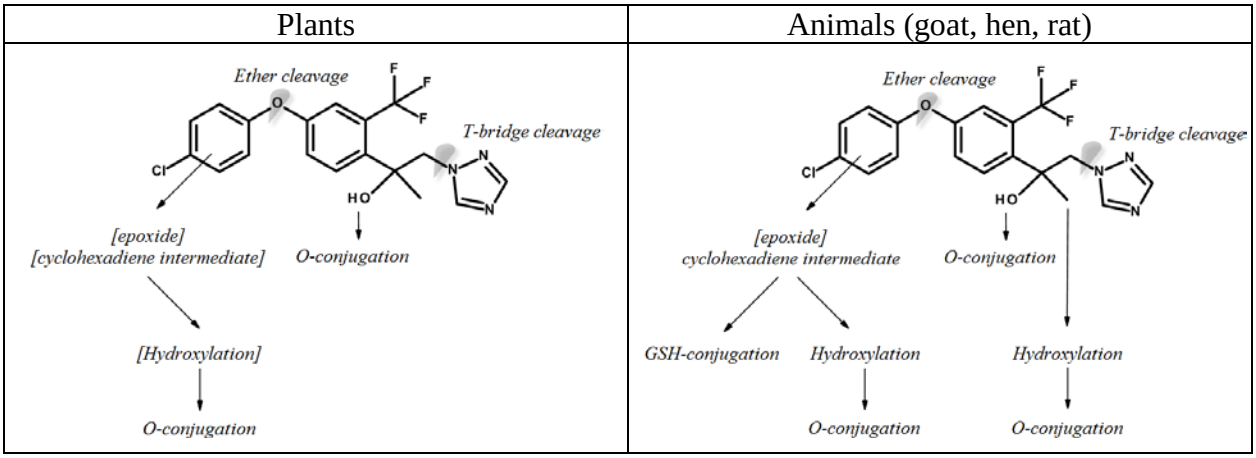
According to Comm Reg. (EU) 283/2013, these studies shall be performed for active substances of similar or related structures to those capable of inducing delayed polyneuropathy such as organophosphorus compounds. BAS 750 F does not belong to the chemical classes suspected to cause delayed neurotoxicity. Therefore, no acute delayed neurotoxicity study was performed.

CA 5.8 Other Toxicological Studies

CA 5.8.1 Toxicity studies of metabolites

BAS 750 F is extensively metabolized in plants and animals, following straight-forward and uniform principles across all species (see Figure 5.8.1-1). The metabolism of BAS 750 F comprises hydroxylation mainly at the chlorophenyl ring with or without subsequent conjugate formation, and cleavage of the three-ring molecule either at the ether bridge or by release of the triazole-ring. Some of the resulting smaller molecules themselves are hydroxylated/oxidated and/or conjugated.

Figure 5.8.1-1: BAS 750 F transformation reactions



Following grouping of metabolites in feed and in edible food items according to chemical similarity and common metabolic pathways, the extent of their occurrence in BAS 750 F studies of rat metabolism was determined. With one exception (M750F022, see below), all metabolites were shown to be formed in the rat at significant proportions (> 10% of the administered dose) and were therefore considered to be sufficiently addressed in toxicological studies performed with BAS 750 F.

M750F022 (*syn.* Reg.No. 6011210) is a metabolite that was identified as residue in a hen metabolism study. Compared to BAS 750 F, Reg.No. 6011210 has a hydroxyl group instead of the triazole ring as a result of cleavage. In the rat metabolism studies with BAS 750 F, Reg.No. 6011210 was not found at significant amounts, although related metabolites have been found also in the rat. Therefore, toxicological studies were carried out with Reg.No. 6011210. Detailed study summaries are found at the end of the toxicological relevance assessment performed for animal and plant metabolites.

Reg.No. 6011210 was of low acute toxicity by the oral route in rats. Three *in vitro* genotoxicity assays were performed with Reg.No. 6011210 (Ames test, mouse lymphoma assay and micronucleus assay with human lymphocytes) all of which did not indicate a genotoxic concern. As presented in section 5.8.2 in more detail, M750F022 showed considerably lower potential for aromatase inhibition than BAS 750 F in an *in vitro* aromatase inhibition assay.

Reg.No. 6011210 was tested in a 28-day dietary study in C57BL/6JRj mice at dose levels of 2500 ppm (selected as top dose on the basis of a 14-day range-finding study), and at dose levels of 872 and 87 ppm, which are molar equivalent dose levels corresponding to 1000 and 100 ppm BAS 750 F, that was tested in a 28-day mouse study (see section 5.3). Similar to BAS 750 F, the target organ of Reg.No. 6011210 was the liver, based on changes in clinical chemistry parameters, liver weight increases and signs of degenerative changes at 872 and 2500 ppm. The top dose level of 2500 ppm exceeded the MTD causing marked reductions in body weight and body weight gain in female mice.

In view of the comparable toxicological profile of BAS 750 F and M750F022, and lack of concern related to aromatase inhibition, it is proposed to apply the reference values of BAS 750 F for human risk assessment of M750F022.

Table 5.8.1-1: Summary of toxicological studies with M750F022 (*syn.* Reg.No. 6011210)

Study **Batch / purity** **Dose levels (ppm)** **Intakes (mg/kg bw/d)**	**NOAEL (mg/kg bw/d)**	**Main results**	**Reference (BASF DocID)**
Acute oral toxicity Female Wistar rat L85-116 / 99.0%		Rat LD_{50} oral: > 2000 mg/kg bw	[redacted] 2015a (2015/1175551)
Mutagenicity in bacteria Ames test L85-106 / 98.3%		Not mutagenic in bacteria with and without metabolic activation (S9 mix)	Woitkowiak, 2015b (2015/1174564)
Mutagenicity in mammalian cells L5178Y mouse lymphoma assay L85-116 / 99.0%		Not mutagenic in the mouse lymphoma assay with and without metabolic activation (S9 mix)	Schulz & Landsiedel, 2015a (2015/1174532)
Clastogenicity in mammalian cells Micronucleus test in human lymphocytes L85-106 / 98.3%		Not mutagenic in the in-vitro micronucleus test in human lymphocytes with and without metabolic activation (S9 mix)	Sokolowski, 2015b (2015/1038964)
28-day oral (diet) C57BL/6 Rj mouse 5 male and 5females/group L85-116 / 99.0% 0 – 87 – 872 – 2500 ppm ♂: 0 – 20 – 180 – 587 mg/kg bw/d ♀: 0 – 32 – 249 – 718 mg/kg bw/d	♂:: 20 ♀: 249	<u>> 872 ppm:</u> 🡻 Triglycerides (♂) 🡹 slight liver wt (+9%) with hypertrophy 🡹 multifocal necrosis in 2 of 5 males <u>2500 ppm:</u> 🡻 bw (♀), bw gain (♂+♀) and food intake (♀) 🡹 ALP (♂+♀), ALT (♂) 🡻 Cholesterol, total protein and albumin (♂) 🡹 liver wt (rel. +52%♂ +64%♀) with heptatocell. Hypertrophy /fine granular eosinophilic cytoplasm; multifocal necroses in 4 ♂ and 1 ♀	[redacted] 2015a (2016/1000646)

ASSESSMENT OF METABOLITES IN FOOD OF PLANT AND ANIMAL ORIGIN

INTRODUCTION

For the toxicological relevance assessment of BAS 750 F metabolites that were identified in food commodities, a grouping approach was applied, and, if applicable and considered necessary, key structures were selected for further in-depth evaluation.

The applied grouping of metabolites took into account

A) Chemical similarity and common metabolism pathways

B) Coverage by mammalian toxicity studies conducted with the parent BAS 750 F

A Chemical Similarity

With regard to evaluation of chemical similarity the general proposals given by e.g. by the EFSA Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment [EFSA Journal 2012;10(07):2799] were followed, taking into consideration:

- Metabolic steps that were identified to probably not cause additional toxicity of the metabolites:
  - Simple demethylation of the ring or side chain
  - Simple hydroxylation of the ring system without any cleavage of the ring
  - Hydroxylation of another ring position than the parent molecule
  - Conjugation of metabolite with amino acid
- Consideration of conjugated metabolites being bioavailable as their unconjugated products (cleavage of glutathione, sulphate, O-glucuronids or sugar conjugates in the human gastrointestinal tract)

Comparison was made to parent as well as to the grouped metabolites in order to select key metabolites for testing. In addition consideration of increased hydrophilicity and thus considered faster excretion of the grouped metabolites as compared to the tested key metabolites and/or parent was taken into account.

B Coverage of metabolites of concern by mammalian toxicity studies

The species comparison showed a remarkable overlap in the metabolism of BAS 750 F in animals and plants. Therefore, general conclusions drawn from the animal metabolism of BAS 750 F as well as overlaps of metabolite structures were taken into consideration when grouping the metabolites with regard to human exposure from the source livestock, plant or rotational crop. It was considered whether either the metabolites under consideration or similar structures were formed in the metabolism studies conducted in mammals. Moreover, it was considered that the uptake of food commodity metabolites of BAS 750 F could subsequently be transformed by known metabolic pathways into structures that have been identified in the mammalian metabolism studies.

Phase II conjugation of hydroxylates (O-bound sugar conjugation, glucuronidation or sulfatation were considered to generally increase the excretability of the metabolite of concern. As shown in section MCA 5.1, hydroxylated, glucuronidated and sulfate-conjugated metabolites of BAS 750 F are commonly found in excreta of rats, and also glutathione conjugation was demonstrated to occur in rat metabolism. Thus, these conjugates are covered by the rat metabolism of the parent molecule. With regard to O-bound sugar conjugates, which are specific to plant metabolism, these O-glycosylated conjugates are known to be readily cleaved in the mammalian gastrointestinal tract, back-transforming the conjugated metabolite to the respective hydroxylates, which are assessed accordingly.

GROUPING

The BAS 750 F molecule consists of three ring substructures:

- 1,2,4-triazole ("T-ring")
- Chlorophenyl-ring ("C-ring") at the opposite side of the molecular backbone, and the
- Trifluoromethylphenyl-ring ("TFMP-ring") in between T-ring and C-ring

The TFMP-and C-rings of BAS 750 F are connected via an ether bridge, and the T-ring is linked to the TFMP-ring via a propan-2-ol rest in ortho position to the trifluoromethyl side chain.

BAS 750 F is metabolized in both plants and in animals and principally follows straight-forward pathways that are common to both plant and animal (rat and livestock) metabolism. Hence, the metabolic pathways of BAS 750 F in both animals and plants mainly proceed according to one of the three following routes …

a) Conjugation of the parent molecule

b) Hydroxylation at the C-Ring with/without subsequent conjugation

c) Cleavage of the T-ring or the C-ring from the molecule, followed by subsequent hydroxylation, oxidation and conjugation.

… with the cleavage at the ether bridge being of minor importance for plant metabolism of BAS 750 F.

In livestock metabolism (and also in rats), the parent molecule or hydroxylated metabolites are subjected to subsequent conjugation, i.e. with glutathione-conjugation, O-glucuronidation, sulfatation and fatty acid ester formation, while in plants, a range of O-glycosylated metabolites are formed.

The identified metabolites in food commodities of plant and animal origin (see also section 6.2) were grouped according to their chemical structure together with their respective conjugates. An overview of the grouping is presented in Table 5.8.1-2. The non-conjugated metabolites that were identified in food commodities are underlined.

Table 5.8.1-2: Grouping of BAS 750 F metabolites in food of plant or animal origin

Group Definition	**(Non-conjugated) Group metabolite members in**			
	PLANTS		**LIVESTOCK**	
	≥ 10% TRR	**< 10% TRR**	**≥ 10% TRR**	**< 10% TRR**
a) Parent and conjugates				
	BAS 750 F	M750F011 M750F012 M750F013 M750F014 M750F028	**BAS 750 F** M750F068	M750F072
b) "C-Ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates				
	M750F018 M750F019 M750F020 M750F026 M750F027	–	**M750F016** M750F034	**M750F015** **M750F041** M750F063
c) Cleavage products & downstream metabolites / conjugates				
Metabolites without the C-Ring	–	M750F009 M750F010	–	**M750F003**
Metabolites without 1,2,4-T-ring	–	–	**M750F022** M750F023 M750F024 M750F025 **M750F038** M750F043 M750F064	–
1,2,4-triazole and triazole-derived metabolites	**1,2,4-T** **TA** **TAA**	**TLA**	**1,2,4-T**	–

The conjugated metabolites (O-glycosylates, sulfates, O-glucuronides, and glutathione conjugates are considered to be unstable under acidic conditions within the human gastrointestinal tract resulting in cleavage to the predecessor (hydroxy) metabolites.

OUTCOME OF TOXICOLOGICAL RELEVANCE ASSESSMENT

The following reference Table 5.8.1-3 gives a comprehensive overview of the assessed metabolites sorted according to metabolite code, including a depiction of the molecular structure, type of conjugate (if applicable), origin of food commodity as major or minor metabolite, and extent of coverage in the rat metabolism study. Details on the toxicological assessment are given in the subsequent sections for each group of metabolites as defined in Table 5.8.1-2, or if necessary, for metabolites not sufficiently covered by rat metabolism of the parent molecule BAS 750 F.

Following grouping of metabolites in feed and in edible food items according to chemical similarity and common metabolic pathways, the extent of their occurrence in BAS 750 F studies of rat metabolism was determined. With one exception (M750F022, see below), all metabolites were shown to be formed in the rat at significant proportions (> 10% of the administered dose) and were therefore considered to be sufficiently addressed in toxicological studies performed with BAS 750 F.

Table 5.8.1-3: BAS 750 F metabolites in food of plant or animal origin

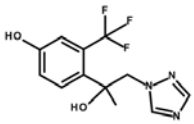
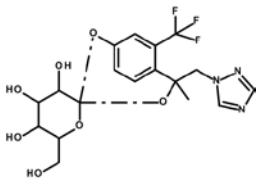
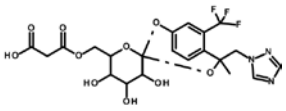
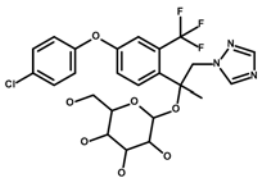
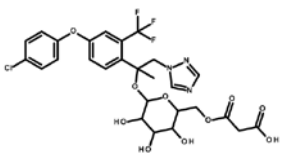
Code	Molecular structure	Conjugation type	Livestock / Plant	≥ 10% TRR (Y / N)	rat metabolism coverage [% AD]
M750F001 (1,2,4-T)		Non-conjugated	Plant	N	up to 20%
			Livestock	Y	
M750F003		Non-conjugated	Livestock	N	> 10%
M750F009		Sugar	Plant	N	See M750F003
M750F010		Sugar	Plant	N	See M750F003
M750F011		Sugar	Plant	N	See BAS 750 F
M750F012		Sugar	Plant	N	See BAS 750 F

Table 5.8.1-3 BAS 750 F metabolites in food of plant or animal origin (cont'd)

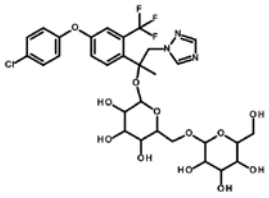
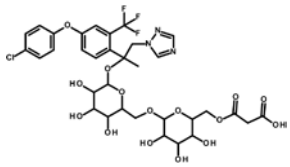
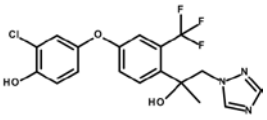
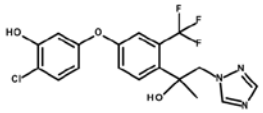
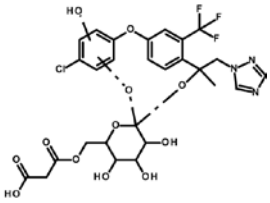
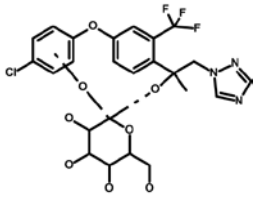
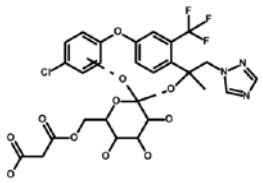
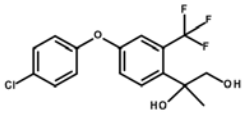
Code	Molecular structure	Conjugation type	Livestock / Plant	≥ 10% TRR (Y / N)	rat metabolism coverage [% AD]
M750F013		Sugar	Plant	N	See BAS 750 F
M750F014		Sugar	Plant	N	See BAS 750 F
M750F015		Non-conjugated	Livestock	N	>50%
M750F016		Non-conjugated	Livestock	Y	>50%
M750F018		Sugar	Plant	Y	See M750F016
M750F019		Sugar	Plant	Y	See M750F016
M750F020		Sugar	Plant	Y	See M750F016
M750F022		Non-conjugated	Livestock	Y	<<10%

Table 5.8.1-3 BAS 750 F metabolites in food of plant or animal origin (cont'd)

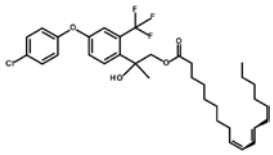
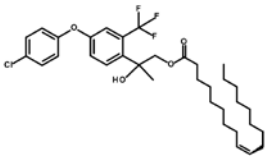
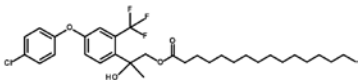
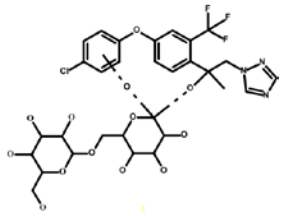
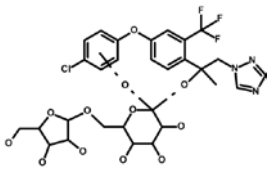
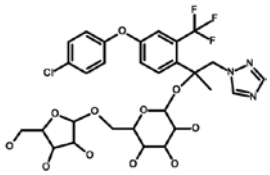
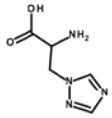
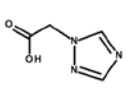
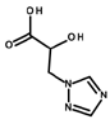
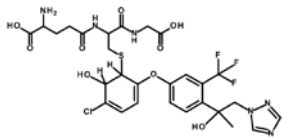
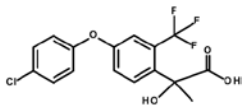
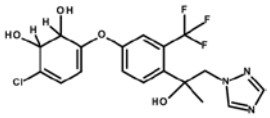
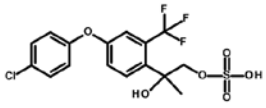
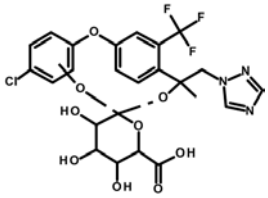
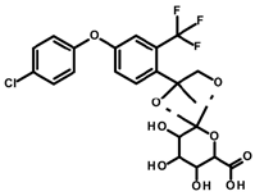
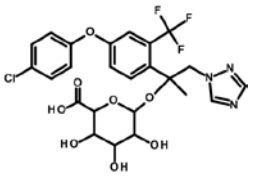
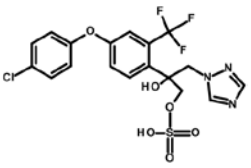
Code	Molecular structure	Conjugation type	Livestock / Plant	≥ 10% TRR (Y / N)	rat metabolism coverage [% AD]
M750F023		Fatty acid	Livestock	Y	See M750F022
M750F024		Fatty acid	Livestock	Y	See M750F022
M750F025		Fatty acid	Livestock	Y	See M750F022
M750F026		Sugar	Plant	Y	See M750F016
M750F027		Sugar	Plant	Y	See M750F016
M750F028		Sugar	Plant	N	See BAS 750 F
M750F029 (TA)		Non-conjugated	Plant	Y	Not found
M750F030 (TAA)		Non-conjugated	Plant	Y	Not found
M750F031 (TLA)		Non-conjugated	Plant	N	Not found

Table 5.8.1-3 BAS 750 F metabolites in food of plant or animal origin (cont'd)

Code	Molecular structure	Conjugation type	Livestock / Plant	≥ 10% TRR (Y / N)	rat metabolism coverage [% AD]
M750F034		Glutathione	Livestock	Y	See M750F016
M750F038		Non-conjugated	Livestock	Y	See M750F022
M750F041		Non-conjugated	Livestock	N	At least 12%
M750F043		Sulfate	Livestock	Y	See M750F022
M750F063		Glucuronide	Livestock	N	See M750F016
M750F064		Glucuronide	Livestock	Y	See M750F022
M750F068		Glucuronide	Livestock	Y	See BAS 750 F
M750F072		Sulfate	Livestock	N	See BAS 750 F

DETAILS OF TOXICOLOGICAL RELEVANCE ASSESSMENT FOR EACH METABOLITE GROUP

a) Parent and conjugates	
RESIDUES IN PLANT COMMODITIES	RESIDUES IN LIVESTOCK COMMODITIES
BAS 750 F (major residue, >10% TRR) M750F011 (< 10% TRR), sugar conjugate M750F012 (< 10% TRR), sugar conjugate M750F013 (< 10% TRR), sugar conjugate M750F014 (< 10% TRR), sugar conjugate M750F028 (< 10% TRR), sugar conjugate	BAS 750 F (major residue, >10% TRR) M750F068 (>10% TRR), O-glucuronide M750F072 (< 10% TRR), sulfate

In plants, the unchanged parent BAS 750 F is the only major residue component, besides triazole-derivative metabolites. The 50:50 enantiomer ratio of the TGAI remains unchanged (racemic mixture). In addition, different types of O-bound sugar conjugates may occur. In the gastrointestinal tract, the conjugates are hydrolyzed under acidic conditions and the non-conjugated parent is released.

In goat metabolism, BAS 750 F was identified as the major residue component. However, chiral analysis revealed a significant shift in the enantiomer ratio in most matrices towards higher levels of the R-enantiomer (50:50 → 80:20). BAS 750 F was also a major residue component in hens, but there was no significant change in the enantiomer ratio in hen metabolism. In addition, M750F068 (O-glucuronide of unchanged parent) was found in goat liver and kidney and minor amounts of M750F072 were found in milk. The sulfate conjugation occurs at the methyl group of BAS 750 F. In the gastrointestinal tract, the conjugates are hydrolyzed under acidic conditions and the non-conjugated parent is released in cases of the sugar and glucuronide conjugate, and hydroxylated parent in case of the sulfate conjugate, resulting in a "diol" structural motif at the methyl group. A similar diol motif is present in the metabolite M750F022, which was subjected to genotoxicity testing and did not give rise to toxicological concern.

The conjugated metabolites of the unchanged parent are considered to be covered by rat studies performed with BAS 750 F due to the expected formation of unchanged (or in the case of the sulfate, hydroxylated) parent in the human gastrointestinal tract as a result of hydrolysation of the sugar-, sulfate or O-glucuronide conjugate under acidic conditions. The enantiomer shift from a racemic 50:50 ratio towards higher abundance of the R-enantiomer as seen in goat metabolism is not considered to be of toxicological concern. This enantiomer shift occurs in rats to a similar extent (up to 86:15 within 1 hour after dosing) Moreover, results of a comparative aromatase inhibition assay indicated the R-enantiomer to have a lower potential to inhibit aromatase as compared to the depleted S-enantiomer, and thus the observed shift might reflect preferential metabolisation and/or translocation resulting in de-toxification.

b) "C-Ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates
RESIDUES IN PLANT COMMODITIES
M750F018 (> 10% TRR) O-malonyl-glucosyl-conjugate with a second hydroxylation of the C-ring M750F019 (> 10% TRR) O-glucosyl-conjugate M750F020 (> 10% TRR) O-malonyl-glucosyl-conjugate M750F026 (> 10% TRR) O-glycosyl-glucosyl-conjugate M750F027 (> 10% TRR) O-pentosyl-glucosyl-conjugate

In food commodities from **plants**, a range of O-glycosylated conjugation products were identified, derived from (assumed) intermediates with hydroxylation of the C-ring at different ring positions; these sugar conjugates form a structurally highly comparable group of compounds. The expected hydrolysis in the human digestive system will release a (C-ring) hydroxy-metabolite.

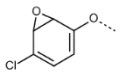
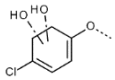
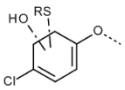
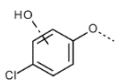
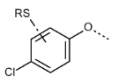
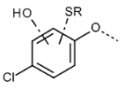
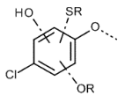
These compounds are common metabolites of BAS 750 F, also abundantly found in animal metabolism. In the rat, the hydroxy metabolites M750F016 and M750F017 and their glucuronide conjugates are found in significant amounts (typically >50% of dose, see **Table 5.8.1-4**). It can therefore be assumed the toxicity of this metabolite group is adequately covered by existing toxicity studies with BAS 750 F.

RESIDUES IN LIVESTOCK COMMODITIES
M750F016 (>10% TRR); non-conjugated metabolite M750F034 (> 10% TRR); glutathione-conjugated metabolite M750F015 (< 10% TRR); non-conjugated metabolite M750F041 (< 10% TRR); non-conjugated metabolite M750F063 (< 10% TRR); O-glucuronide of parent. M750F016 or M750F017

M750F016 is a C-ring hydroxylated metabolite of the unchanged parent backbone. It is the most abundant member of a group of isomers, including also M750F015 (minor metabolite, see below) and M750F017 (quantified only in excreta, see section CA 6.2). These hydroxy-metabolites are abundantly present in rat (representing up to 12% of dose). Together with conjugates forms and intermediate transformation products (notably M750F041 and M750F034, see below) of this C-ring hydroxylation branch, they account for up to 72% of dose in rat metabolism (see Table 5.8.1-4). It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies with BAS 750 F (section CA 5).

M750F015 is a regio-isomer, thus closely structurally related, of the C-ring hydroxylated metabolites M750F016 and M750F017, where as part of the transformation reaction at the C-ring, the chlorine was shifted to a neighboring position (NIH shift). As detailed above, these C-ring hydroxylated metabolites as a group are abundantly present in rat metabolism (typically >50% of dose). It is occurring in goat only in low amounts (<3% TRR, 0.011 mg/kg, only TFMP-label). Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.

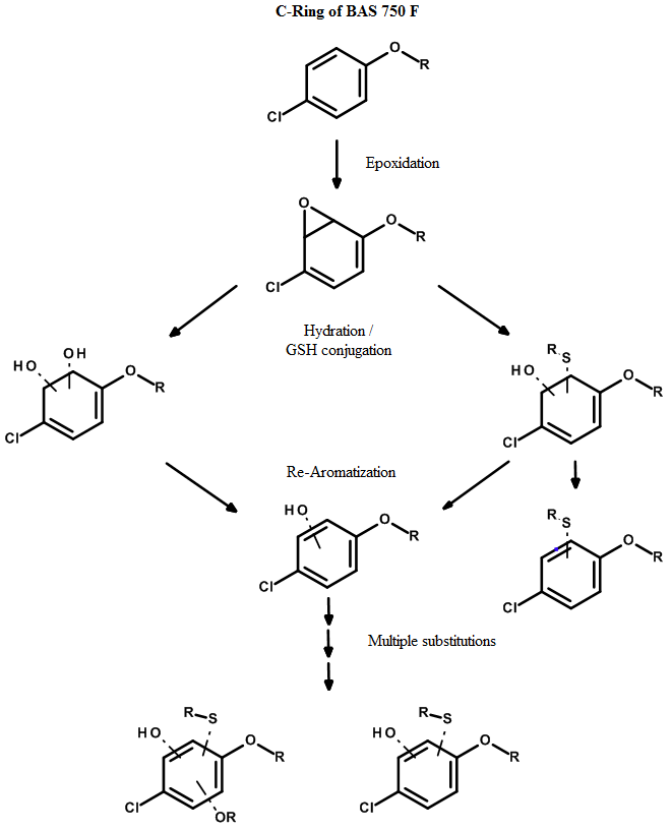
Table 5.8.1-4: Abundance of C-ring metabolites of BAS 750 F in rat metabolism

RAT C-ring metabolites		maximal dose recovery (per metabolite group)					
		3-ring molecules: C , TFMP, T		2-ring molecules: C , TFMP		1-ring molecule: C	
		Metabolite code	*1)* % dose	Metabolite code	*1)* % dose	Metabolite code	*1)* % dose
ether intermediate		-	n.d.	-	n.d.	-	n.d.
cyclohexadiene intermediates		-	n.d.	-	n.d.	-	n.d.
		**F087** F102 F085 F084 F091 F069	>10 (41)	F065 F106	<1	-	n.d.
C-ring hydroxylation		**F015 F035** F005 **F016 F044** F092 **F017 F045**	>12 (53)	F082 F105 F077 F078 F066	<1%	F094 F081	n.d.
C-ring conjugate		F053 F075 F104	>11	-	n.d.	F050 F079 F052 F083 F055	2
Multiple substitutions	 	**F049** F089 F108 F059 F110 F090 F062 F093 F098 F057 F088 F096 F063 F060 F097 F107	<1	F099 F067 F100 F061 F046	1.9	F048 F080	<1
Entire metabolic branch *2)*		Σ (F085… F107)	**72**	-	-	-	-

Note
Data on occurrence of individual metabolites are given in section MCA 5.1.1 (see tables 5.1-56 to -60). Coverage was calculated within a dose group (high dose/female, high dose/male, low dose/female, low dose /male) considering amounts detected in urine and bile. Additional amounts in tissues were not included due to differences in sampling time points.
1) the maximal value in any dose group, 2) the maximal value in any dose group (Σ F085 – F107, sum of all "downstream metabolites"). Metabolite codes are abbreviated, e.g. "F085" denotes "M750F085" etc., particularly abundant metabolites are indicated by bold typing. The recovery for the metabolic branch (C-ring hydroxylation) was typically >50% of dose. The highest recovery was 71.9% of dose (dose group: T-label, female, low dose) considering urine and bile including metabolites M750F049, M750F075, M750F087, M750F044, M750F045, M750F035, M750F015, M750F016, M750F017, M750F059.

M750F034 is an intermediate in the CYP mediated oxygenation of the C-ring (similar to goat metabolite M750F041, see below). The dihydrodiol structure of the C-ring is the precursor preceding the re-aromatization to more stable hydroxy metabolites, such as the regio-isomers M750F015 and M750F016 (C-OH metabolites, see Figure 5.8.1-2).

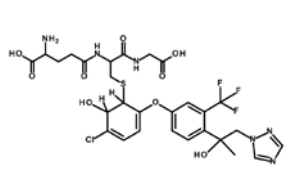
Figure 5.8.1-2: BAS 750 F transformation reactions: C-ring hydroxylation



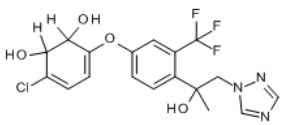
Dihydrodiol-cyclohexadiene structures, as such, are unstable under acid conditions (e.g. *Jerina et al.1970, Biochemistry 9(1):147-56*), known to re-arrange non-enzymatically to the hydroxylated aromatic ring. Likewise, M750F034 is considered unstable under dietary conditions, and to re-arrange, upon exposure to stomach acid, to a "C-OH metabolite" (namely M750F015, M750F016 and M750F017), thereby eliminating actual dietary exposure.

Furthermore, such cyclohexadiene intermediates, namely M750F087 (cyclohexadiene structure with -OH/-SR, see Figure 5.8.1-2) are also abundantly present in rat as a group amounting to at least 10.4% of dose and amounting to up to 53% of dose when considering also amounts co-eluting with structurally related down-stream metabolites (see Table 5.8.1-4). The latter C-ring hydroxy-metabolites and their glucuronic acid conjugates M750F035, M750F044, M75F045 (designated "C-OH group"), are found abundantly in rat amounting to at least 12% of dose. If structurally related co-elutes are additionally taken into account, namely M750F049 (C-ring di-hydroxylated metabolite) and M750F087, significantly higher dose coverages are obtained (see Figure 5.8.1-3).

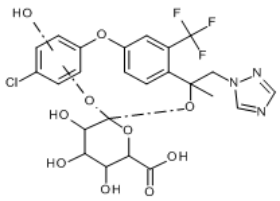
Figure 5.8.1-3: Structure relationship of M750F034, …F041, …F049 and …F087



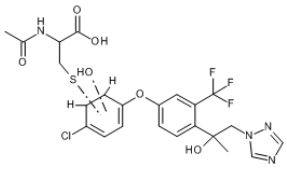
M750F034



M750F041



M750F049



M750F087

For example, the following recoveries were found in the rat metabolism study:

- 22% of dose for M750F044 co-eluting with M750F087 (high dose TFMP-label, male)
- 23% of dose for "C-OH group" co-eluting with M750F049 (high-dose T-label, male)
- 49-53% of dose for "C-OH group" co-eluting with M750F087/M750F049 (low dose, C-label, male/female)

M750F041 was found in ruminant milk (goat metabolism study) at non-major amounts (6.0 - 7.2% TRR, corresponding to <0.005 mg/kg). In practice, the concentration is expected to be <0.001 mg/kg in milk (see also CA 6.7). Similar to M750F034 (discussed above), the dihydrodiol-cyclohexadiene structure of M750F041 is inherently instable and expected to re-aromatize and re-arrange under the acidic conditions in the human stomach to a "C-OH metabolite" (namely M750F015, M750F016 and M750F017), thereby eliminating actual dietary exposure (see discussion on M750F034). In conclusion, based on abundant coverage in rat metabolism, only traces occurring in livestock commodities, as well as elimination under dietary conditions (stomach acid), this metabolite has no practical relevance for human consumption.

M750F063 is a glucuronide conjugate of the C-ring hydroxylated metabolite group. Hydrolytic removal of the glucuronide group in the human digestive system is expected to release the corresponding C-ring hydroxy-metabolite (M750F016 or M750F017). M750F063 was found in C-labelled goat samples co-eluting with M750F022, together accounting for less than 7% TRR (or less than 0.05 mg/kg). Since C-ring hydroxy-metabolites are much more abundant, the quantitative contribution of M750F063 is low. Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.

When taken together, structurally related metabolites of this metabolic branch amount to 72% of dose. It can therefore be considered that the toxicity of this metabolite group is covered by the existing toxicology studies with BAS 750 F.

c) Cleavage products & downstream metabolites / conjugates	
RESIDUES IN PLANT COMMODITIES	RESIDUES IN ANIMAL COMMODITIES
Metabolites without the C-Ring (all < 10% TRR) M750F009, O-glucoside conjugate of M750F003 M750F010, O-malonyl conjugate of M750F003	***Metabolites without the C-Ring*** (all >10% TRR) M750F003, non-conjugated

In food commodities from **plants**, products resulting from cleavage at the ether bridge followed by sugar conjugation, namely **M750F009** and **M750F010**, are found only in minor amounts (together accounting for <3% TRR in straw).

In food commodities from **animals**, the main product of cleavage at the ether bridge is **M750F003**, which was determined in quantifiable amounts in goat kidney (only with TFMP-label at 3.2% TRR, 0.14 mg/kg, absent in studies with triazole-labeled BAS 750 F). Under realistic exposure conditions, the extent of recoverable residue is expected to be less than 0.01 mg/kg.

M750F003 was identified in rats at >1.9% of the administered dose. Together with the conjugates M750F071 (sulfate conjugate) and M750F054 (glucuronide conjugate) it represents up to 12.5% of the dose in BAS 750 F treated rats.

It can therefore be considered that **the toxicity of this metabolite group (M750F003, M750F009, M750F010) is adequately covered by the existing toxicology studies with BAS 750 F in rats.** Thus, both from a toxicological as well as from an exposure point of view this metabolite group does not have any relevance for human consumption.

RESIDUES IN PLANT COMMODITIES	RESIDUES IN ANIMAL COMMODITIES
Metabolites without 1,2,4-T-ring -	***Metabolites without 1,2,4-T-ring*** (all >10% TRR) M750F022, non-conjugated M750F023, fatty acid conjugate of M750F022 M750F024, fatty acid conjugate of M750F022 M750F025, fatty acid conjugate of M750F022 M750F038, non-conjugated M750F043, sulfate-conjugate of M750F022 M750F064, O-glucuronide conjugate of M750F022

M750F022 (Reg.No. 6011210) is generated by cleavage of the T-bridge. Elimination of the triazole-ring leaves the ether structure, consisting of C-ring and TFMP-ring unchanged, therefore resulting in a cleavage product structurally related to the parent molecule.

In food commodities originating from **plants**, quantifiable amounts of residues lacking the 1,2,4-triazole ring were not found.

In ruminant **livestock**, M750F022 occurs only in kidney at major amounts (up to 10.7% TRR), otherwise in milk, muscle, liver and fat at <7.6% TRR. In contrast, in hen matrices this metabolite generally accounted for the predominant proportion; in fat M750F022 made up almost the complete residue when including also fatty acid conjugates (M750F023, M750F024, M750F025, see below). In **rats**, comparable downstream transformation products are seen for BAS 750 F and M750F022, including conjugates with glucuronic acid, sulfate and C-ring hydroxy metabolites. T-bridge cleavage is a key transformation step of BAS 750 F metabolism, as reflected by numerous downstream metabolites, which however were not measured at appreciably high quantities at the chosen sampling time points. M750F022 itself was not detectable under the study conditions.

Therefore, M750F022 was subjected to toxicological testing in order to confirm the lack of a genotoxic potential and to allow a comparison of the toxicological profile. An overview of the available studies, results and references is summarized in Table 5.8.1-1. Briefly, M750F022 (Reg.No. 6011210) was of low acute toxicity in rats after single oral dosing (LD_{50}: > 2000 mg/kg bw). M750F022 was assessed for *in vitro* genotoxicity in the Ames and the mouse Lymphoma assay, and for chromosomal aberration in the *in vitro* micronucleus test in human lymphocytes. No genotoxicity or chromosome damaging potential was observed in these studies. As the metabolic follow up reactions (GSH-conjugation, glucuronidation and sulfatation) are the same as those observed for parent and are normally considered to be detoxification reactions, it can be assumed that they do not contribute in any way to a putative genotoxic potential. In an *in vitro* aromatase inhibition assay, M750F022 showed considerably lower potential for aromatase inhibition than BAS 750 F (for details see section 5.8.3). Finally, in a 28-day oral diet study with mice, M750F022 showed comparable toxicity as BAS 750 F. The data clearly indicated that the two-ring molecular structure shared by BAS 750 F and M750F022 is determining the liver toxicity of both molecules. The derived NOAEL in the 28-day mouse study with M750F022 was 87 ppm (ca. 20 mg/kg bw/d).

In view of the comparable toxicological profile of BAS 750 F and M750F022, and lack of concern related to aromatase inhibition, it is proposed to apply the reference values of BAS 750 F for human risk assessment of M750F022.

M750F023, M750F024 M750F025 are fatty acid conjugates of M750F022, which upon hydrolytic removal of the fatty acid group will release metabolite M750F022. Consequently, it is appropriate to apply the toxicological reference values of M750F022 to this group of conjugates. While non-detectable in fish and goat, this group of metabolites accounted for a significant proportion of the residue in hen metabolism study (>25% TRR in fat, egg yolk, less in liver and kidney, and not more than 0.01 mg/kg in muscle). This observation is confirmed by *magnitude of the residue* study in hen (see section CA6.4) showing that the residue level for "*sum of M750F022 and fatty acid conjugates*" is higher than the residue level of "*M750F022*" (in fat by a factor of 4, in egg and muscle by a factor of 1.5, see MCA 6.7.1). Therefore, fatty acid conjugates are, from a toxicological perspective considered equivalent to M750F022, and from an exposure perspective considered to contribute to M750F022 residue in several hen matrices, namely fat (including skin), muscle and egg.

M750F038 is generated by conversion of the hydroxyl group of M750F022 to a carboxy group, leaving the remaining molecular structure unchanged. Consequently, toxicological properties equivalent to M750F022 can be assumed. By increasing hydrophilicity, excretability of the molecule can be assumed to be increased (compared to M750F022). This is consistent with the fact, that M750F038 occurrence in livestock is not only restricted to excretory organs (goat liver, kidney) but also found in significant amounts in excreta of these animals (notably in bile up to 11% TRR, in feces up to 6% TRR). Additional amounts are to be assumed for urine, where co-elution with M750F042 accounts for up to 28-47% of dose. Based on the overall high comparability of metabolic routes in ruminants and rat, a similar excretion behaviour is to be expected for rat (and thus also for humans). Therefore, it is appropriate to apply toxicological reference values of M750F022 to M750F038.

M750F043 is a sulfate conjugate of M750F022. Hydrolytic removal of the sulfate group in the human digestive system is expected to release M750F022, thus same toxicological properties apply to M750F043. It is occurring in goat milk at 14.2% TRR (C-label) and 25% TRR (T-label) albeit corresponding to only very low amounts (0.004 mg/kg and 0.016 mg/kg respectively). Under realistic exposure conditions, the concentration is expected to be < 0.01 mg/kg. Thus, both from a toxicological as well as exposure point of view M750F043 does not have any relevance for human consumption.

M750F064 is a glucuronide conjugate of M750F022. Hydrolytic removal of the glucuronide group in the human digestive system is expected to release M750F022, thus same toxicological properties apply to M750F064. It is occurring in goat kidney (C-label), co-eluting with M750F038, another M750F022 structurally related metabolite, together accounting for 26.6% TRR (0.09 mg/kg).

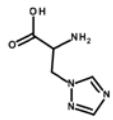
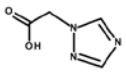
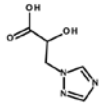
RESIDUES IN PLANT COMMODITIES	RESIDUES IN ANIMAL COMMODITIES
1,2,4-triazole and triazole-derivative metabolites (TDMs) M750F001 = 1,2,4-triazole (< 10% TRR) M750F029 = triazolyl alanine (> 10% TRR) M750F030 = triazolyl acetic acid (> 10% TRR) M750F031 = triazolyl lactic acid (< 10% TRR)	***1,2,4-triazole and triazole-derivative metabolites (TDMs)*** M750F001 = 1,2,4-triazole (> 10% TRR)

M750F001 (1,2,4-triazole) is generated by cleavage of BAS 750 F at the T-bridge. It is a metabolite common to other triazole containing fungicides. In **plants**, 1,2,4-triazole is found only at low levels (< 2% TRR) due to subsequent amino acid conjugation and oxidation. In **livestock**, 1,2,4-triazole was found in major amounts in most tissues, namely in goat (>60% TRR in milk, muscle, kidney, lower in liver and fat), hen (>60% TRR in egg white, muscle, liver, kidney and fat), fish (> 10% TRR in filet and filet-skin) as well as in rat (up to 20% of dose).

Further **plant** metabolites of 1,2,4-triazole are **M750F029** (= triazole alanine, TA), which is a conjugation product of 1,2,4-triazole and occurs in major amounts in wheat grain and in soybean seed. **M750F030** (triazolyl acetic acid, TAA) was also identified as major metabolite in wheat grain, while **M750F031** (TLA) was present at minor amounts (<2% TRR). TA, TAA and TLA were not observed in animal matrices or in rat metabolism.

The toxicological data for 1,2,4-triazole, TA, TAA and TLA were assessed and reference values were derived for consumer risk assessment in 2007 (PRAPeR 14 Expert meeting). Since then, additional data for 1,2,4-T and the TDMs have become available, which were recently reviewed by the Rapporteur Member State United Kingdom. On the basis of this review, UK CRD has proposed to revise the reference values for 1,2,4-T and the TDMs. At the time this dossier was prepared, the EU peer review of the new data was pending.

Table 5.8.1-5: Proposed reference values for 1,2,4-triazole and triazole-derived metabolites (UK CRD, DAR, 2015)

M750F001 = 1,2,4-T		Livestock, plant and rotational crop metabolite	Proposed reference values for **1,2,4-triazole** (DAR, 2015): ADI = 0.05 mg/kg bw (rat 2-gen) SF 300 ARfD = 0.1 mg/kg bw (rat dev tox), SF 300
M750F029 = TA		Plant and rotational crop metabolite	Proposed reference values for **triazolyl alanine** (DAR, 2015): ADI = 1 mg/kg bw (rat 2-gen and rat dev tox), SF 100 ARfD = 1 mg/kg bw (rat 1-gen, rat dev tox), SF 100
M750F030 =TAA		Plant and rotational crop metabolite	Proposed reference values for **triazolyl acetic acid** (DAR, 2015): ADI = 1 mg/kg bw (rat 2-gen and rat dev tox), SF 100 ARfD = 1 mg/kg bw (rat 1-gen, rat dev tox), SF 100
M750F031 =TLA		Plant and rotational crop metabolite	Proposed reference values for **triazolyl lactic acid** (DAR, 2015): ADI = 1 mg/kg bw (rat 2-gen and rat dev tox), SF 100 ARfD = 1 mg/kg bw (rat 1-gen, rat dev tox), SF 100

The group of TDMs are common to other triazole containing fungicides. Within the frame of the ongoing evaluation of these common metabolites, it is proposed to set separate residue definitions for the TDMs.

Toxicological Studies with metabolite M750F022 (Reg.No. 6011210)

Report:	CA 5.8.1/1 [redacted] 2015 a Reg.No. 6011210 - Acute oral toxicity study in rats 2015/1175551
Guidelines:	OECD 423 (2001), EPA 870.1100, JMAFF No 12 Nosan No 8147, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)
Report:	CA 5.8.1/2 Schmitt D., 2015 a Concentration control analysis and homogeneity control analysis of Reg.No. 6011210 in vehicle corn oil 2015/1186900
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In an acute oral toxicity study two groups of 3 young adult female Wistar rats were sequentially administered a single oral dose via gavage of Reg.No. 6011210 (Batch: L85-116; Purity: 99.0%) suspended in corn oil at a dose level of 2000 mg/kg bw. Animals were observed for 14 days.

After administration of 2000 mg/kg bw of BAS 750 F all animals survived until the termination of the study. Accordingly, the oral LD_{50} was found to be greater than 2000 mg/kg bw.

Rat acute oral LD_{50} > 2000 mg/kg bw

Clinical signs were noted from 2 hours up to 3 days after treatment and comprised impaired general state and piloerection in all animals; three rats additionally showed apathy and cowering on the day of treatment and dyspnea on the next day. There were no apparent effects on body weight development throughout the 14-day post-exposure period. No apparent abnormalities were observed in any animal at necropsy performed at the termination of the study.

According to classification criteria of UN-GHS and EU CLP Regulation 1272/2008, no classification is warranted as to acute oral toxicity for Reg.No. 6011210.

(DocID 2015/1175551)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Reg.No. 6011210

Description:	solid / amber turbid
Batch #:	L85-116
Purity / content:	99.0%
Stability of test compound:	The stability was guaranteed for the duration of the study.

2. Vehicle: Corn oil

3. Test animals:

Species:	Rat
Strain:	Wistar / Crl:WI (Han) SPF
Sex:	female
Age (day 0):	ca. 10 weeks
Weight at dosing (mean):	180.7 ± 0.58 g (I); 176.3 ± 1.53 g (II)
Source:	Charles River Wiga GmbH, Germany
Acclimation period:	At least 5 days
Diet:	VRF1(P); SDS Special Diets Services, 67122 Altrip, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cage type III

Environmental conditions:

Temperature:	22 ± 3°C (continuous control and recording)
Humidity:	30 to 70% (continuous control and recording)
Air changes:	Fully air-conditioned rooms, approx. 10 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of work: 10-Jun-2015 to 08-Jul-2015 [in life phase: 16-Jun-2015 (treatment) to 07-Jul-2013 (day of last observation)]

2. Animal assignment and treatment:

Two groups of 3 female rats received a single starting dose of 2000 mg/kg bw of the test item administered as corn oil suspension (40 g / 100 mL) by oral gavage. The test item suspension was prepared shortly before administration; stability and homogeneity of the test item in the vehicle was verified indirectly by concentration control analysis. The animals were deprived of food at least 16 hours before dosing, but had free access to water. Food was offered again approximately 4 hours after administration. Test substance was given to the animals at a volume of 5 ml/kg bw. The dosing was performed sequentially, and as no deaths occurred after administration of the first group of three animals, the results were confirmed in three other females at the same dose level.

A check for any dead or moribund animals was made at least once each workday. Observations for clinical signs were performed several times on the day of administration. Thereafter, the animals were observed at least once a day for a total of 2 weeks.

Body weights were recorded at day 0 (prior to dosing), weekly thereafter and on the last day of observation.

The animals were sacrificed by carbon dioxide inhalation and subsequently subjected to macroscopic examination on the last day of the observation period.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred.

B. CLINICAL OBSERVATIONS

Clinical observations are summarized in Table 5.8.1-6. Clinical signs in the first 2000 mg/kg bw test group comprised impaired general state and piloerection, starting from hour 5 and lasting until day 3 after treatment, in all three animals.

In the second 2000 mg/kg bw test group, all animals showed the same symptoms consisting of impaired general state and piloerection 2 hours after administration, cowering and between hours 2 and 4 and apathy at the 3-hour observation timepoint. Impaired general state and piloerection were noted up to day 2 after treatment.

Table 5.8.1-6: Acute oral toxicity in rats - clinical observations

Observation	Group 1: 2000 mg/kg bw (n=3) Animals with signs	Observed	Group 2: 2000 mg/kg bw (n=3) Animals with signs	Observed
Impaired general state	3	h5 – d3	3	h2 – d2
Piloerection	3	h5 – d3	3	h2 – d2
Apathy	0	–	h3	h3
Cowering position	0	–	h2 – h4	h2 – h4

C. BODY WEIGHT

The mean body weight of the test groups increased throughout the study period within the normal range.

D. NECROSCOPY

There were no macroscopic pathological findings in the animals sacrificed at the end of the observation period.

E. CONCENTRATION CONTROL ANALYSES

Three samples of the test substance preparation in corn oil (nominal concentration: 40 g / 100 mL) were analyzed for BAS 750 F content with HPLC. The mean analyzed content was found to be 41.7g/100 mL (RSD: 1.77%), corresponding to 104.3% relative to the declared nominal concentration.

III. CONCLUSION

Under the conditions of this study the median lethal dose of Reg.No. 6011210 after oral administration was found to be greater than 2000 mg/kg bw in rats. Clinical signs of general toxicity were seen up to three days after exposure. No effects on body weight development or necroscopy findings were observed.

Report: CA 5.8.1/3
Lawson S., 2015a
Toxicological analysis of BAS 750 F and a metabolite using Derek Nexus
2015/1112684
Guidelines: <none>
GLP: no

EXECUTIVE SUMMARY

BAS 750 F and Reg. No. 6011210 were subjected to *in silico* analysis for toxicity over a range of endpoints using Derek Nexus in a number of bacterial and mammalian species (including organ toxicity, mutagenicity, carcinogenicity, respiratory and skin sensitisation and reproductive toxicity). No alerts were triggered for chromosome damage, genotoxicity or mutagenicity by any structure analysed. Furthermore, Derek Nexus has indicated that these structures contain no misclassified or unknown structures and as such are predicted to be inactive for in vitro bacterial mutagenicity. This being the case these structures should be regarded as negative for these endpoints. In addition, Reg. No. 6011210, triggered no alerts for carcinogenicity.
Due to a phenylethyltriazole or analogue moiety BAS 750 F triggered an alert for liver carcinogenicity and hepatotoxicity. In humans the assigned likelihood of carcinogenicity occurring is deemed to be equivocal as the evidence suggests that the mechanism of carcinogenicity is non-genotoxic and of low relevance to humans. The alert for hepatotoxicity was deemed to be plausible in humans.

BAS 750 F triggered an equivocal “rapidprototype alert” for adrenal gland toxicity, classified on the basis of the presence or absence of histopathologic lesions in the adrenal gland in oral rat repeat dose studies mostly of 28-days duration. This type of alter represents only an indication of potential toxicity and does not carry the same weight as a full alert.

Reg. No. 6011210 triggered an equivocal “rapidprototype alert” for nephrotoxicity; this alert describes the nephrotoxicity of 1,2-ethyleneglycol and its derivatives. Again, this alert type of DEREK Nexus represents only an indication of potential toxicity and does not carry the same weight as a full alert. The validity of the latter alert is expected to be low. No other human relevant endpoints were triggered by these structures.

(DocID 2015/1112684)

Report:	CA 5.8.1/4 Woitkowiak C., 2015 b Reg.No. 6011210 - Salmonella typhimurium / Escherichia coli - Reverse mutation assay 2015/1174564
Guidelines:	OECD 471 (1997), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142, EPA 870.5100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 5.8.1/5 Becker M., Kamp M., 2015 a Reg.No. 6011210 - Stability analysis in dimethylsulfoxide 2015/1186975
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

S. typhimurium and *E. coli* strains were exposed to Reg.No. 6011210 (Batch: L85-106, Purity: 98.3%) using dimethyl sulfoxide (DMSO) as a solvent in the presence and absence of metabolic activation (hepatic S9-mix of phenobarbital/ß-naphthoflavone induced rats) in standard plate (SPT) and pre-incubation tests (PIT). Triplicate plates were used per dose and per test condition. Vehicle and positive controls were included in each experiment. Tested concentrations ranged from 1.0 to 5000 µg/plate and from 1.0 to 1000 µg/plate for SPT and PIT, respectively.
A bacteriotoxic effect (reduced his^- or trp^- background growth, decrease in the number of his^+ or trp^+ revertants) was observed in SPT and PIT from about 333 and 100 µg/plate onward, respectively. No precipitation was observed with and without addition of S9-mix. A biologically relevant increase in the number of revertant colonies was not noticed in any of the strains tested in the presence or absence of metabolic activation in any of the experiments. The number of revertant colonies in the solvent controls was within the range of the historical negative control data for each tester strain in the presence or absence of S9-mix. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

According to the results of the study, Reg.No. 6011210 was not mutagenic in the *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay under the experimental conditions of the study. (DocID 2015/1174564)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Reg.No. 6011210

Description: Liquid viscous, yellowish

Lot/Batch #: L85-106

Purity: 98.3% (tolerance ±1.0%)

Stability of test compound: The test substance was stable over the study period under the storage conditions (Expiration date: 01-Feb-2017). The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control, without bacteria) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	***Mutagen***	***Solvent***	***Concentration***
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60.0 µg/plate

In order to demonstrate the efficacy of the S9-mix in this assay, the S9 batch was further characterized with benzo(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

3. Activation:

S9 was produced from the livers of at least 5 induced male Wistar (Crl:WI(Han)] rats. The rats received phenobarbital (80 mg/kg bw; i.p.) and β-naphthoflavone (80 mg/kg bw; orally) each on three consecutive days. 24 hours after the last administration the animals were sacrificed and the liver S9 fraction were prepared at stored at ≤ -70°C. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.

The S9-mix was prepared immediately before use and had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10 %

4. Test organisms:

S. typhimurium strains TA98, TA100, TA1535, TA1537; *E. coli* strain: WP2 uvrA

The *Salmonella* strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (Δ uvrB); ampicillin resistance (R factor plasmid).

E. coli WP2 uvrA is checked for UV sensitivity.

PERFORMANCE:

Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

SPT:

- all strains (±S9): 0, 33, 100, 333, 1000, 2500, 5000 µg/plate
- TA strains (± S9): 0, 1.0, 3.3, 10, 33, 100, 333 µg/plate (due to strong bacteriotoxicity observed in the first experiment)

PIT:

- TA strains (± S9): 0, 1.0, 3.3, 10, 33, 100, 333 µg/plate
- WP2 uvrA (± S9):: 0, 3.3, 10, 33, 100, 333, 1000 µg/plate

B. TEST

1. Dates of experimental work: 23-Jun-2015 to 10-Jul-2015

2. Plate incorporation assay:

To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto minimal glucose agar plates. In the experiments with *E. coli* the warm soft agar contains 0.5 mM tryptophan instead of histidine + 0.5 mM biotin. After incubation for 48-72 h at 37°C, his^+ or trp^+ revertants were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perceptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System. Three test plates per concentration or per control incubation were carried out.

3. Pre-incubation assay:

100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9-mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C the bacterial colonies were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perceptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System. Three test plates per concentration or per control incubation were carried out.

4. Statistics:

No special statistical tests were performed.

5. Evaluation criteria:

Solubility: Precipitation of the test material was recorded and indicated in the tables. As long as precipitation did not interfere with the colony scoring, 5 mg/plate was generally selected and analyzed (in cases of nontoxic compounds) as the maximum dose at least in the 1st experiment even in the case of relatively insoluble test compounds to detect possible mutagenic impurities. Furthermore, doses > 5 mg/plate might also be tested in repeat experiments for further clarification or substantiation.

Toxicity: Toxicity detected by a

- Decrease in the number of revertants (factor ≤ 0.6)
- Clearing or diminution of the background lawn (reduced his^- or trp^- background growth)

was recorded for all test groups both with and without S9-mix in all experiments and indicated in the tables.

Mutagenicity: The test substance is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and *E.coli* WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9-mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance Reg.No. 6011210 at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (Project No. 01Y0077/15Y001, see DocID 2015/1186975).

B. TOXICITY AND SOLUBILITY

Bacteriotoxic effect, evident by reduced his^- or trp^- background growth and/or decrease in the number of his^+ or trp^+ revertants was observed depending on the strain and test conditions from about 333 and 100 µg/plate onward in SPT and PIT, respectively.

No test substance precipitation was found with and without S9 mix.

C. MUTATION ASSAYS

Neither in the SPT (see Table 5.8.1-7) nor in the PIT experiments (see Table 5.8.1-8) with and without metabolic activation a biologically relevant increase in number of revertants was observed in any strain tested. The number of revertant colonies in the solvent controls was within the range of the historical negative control data for each tester strain in the presence or absence of S9-mix.The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system.

Table 5.8.1-7: Ames test (SPT) with Reg.No. 6011210 - Mean number of revertants

Experiment 1: Plate incorporation assay[#]										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	21	21	110	74	10	10	7	6	18	16
Test substance										
33 µg/plate	12	20	107	77	8	10	8	8	19	20
100 µg/plate	8	18	123	89	8	6	7	6	15	15
333 µg/plate	5 B	0 B	94 B	83 B	6 B	7 B	0 B	0 B	18 B	17 B
1000 µg/plate	0 B	0 B	0 B	0 B	0 B	4 B	0 B	0 B	17 B	17 B
2500 µg/plate	0 B	0 B	0 B	0 B	0 B	0 B	0 B	0 B	11 B	3 B
5000 µg/plate	0 B	0 B	0 B	0 B	0 B	0 B	0 B	0 B	7 B	2 B
Pos. control[§]	109	343	1830	4454	173	5221	109	905	69	908
Experiment 2: Plate incorporation assay[#]										
Strain	TA 98		TA 100		TA 1535		TA 1537			
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		
Neg. control (DMSO)	30	16	102	101	9	8	8	6		
Test substance										
1.0 µg/plate	21	19	98	89	12	7	8	8		
3.3 µg/plate	21	17	94	100	9	13	6	4		
10 µg/plate	23	19	95	95	8	7	7	6		
33 µg/plate	20	17	103	93	10	9	8	5		
100 µg/plate	16	17	96	83	8	10	6	5		
333 µg/plate	14 B	14 B	87 B	84 B	12 B	6 B	5 B	0 B		
Pos. control[§]	1701	443	1996	4274	293	4996	151	1238		

[#]: Numbers may differ from original data due to rounding
[§] = Compound and concentrations see Material and Methods (I.A.2.) above
B = reduced background growth

Table 5.8.1-8: Ames test (PIT) with Reg.No. 6011210 - Mean number of revertants

Experiment 3: Pre-incubation assay [#]										
Strain	TA 98		TA 100		TA 1535		TA 1537			
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		
Neg. control (DMSO)	24	18	111	96	10	11	9	6		
Test substance										
1.0 µg/plate	24	20	102	103	10	11	8	7		
3.3 µg/plate	31	15	104	97	8	12	10	7		
10 µg/plate	34	16	103	95	8	11	11	8		
33 µg/plate	30	15	96	95	8	10	6	8		
100 µg/plate	30	15 [B]	92	59 [B]	8	9 [B]	7 [B]	2 [B]		
333 µg/plate	21 [B]	0 [B]	66 [B]	0 [B]	4 [B]	0 [B]	0 [B]	0 [B]		
Pos. control [§]	1702	375	1616	2728	194	2145	139	803		
Experiment 3: Pre-incubation assay [#]										
Strain									E. coli	
Metabol. activation									+S9	-S9
Neg. control (DMSO)									21	16
Test substance										
3.3 µg/plate									26	20
10 µg/plate									19	18
33 µg/plate									17	19
100 µg/plate									23	21
333 µg/plate									21	23 [B]
1000 µg/plate									17 [B]	17 [B]
Pos. control [§]									87	1145

[+]: Data from repeated experiments were included in the table for TA 1537
[#]: Numbers may differ from original data due to rounding
[§] = Compound and concentrations see Material and Methods (I.A.2.) above
B = reduced background growth

III. CONCLUSION

According to the results of the present study, the test substance Reg.No. 6011210 is not mutagenic in the bacterial reverse mutation assay (Ames test) in the absence and the presence of metabolic activation, under conditions applied.

Report:	CA 5.8.1/6 Schulz M., Landsiedel R., 2015 a Reg.No. 6011210 - In vitro gene mutation test in L5178Y mouse lymphoma cells (TK+/- Locus assay, microwell version) 2015/1174532
Guidelines:	OECD 476, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.17 No. L 142, EPA 870.5300
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Reg.No. 6011210 (Batch: L85-116, purity: 99.0%) was tested *in vitro* in L5178Y $TK^{+/-}$ mouse lymphoma cells (MLTK) for its ability to induce forward mutations of the heterozygous autosomal thymidine kinase (TK) locus and structural chromosome aberrations at chromosome 11 carrying the functional TK gene. Two independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. Based on the results of a preliminary cytotoxicity assay, concentrations from 1.56 to 50 µg/mL were used in the main experiments. The treatment intervals for both experiments in the presence and absence of metabolic activation were generally 4 hours, except in experiment II (in the absence of metabolic activation) where a treatment interval of 24 h was applied. Methylmethanesulfonate (MMS) or 7,12-dimethylbenz[a]anthracene (DMBA) and Cyclophosphamide (CPA) served as positive controls in the experiments without or with metabolic activation, respectively and DMSO was used as the vehicle control. After the incubation period, treatment media were replaced by culture medium in both experiments and the cells were incubated for 48 h for expression of mutant cells. This was followed by incubation of cells in selection medium containing TFT for about 10 days.
No biological relevant increase in mutant colony numbers was observed either with or without metabolic activation in both main experiments. Appropriate reference mutagens (MMS and DMBA/CPA) were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid. Furthermore, the mutation frequencies of the vehicle control groups were within the range of historical negative control data.

Thus, under the experimental conditions described, Reg.No. 6011210 did not induce forward mutations or structural chromosome aberrations in vitro in the mouse lymphoma assay with L5178Y $TK^{+/-}$ cells in the absence and the presence of metabolic activation.

(DocID 2015/1174532)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material Reg.No. 6011210

Description:	Solid, amber turbid
Lot/Batch #:	L85-116
Purity:	99.0% (tolerance ± 1.0%)
Stability of test compound:	The test substance was stable over the study period under the storage conditions (Expiration date: 01-Apr-2017). The homogeneity of the test substance preparation was ensured by mixing and ultrasonic treatment prior to application. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified using a comparable batch (L85-106).
Solvent used:	Dimethylsulfoxide (DMSO),

2. Control Materials:

Negative control:	A negative control was not employed in this study
Solvent control:	1% (v/v) DMSO in culture medium
Positive control -S9:	Methylmethanesulfonate (MMS): 15 µg/mL (4 h) and 5 µg/mL (24 h), dissolved in culture medium
Positive control +S9:	Cyclophosphamide (CPA) 2.5 µg/mL dissolved in culture medium 7,12-dimethylbenzo[a]anthracene (DMBA): 2.5 and 4.0 µg/mL dissolved in DMSO

3. Activation: S9 was produced from the livers of at least 5 male Wistar rats ([Crl:WI (Han)], 200 - 300 g; supplied by Charles River Laboratories Germany GmbH, Sulzfeld, Germany) that received phenobarbital (80 mg/kg bw; i.p.) and β-naphthoflavone (80 mg/kg bw; orally) each on three consecutive days. 24 hours after the last administration the animals were sacrificed and the liver S9 fraction were prepared at stored at ≤ -70°C. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.

The S9-mix had the following composition:

Component	***Concentration***
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9-fraction	10%

4. Test organism:

L5178Y mouse lymphoma cells were used. They have a high proliferation rate (doubling time about 9-10 h), a high plating efficiency (about 90%) and a stable karyotype with a near diploid (40 ± 1) chromosome number. Stocks of the L5178Y cell line in culture medium supplemented with 7% (v/v) DMSO were maintained at -196°C in liquid nitrogen. Each batch used for mutagenicity testing was checked for mycoplasma contamination. During the week prior to treatment, spontaneous TK-deficient mutants were eliminated from the stock cultures by growing the cells for one day in pretreatment medium 1 and the following three days in pretreatment medium 2 (see below).

5. Culture media

Culture medium: RPMI 1640 medium with 1% of 10000 IU/10000 µg/mL penicillin/streptomycin and 1% (v/v) sodium pyruvate (10 mM)

Pretreatment medium 1: ("THMG" medium): culture medium supplemented with 10% (v/v) fetal calf serum (FCS), containing thymidine (3.0 µg/mL), hypoxanthine (5.0 µg/mL), methotrexate (0.1 µg/mL), and glycine (7.5 µg/mL).

Pretreatment medium 2: ("THG" medium): culture medium supplemented with 10% (v/v) FCS, containing thymidine (3.0 µg/mL), hypoxanthine (5.0 µg/mL), and glycine (7.5 µg/mL).

Selection medium: Culture medium supplemented with 20% (v/v) FCS and 4 µg/mL trifluorothymidine (TFT).

6. Locus examined: thymidine kinase (TK)

7. Test concentrations:

Preliminary toxicity assay:	Nine concentrations ranging from 13.7 to 3500 µg/mL (corresponding to a max. concentration of ca. 10 mM)
Mutation assay:	The dose range of the main experiments was set according to data generated in the pre-experiment. In both main experiments the individual concentrations were generally spaced by a factor of 2.0 in the lower range. At least four concentrations were evaluated to describe a possible dose-response relationship.
- Experiment I (4 h/ ± S9):	**0, 3.13, 6.25, 12.5, 25, 50**, 100, 200 µg/mL (evaluated concentrations are indicated in bold)
- Experiment II (24 h/ - S9):	**0, 1.56, 3.13, 6.25, 12.5, 25, 50**, 100 µg/mL (evaluated concentrations are indicated in bold)
(4 h/ - S9):	**0, 2.34, 4.69, 9.38, 18.75, 37.50**, 75, 150 µg/mL (evaluated concentrations are indicated in bold)

B. TEST PERFORMANCE:

1. Dates of experimental work: 22-Jun-2015 to 11-Sep-2015

2. Preliminary cytotoxicity assay

A pre-test was performed in order to determine the concentration range of the mutagenicity experiments. Following the requirements of the current international guidelines a test substance should be tested up to a maximum concentration of 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. In case of toxicity, the top dose should result in approximately 10 - 20% relative suspension growth (RSG, pretest) or relative total growth (RTG, main experiments), but not less than 10%. For relatively insoluble test substances the highest applied concentration should be at the border of test substance precipitation in culture medium at the end of exposure period determined with the unaided eye.

In the pretest for toxicity Reg.No. 6011210 concentrations in the range from 13.7 to 3500 µg/mL (approx. 10 mM) were used both with and without S9 mix at 4-hour exposure time and without S9 mix at 24-hour exposure time.

The pretest was performed following the method described for the main experiment (see below). The RSG was determined as toxicity indicator for dose selection and various parameters were checked for all or at least for some selected doses.

3. Mutation Assay:

Cell treatment and expression period:

Logarithmically growing cells in suspension culture (3 x 10^5 cells per 75 cm² flask in a total volume of 30 mL in exponential growth per treatment group required) were incubated 4 - 5 days prior to the start of the experiment. Following centrifugation and resuspension the cells were dispensed into 75 cm² flasks (4-hour exposure: 1.5 x 10^7 cells per culture; 24-hour exposure: 1 x 10^7 cells per culture).

The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment period of 24 hours in the absence of metabolic activation and with a treatment period of 4 hours in the presence of metabolic activation. During exposure, cells were cultivated in culture medium supplemented with 5% and 10% FCS with and without addition of S9-mix, respectively.

At the end of the exposure period, the cells were transferred in tubes, centrifuged for 5 minutes at 1000 rpm (173 g) and were resuspended in culture medium with 5% FCS. The washing of the cells was repeated at least once. Then the cells were centrifuged at 1000 rpm (173 g, 5 min) and were resuspended in culture medium with 10% FCS. From each culture a sample of treated cells (2 x 105 cells/mL or 6 x 106 cells/flask) was pipetted in 75 cm² flasks and was incubated for a 2-day expression period.

To maintain exponential growth during this phase, each culture was counted daily and the cell numbers were adjusted at each day to 2 x 105 cells/mL in 30 mL RPMI-10 medium. The cell numbers were determined using a cell counter (CASY®, Roche Applied Science, Mannheim, Germany).

Seeding for selection and cloning efficiency

After the expression period the cultures were selected. Cells were centrifuged (173 g, 5 min) and 5 x 10^5 cells from each culture were resuspended in 50 mL selection medium (“TFT" medium; 1 x 10^4 cells/mL). Per culture 200 µL were dispensed in each well of two 96-well plates (2000 cells/well). After incubation for at least 9 days, both the number of negative wells and the number of wells containing small or large colonies were scored for calculation of the mutant frequency (MF).

Cytotoxicity determination:

- Cloning efficiency 1 (survival)

  At the end of the exposure period, the cells were centrifuged (173 g, 5 min) and 400 cells from each test group were resuspended in 50 mL RPMI-20 medium (8 cells/mL). Per culture 200 µL were dispensed in each well of two 96-well plates (1.6 cells/well). After incubation for 9 - 11 days the plates were scored for empty wells.

- Cloning efficiency 2 (viability)

  After the expression period, 2 days after end of exposure, the cells were centrifuged (173 g, 5 min) and 400 cells from each culture were resuspended in 50 mL RPMI-20 medium (8 cells/mL). Per culture 200 µL were dispensed in each well of two 96-well plates (1.6 cells/well). After incubation for at least 9 days the plates were scored for empty wells.

- Suspension growth (RSG) and relative total growth (RTG)

  For calculation of the suspension growth (SG) and the relative total growth (RTG) the cell counts determined within the expression period at 2nd and 3rd passage after exposure in the case of 4-hour exposure and 1st, 2nd and 3rd passage after exposure in the case of 24-hour exposure were used.

4. Evaluation:

Cytotoxicity

The number of empty wells of the 96-well plates was scored and recorded.

- Cloning efficiency 1 (survival)

  The cytotoxicity of the test substance after the exposure period was determined for each test group and is given as absolute and relative cloning efficiency (CE_1 and RCE_1, respectively).

- Cloning efficiency 2 (viability)

  The cytotoxicity of the test substance at the end of the expression period was determined for each test group and is given as absolute and relative cloning efficiency (CE_2 and RCE_2, respectively).

The cloning efficiency (CE, %) was calculated for each test group as follows:

$$CE_x = \frac{-\ln \frac{\text{Total number of empty wells}}{\text{Total number of seeded cells (96)}}}{\text{Number of seeded cells per well (1.6)}} \times 100$$

$$RCE_x = \frac{CE_x \text{ (test group)}}{CE_x \text{ (control group)}} \times 100$$

- Relative total growth (RTG)

  The relative total growth is the standard measure of cytotoxicity. This measure includes the relative growth in suspension (RSG) during the expression period and the relative cloning efficiency (RCE_2; viability) at the time the mutants are selected.

  For taking into account any loss of cells during the 4-hour treatment period a relative growth during treatment factor (RGDT, %) was calculated by comparing the growth of each treated culture relative to the control:

$$RGDT = \frac{\text{Cell count (test group) after 4 h}}{\text{Cell count (control group) after 4 h}} \times 100$$

  The total suspension growth (SG) and the relative suspension growth (RSG, %) were calculated for each test group as follows:

  Total suspension growth (SG) after 4 hour-exposure

$$SG = \frac{\text{Cell count after 24 h}}{5 \times 10^5 \text{ cells/mL}} \times \frac{\text{Cell count after 48 h}}{2 \times 10^5 \text{ cells/mL}} \times \frac{RGDT}{100}$$

  Total suspension growth (SG) after 24 hour-exposure:

$$SG = \frac{\text{Cell count after 24 h}}{5 \times 10^5 \text{ cells/mL}} \times \frac{\text{Cell count after 48 h}}{2 \times 10^5 \text{ cells/mL}} \times \frac{\text{Cell count after 72 h}}{2 \times 10^5 \text{ cells/mL}}$$

$$RSG = \frac{\text{SG of the test group}}{\text{SG of the negative / vehicle control}} \times 100$$

  The relative total growth (RTG, %) was calculated for each test group as follows:

$$RTG = \frac{RSG \times RCE_2}{100}$$

Mutant frequency

The number of empty wells and the number of wells containing colonies were scored and reported. The colonies are classified into large colonies (indication of gene mutation) and small colonies (indication of chromosome breakage). Small colonies are defined as less than 1/4 of the diameter of the well. Size is the key factor and morphology (the optical density of the small colonies is considerably higher) should be secondary.

- Uncorrected mutant frequency (MFuncorr.)

  The uncorrected mutant frequency per 10^6 cells was calculated for each test group as follows:

$$MF_{uncorr} = \frac{-\ln \frac{\text{Total number of empty wells}}{\text{Total number of seeded cells (96)}}}{\text{Number of seeded cells per well (200)}} \times 10^6$$

- Corrected mutant frequency (MFcorr.)

  The corrected mutation frequency was calculated considering the values of the cloning efficiency 2 (CE_2):

$$MF_{corr} = \frac{MF_{uncorr}}{CE_2} \times 100$$

5. Statistics:

An appropriate statistical trend test (MS EXCEL function RGP; 9) was performed to assess a possible dose-related increase of mutant frequencies. The number of mutant colonies obtained for the test substance treated groups was compared with that of the respective negative/vehicle control groups. A trend was judged as statistically significant whenever the one-sided p-value (probability value) was below 0.05 and the slope was greater than 0. However, both, biological and statistical significance has been considered together.

6. Acceptability criteria:

The MLTK assay is considered valid if the following criteria are met considering the international guidelines and the current recommendations of the IWGT:

- The absolute cloning efficiency obtained at the time of mutant selection (CE_2) of the negative/vehicle controls should fall in the range of 65 - 120%.

- The suspension growth (SG) of the negative/vehicle controls referring to the expression period following treatment should fall in the range of 8 - 32 for 4-hour exposure and 32 - 180 for 24-hour exposure.

- The mutant frequency of the negative/vehicle controls should fall within the range of 50 - 170 x 10^{-6} colonies.

- The positive controls should yield an absolute increase in total MF that is an increase above the spontaneous background MF (an induced MF [IMF]) of at least 300 x 10^{-6} colonies. The small colony MF should account for at least 40% of that IMF, means a small colony IMF of at least 120 x 10^{-6} colonies. Alternatively, the positive controls should induce at least 150 small colonies. The upper limit of cytotoxicity observed in the positive controls should have a relative total growth (RTG) that is greater than 10%.

- The highest applied concentration of the test substance should be 5 mg/mL, 5 µL/mL or 10 mM, unless limited by cytotoxicity or solubility of the test substance. If toxicity occurs, the highest concentration should lower the cloning efficiency 1 (CE_1) or the relative total growth (RTG) to 10 to 20% of survival. If precipitation occurs, the highest evaluated concentration should be the lowest concentration where precipitation is observed by the unaided eye.

7. Evaluation criteria:

A test substance is classified as mutagenic if all of the following criteria is met:

- The induced mutation frequency exceeds a threshold of 126 colonies per 10^6 cells above the corresponding solvent control.
- Evidence of reproducibility of any increase in mutant frequencies, meaning the mutagenic response occurs at least in both parallel cultures of one experiment.
- A statistically significant dose-related increase in mutant frequencies using an appropriate statistical trend test.

The test substance is considered non-mutagenic if at least one of the following criteria is met:

- The mutation frequency is below a threshold of 126 colonies per 106 cells above the concurrent solvent control value.
- No evidence of reproducibility of an increase in mutant frequencies is obtained.
- No statistical significant dose-related increase in mutant frequencies using an appropriate statistical trend.

However, in the evaluation of the test results the historical variability of the mutation rates in negative and vehicle controls and the mutation rates of all negative and vehicle controls of this study were taken into consideration.

Results of test groups have been rejected if the relative total growth (RTG) and/or the cloning efficiency 1 (CE_1) were less than 10% of the respective negative/vehicle control.

Whenever a test substance is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose-related shift in the ratio of small versus large colonies clastogenic effects are indicated.

II. RESULTS AND DISCUSSION

A. ANALYTICS

The test substance was stable over the study period under the storage conditions. The stability of the test substance Reg.No. 6011210 at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically using a comparable batch (Batch: L85-106; Project No. 01Y0077/15Y001, see DocID 2015/1186975).

B. PRELIMINARY CYTOTOXICITY ASSAY

The pH value and osmolarity were not relevantly influenced by the addition of the test substance preparation to the culture medium at the concentrations measured.

In addition, no test substance precipitation in the vehicle DMSO was observed in the stock solution (3500 µg/mL). In culture medium test substance precipitation occurred at 218.8 µg/mL and above at the end of treatment in the absence and the presence of S9 mix.

After 4 hours treatment in the absence and presence of S9 mix and after 24 hours treatment in the absence of S9 mix cytotoxicity indicated by reduced RSG of about or below 20% was observed at 54.7 µg/mL and above.

C. MUTAGENICITY ASSAYS

Results are summarized in Table 5.8.1-9 below.

No precipitation in culture medium was observed up to the highest test substance concentrations applied.

Relevant cytotoxic effects indicated by a relative total growth of less than 50% and/or reduced cloning efficiency of less than 80% as compared with the respective vehicle control were observed in the first experiment at 50 µg/mL with and without metabolic activation. In the second experiment cytotoxic effects as described above were noted at 25 µg/mL after 24 h exposure without metabolic activation.

The test substance Reg.No. 6011210 did not lead to a biologically relevant increase in the number of mutant colonies either without S9-mix or after the addition of a metabolizing system in two experiments performed independently of each other. The mutant frequencies at all test concentrations were within the range of the historical negative control data and did not exceed the respective threshold value.

The mutation frequencies of the vehicle control groups were within the historical negative control data range, verifying the validity of the performed experiments. All positive control substances induced a clear and substantial increase of the mutation frequencies, demonstrating the sensitivity of the test system toward known mutagens that do or do not require metabolic activation.

III. CONCLUSION

Thus, under the experimental conditions chosen, Reg.No. 6011210 does not induce forward mutations or chromosomal aberrations in vitro in the mouse lymphoma assay with L5178Y $TK^{+/-}$ cells in the absence and the presence of metabolic activation.

Table 5.8.1-9: Results of Mouse Lymphoma Assay - Main experiments

Test group	conc. [µg/mL]	S9 mix	Cytotoxicity[#]		Genotoxicity	
			RCE_1 [%]	RTG [%]	$MF_{corr.}$ [colonies/ 10^6 cells]	Threshold[##]
Experiment I / 4 h treatment						
DMSO	-	-	100	100	51.8	
Reg.No. 6011210	3.13	-	111.9	85.2	46.7	
	6.25	-	122.9	96.9	53.7	
	12.50	-	98.5	83.6	67.3	
	25.00	-	95.0	72.9	36.0	178
	50.00	-	91.0	40.3	78.1	
	100.00	-	2.2	n.c.	n.c.	
	200.00	-	n.c.	n.c.	n.c.	
MMS	15.0	-	80.0	21.2	1218.9	
Experiment I / 4 h treatment						
DMSO	-	+	100.0	100	50.3	
Reg.No. 6011210	3.13	+	108.3	94.0	40.2	
	6.25	+	99.2	95.9	47.5	
	12.50	+	98.5	89.0	45.0	
	25.00	+	100.8	70.8	50.7	
	50.00	+	68.3	29.3	61.0	176
	100.00	+	n.c.	n.c.	n.c.	
	200.00	+	n.c.	n.c.	n.c.	
CPA	2.5	+	100.0	52.8	362.5	
DMBA	2.5	+	89.9	52.8	354.2	
	4.0	+	85.4	55.6	315.2	
Experiment II / 24 h treatment						
DMSO	-	-	100	100	40.8	
Reg.No. 6011210	1.56	-	79.3	82.7	32.3	
	3.13	-	116.9	78.5	56.3	
	6.25	-	121.7	81.1	51.7	
	12.50	-	92.0	56.4	48.0	167
	25.00	-	79.3	43.7	53.1	
	50.00	-	12.2	n.c.	n.c.	
	100.00	-	n.c.	n.c.	n.c.	
MMS	5.0	-	90.5	31.7	588.6	
Experiment II / 4 h treatment						
DMSO	-	+	100	100	50.0	
Reg.No. 6011210	2.34	+	97.0	114.6	36.3	
	4.69	+	114.4	110.2	33.8	
	9.38	+	110.7	105.5	37.3	
	18.75	+	92.1	91.2	39.2	
	37.50	+	92.1	76.8	37.6	176
	75.00	+	2.4	n.c.	n.c.	
	150.00	+	n.c.	n.c.	n.c.	
CPA	2.5	+	88.1	62.2	399.9	
DMBA	2.5	+	93.4	61.0	331.9	
	4.0	+	100.0	71.5	421.0	

[#] = cytotoxicity related to the respective vehicle control
[##] = number of mutant colonies per 10^6 cells of current vehicle control plus 126 (rounded value)
n.c. = Culture not continued due to strong cytotoxicity

Report: CA 5.8.1/7
Sokolowski A., 2015 b
Reg.No. 6011210: Micronucleus test in human lymphocytes in vitro
2015/1038964
Guidelines: OECD 487 (2014)
GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

EXECUTIVE SUMMARY

Reg.No. 6011210 (Batch: L85-106; Purity: 98.3%) was tested for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of hepatic S9-mix from induced rats (metabolic activation). Two independent experiments were performed where the cells were incubated for 4 (±S9-mix) or 20 hours (-S9-mix) with the test substance at concentrations in the range of 0.3 to 100 µg/mL, whereas concentrations from 6.1 to 32.7 µg/mL were evaluated. The vehicle DMSO served as negative control, mitomycin C (4 h) and demecolcin (20 h) as positive controls in the absence of metabolic activation and cyclophosphamide as positive control in the presence of metabolic activation. Exposure was started after a 48-hour stimulation period with phytohemeagglutinine. Thereafter, cytochalasin B was added and the cultures were fixed and stained finally after another 20 hours. Cytokinesis-block proliferation index (CBPI) and cytostasis were determined in 500 binucleated cells/culture as cytotoxicity parameters and number of micronucleated cells were determined in 1000 binucleated cells/culture for evaluation of mutagenicity.
Cytotoxicity was observed in both experiments either without or with addition of S9-mix at the highest evaluated concentration of 8.2 and 32.7 µg/mL, respectively. Osmolarity and pH values were not influenced by test substance treatment. No precipitation of the test item in the culture medium at the end of treatment was observed.

The test substance Reg.No. 6011210 did not lead to a relevant increase in the number of micronucleated cells either without S9-mix or after the addition of a metabolizing system in two experiments performed independently of each other. In both experiments, either CPA or demecolcin and MMC showed distinct increases in cells with micronuclei, and thus demonstrating sensitivity of the test system towards known mutagens that do or do not require metabolic activation, respectively. Furthermore, the number of micronucleated cells induced by the vehicle control DMSO was within the range of the historical control data.

In conclusion, Reg.No. 6011210 is considered to be non-clastogenic in this *in vitro* micronucleus test when tested up to cytotoxic concentrations on human lymphocytes.

(DocID 2015/1038964)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material Reg.No. 6011210

Description: liquid (viscous), yellowish

Lot/Batch #: L85-106

Purity: 98.3%

Stability of test compound: The test substance was stable over the study period under the storage conditions (Expiration date: 01-Feb-2017). The homogeneity of the test substance was ensured by mixing prior to preparation of test substance formulations. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified.

Vehicle used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Vehicle/solvent control: DMSO, 0.5% v/v in culture medium

Positive control: Without metabolic activation:

Mitomycin C (MMC, 2 µg/mL; 4 h treatment) dissolved in deionized water;

Demecolcin (125 ng/mL; 20 h treatment) dissolved in deionized water

With metabolic activation:

Cyclophosphamide (CPP, 15.0 and 17.5 µg/mL) dissolved in saline

3. Activation: S9 was produced from the livers of rats pretreated with phenobarbital/β-naphthoflavone. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction (protein content: 35.0 mg/mL) was thawed at room temperature and mixed with a sufficient amount of S9-supplement (cofactors). This preparation, the so called S9-mix, was kept on ice until used.

The concentrations of the co-factors in the S9-mix were as follows:

Component	***Concentration***
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

50 µL S9-mix per mL culture medium were added yielding a final protein concentration of 0.75 mg/mL in the cultures.

4. Test organism: Human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication: female donor (32 years old) for experiment I, and female donor (28 years old) for experiment II.

5. Culture media: Dulbecco´s Modified Eagles medium/Ham´s F12 (1:1) with GlutaMAX™ (200 mM) supplemented with 10% (v/v) fetal bovine serum (FBS), Pen/Strep (100 U/mL / 100 µg/mL), HEPES (10 mM), heparin (125 U.S.P.-U/mL) and phytohemeagglutinine (PHA, 3 µg/mL).

6. Test concentrations:

Micronucleus test, Experiment I

(4-h exposure, -S9): 6.1, 10.7, 18.7 µg/mL

(4-h exposure, +S9): 10.7, 18.7, 32.7, (57.1) µg/mL

Micronucleus test, Experiment II

(4-h exposure, +S9): 6.1, 10.7, 18.7 µg/mL

(20-h exposure, -S9): 10.7, 18.7, 32.7 µg/mL

B. TEST PERFORMANCE

1. Dates of experimental work: 25-Mar-2015 to 27-Mar-2015

2. Dose selection

Dose selection was performed according to the current OECD Guideline for the *in vitro* micronucleus test. The highest test item concentration should be 2000 µg/mL, 2 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

Taking into account purity of the test item (98.3%), 2035 µg/mL was applied as top concentration for treatment of the cultures in the pre-test. Due to strong cytotoxicity, the pre-test was repeated with a top dose of 100 µg/mL. Test item concentrations ranging from 0.6 to 100 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. No precipitation of the test item was observed. Since the cultures fulfilled the requirements for cytogenetic evaluation, this repeated test was designated Experiment I.

Using the reduction of the Cytokinesis-block proliferation index (CBPI) as indicator for toxicity, clear toxic effects were observed after 4 hours treatment with 18.7 µg/mL and above in the absence of S9-mix and with 57.1 µg/mL and above in the presence of S9-mix. Considering the toxicity data and precipitation, 100 µg/mL (with and without S9-mix) was chosen as top concentration in Experiment II.

3. Preliminary cytotoxicity assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay. The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9-mix). The preparation interval was 40 hrs after start of the exposure.

4. Micronucleus test

The cultures were treated according to the following scheme:

	Without S9-mix		With S9-mix	
	Experiment I	Experiment II	Experiment I	Experiment II
Stimulation period	48 h	48 h	48 h	48 h
Exposure time	4 h	20 h	4 h	4 h
Recovery time	16 h	-	16 h	16 h
Cytochalasin B exposure	20 h	20 h	20 h	20 h
Harvest time	40 h	40 h	40 h	40 h
Total culture period	88 h	88 h	88 h	88 h

Pulse exposure (4 h)

About 48 h after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9-mix per mL culture medium was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period, Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous (20 h) exposure

About 48 h after seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Preparation of cells

The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37°C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

5. Cytotoxicity and genotoxicity evaluation

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 1000 binucleated cells per culture were evaluated for cytogenetic damage on coded slides, except for the positive control in Experiment I in the absence of S9-mix, where only 500 binucleated cells were evaluated. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$\text{CBPI} = \frac{(\text{MONC} \times 1) + (\text{BINC} \times 2) + (\text{MUNC} \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mono-nucleated cells
BINC	Bi-nucleated cells
MUNC	Multi-nucleated cells

$$\text{Cytostasis (\%)} = 100 - 100 \times \frac{(\text{CBPI}_T - 1)}{\text{CBPI}_C - 1}$$

T	Test substance
C	Solvent control

6. Test acceptability criteria

The *in vitro* micronucleus assay is considered acceptable if the following criteria are met:

- The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significantly increased and falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significant increased.
- The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.
- The criteria for the selection of top concentration are consistent with: Test item was either tested to a maximum concentration of 10 mM, 2 mg/mL or 2 µL/mL or precipitating concentrations or an acceptable limit of cytotoxicity. Where cytotoxicity occur the applied concentrations should cover a range from no to approximately 55 ± 5% cytostasis.

7. Assessment criteria

A test item can be classified as non-clastogenic and non-aneugenic if:

- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data and
- no statistically significant or concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as clastogenic and aneugenic if:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.

An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS AND TREATMENT CONDITIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance Reg.No. 6011210 at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (Project No. 01Y0077/15Y001, see DocID 2015/1186975).

Osmolarity and pH values were not influenced by test substance treatment. No precipitation of the test item in the culture medium at the end of treatment was observed in the absence and the presence of S9-mix.

B. CYTOTOXICITY

In Experiment I and II in the absence of S9 mix and in Experiment II in the presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentrations.

C. MICRONUCLEUS ASSAY

In both experiments in the absence and presence of S9-mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item. The micronucleus rates of the cells after treatment with the test item (0.20 - 1.10% micronucleated cells) did not exceed the range of the solvent control values (0.45 - 1.30% micronucleated cells) and were within the range of the laboratory historical control data.

In both experiments, either CPA or Demecolcin and MMC were used as positive controls and showed distinct increases in cells with micronuclei, and thus demonstrating sensitivity of the test system towards known mutagens that do or do not require metabolic activation, respectively.

Table 5.8.1-10: Results of the in vitro micronucleus test in human lymphocytes with Reg.No. 6011210

Test group	conc. [µg/mL]	S9 mix	Cytotoxicity[#] Proliferation index (CBPI)	Cytotoxicity[#] Cytostasis [%][a]	Genotoxicity Micronucleated cells [%][b]
Experiment I / 4 h exposure (preparation at 40 h)					
DMSO [0.5% (v/v)]	-	-	1.77		0.45
Reg.No. 6011210	6.1	-	1.12	4.7	0.65
	10.7	-	1.74	28.8	0.55
	18.7	-	1.55	55.8	0.45
MMC	2.0	-	1.12	84.5	**13.30***
DMSO	-	+	1.58		1.30
Reg.No. 6011210	10.7	+	1.62	n. c.	0.75
	18.7	+	1.54	7.4	1.10
	32.7	+	1.34	41.7	0.65
	57.1	+	1.22	62.3	n. e.
CPA	17.5	+	1.28	51.6	**4.50***
Experiment II / 20-h exposure (preparation at 40 h)					
DMSO	-	-	1.93		0.70
Reg.No. 6011210	6.1	-	1.68	26.7	0.45
	10.7	-	1.65	30.0	0.25
	18.7	-	1.36	61.7	0.50
Demecolcin	125.0	-	1.49	47.9	**3.90***
Experiment II / 4-h exposure (preparation at 40 h)					
DMSO	-	+	1.98		0.85
Reg.No. 6011210	10.7	+	1.79	19.7	0.35
	18.7	+	1.61	37.8	0.20
	32.7	+	1.30	69.6	0.25
CPA	15.0	+	1.51	48.3	**6.15***

[a]: the values are related to the solvent controls
[b]: The number of micronucleated cells was determined in a sample of 2000 binucleated cells
*: statistically significantly higher than corresponding control values ($p \leq 0.05$)
n. c.: Not calculated as the CBPI is equal or higher than the solvent control value
n. e.: Not evaluated due to a reduction of the cell number and most of the cells did not show a clear visible cytoplasm area, and therefore did not met the acceptance criteria for evaluation

III. CONCLUSION

Under the experimental conditions reported, the test substance did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, Reg.No. 6011210 is considered to be non-mutagenic in this in vitro micronucleus test when tested up to cytotoxic concentrations.

Report:	CA 5.8.1/8 [redacted] 2016 a Reg.No. 6011210: Repeated-dose 28-day toxicity study in C57BL/6 J Rj mice - Administration via the diet 2016/1000646
Guidelines:	OECD 407 (2008), EPA 870.3050, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.7 No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Reg.No. 6011210 (batch L85-116, purity: 99.0%) was administered via the diet to groups of 5 male and 5 female C57BL/6 J Rj mice at concentrations of 0, 87, 872 and 2500 ppm for at least 28 days (equivalent to mean intakes of 0, 20/32, 180/249 and 587/718 mg/kg bw/d in male/female mice, respectively). The selected low- and the mid-dose levels of Reg.No. 6011210 correspond to equimolar BAS 750 F dietary concentrations of 100 and 1000 ppm that were tested in the same mouse strain for 28 days, to allow for comparison of toxicity. Treatment with 2500 ppm caused marked reductions of body weight and food intake in females, and reduced body weight gain in both sexes. Clinical chemistry changes at 2500 ppm were indicative of impaired liver function in both male and female mice, comprising increased serum alkaline phosphatase (ALP) in both sexes and, additionally in males, increased alanine aminotransferase (ALT), decreased triglycerides, cholesterol, total protein and albumin levels. Liver weights were markedly increased in males (abs. +39% / rel. +52%) and in females (abs. +38% / rel. +64%) and associated with hepatocellular hypertrophy and degenerative changes (fine, granular eosinophilic cytoplasm and multifocal necroses in 1 female and 4 male mice. In male mice at the mid-dose level of 872 ppm, decreased triglycyeride levels were paired with slightly increased liver weights and hypertrophy; two of the five male mice were found with liver cell necrosis. Reduced fat storage in hepatocytes was noted in both sexes at 872 and 2500 ppm but not assessed as an adverse change. Changes in the ovaries and uterus of 3 females from the high-dose group, and cervix and vagina changes seen in one high-dose female could be clearly associated with severe body weight reductions in these individual animals, and were therefore not considered to reflect organs-specific toxicity, in line with published literature. The NOAEL was 87 ppm in males (20 mg/kg bw/d) and 872 ppm in females (249 mg/kg bw/d).

(DocID 2016/1000646)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Reg.No. 6011210

Description:	solid / amber turbid
Batch #:	L85-116
Purity / content:	99.0%
Stability of test compound:	The stability was guaranteed for the duration of the study (expiration date 01 April 2017).

2. Vehicle: Rodent diet

3. Test animals:

Species:	Mouse
Strain:	C57BL/6 J Rj
Sex:	male and female
Age:	42 - 44 days at delivery; 48 - 50 days at start of dosing
Weight at dosing (mean):	♂: 22.0 ± 0.97 g; ♀: 18.3 ± 0.86 g (Day 0)
Source:	Janvier Labs SAS, Le Genest Saint Isle, France
Acclimatization period:	6 days
Diet:	Ground Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water, ad libitum
Housing:	in polycarbonate cages type MII with mesh wire tops (Becker & Co, Castrop-Rauxel, Germany) – 1 male animal/cage and 2 or 3 female animals/cage;, dust free wooden bedding; enrichment: mouse tunnel and nest building material (Plexx B.V., Elst, Netherlands)
Environmental conditions:	
Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	Fully air-conditioned rooms, approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN

1. Dates of work: 16-Jul-2015 to 09-Dec-2015 [in life phase: 22-Jul-2015 (start of treatment) to 20-Aug-2015 (necropsy)]

2. Animal assignment and treatment

Reg.No 6011210 was administered to groups of 5 male and 5 female C57BL/6 J Rj mice at dietary concentrations of 0, 87 (low dose), 872 (mid-dose) and 2500 ppm (top dose) for at least 28 days. The top-dose level of 2500 ppm was chosen on the basis of results from a 14-day range-finding study. The selected low- and the mid-dose levels of Reg.No. 6011210 correspond to equimolar BAS 750 F dietary concentrations of 100 and 1000 ppm that were tested in the same mouse strain for 28 days, to allow for comparison of toxicity. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

3. Test substance preparation and analysis

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer.

Analyses performed prior to the start of the administration period revealed that the test-substance was stable in the diet for 4 days.

Concentration control analyses of all dose levels were determined at the beginning of the study. The homogeneity of the low dose and of the high dose was determined at the beginning of the study. No test article was determined in control diets. The method used for analyzing the test material in the diet involved extraction with a solvent followed by HPLC analysis with an external standard.

Table 5.8.1-11: Analysis of preparations for homogeneity and test-item content

Reg.No 6011210 nominal conc. [ppm]	Date of sampling	Date of analysis	Sample [#]	Analytical concentration [ppm]	% of nominal concentration	Mean ± RSD
87 ppm	22-Jul-2015	23-Jul-2015	3	85.593	98.4	100.0± 1.6
			4	88.297	101.5	
			5	87.044	100.1	
872 ppm	22-Jul-2015	23-Jul-2015	6	903.879	103.7	–
2500 ppm	22-Jul-2015	23-Jul-2015	7	2616.486	104.7	102.7 ± 2.1
			8	2575.857	103.0	
			9	2508.817	100.4	

Relative standard deviations of maximum 2.1% indicated the homogenous distribution of Reg.No 6011210 in the diet preparations. The actual nominal test-item concentrations were in the range of 98.4 to 104.7% of the target nominal concentrations and thus in the acceptable range.

4. Statistics

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
Food consumption (males); body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Statistics for clinical pathology	
Parameter	**Statistical test**
Blood parameters with bi-directional changes	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
Statistics for pathology	
Parameter	**Statistical test**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians

C. METHODS

1. Observations

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Animals in moribund stage were sacrificed under isoflurane anesthesia and necropsied. Observations for overt clinical signs of toxicity were performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this, the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. Abnormal behavior during handling
2. Fur
3. Skin
4. Body posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Feces (appearance/consistency)
16. Urine
17. Pupil size

2. Body weight

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on study day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on study day 0 was calculated as body weight change.

3. Food consumption and test compound intake

Food consumption was determined weekly and calculated as mean food consumption in grams per animal and day.

The mean daily intake of test substance (group means) was calculated based on individual valued for body weight and mean food intake per cage, according to the following equation:

$$\text{Substance intake for Day}_x = \frac{FC_x \times C}{BW_x}$$

with FCx as the mean daily food consumption (in g/day) on Dayx, C as the dose in ppm and BWx as body weight on Dayx of the study (in g).

4. Water consumption

Drinking water consumption was monitored by daily visual inspection of the water bottles for any changes in volume.

5. Ophthalmoscopy

Not performed in this study.

6. Functional observation battery (FOB)

Not performed in this study.

7. Motor activity measurement

Not performed in this study.

8. Hematology and clinical chemistry

On the day of necropsy, blood was withdrawn in the morning from fasted, isoflurane-anesthetized animals from the retro-orbital plexus (for hematology) or after decapitation (for clinical chemistry). The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Differential blood cell count*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Lymphocytes
✓	Hemoglobin (HGB)			✓	Neutrophils
✓	Hematocrit (HCT)		*Clotting Potential*	✓	Eosinophils
✓	Mean corp. volume (MCV)		Prothrombin time (Hepato Quick)	✓	Basophils
✓	Mean corp. hemoglobin (MCH)	✓	Platelet (Thrombocyte) count (PLT)	✓	Large unstained cells
✓	Mean corp. Hb. conc. (MCHC)		Activated partial thromboplastin time	✓	Monocytes
✓	Reticulocytes (RET)				
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bile acids (TBA)	✓	Aspartate aminotransferase (AST)
	Magnesium	✓	Bilirubin (total)	✓	Alkaline phosphatase (ALP)
✓	Phosphate (inorganic)	✓	Cholesterol	✓	γ-glutamyl transferase (GGT)
✓	Potassium	✓	Creatinine		
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis

Not performed in this study.

10. Plasma concentration analysis

All animals were subjected to plasma concentration analysis. EDTA blood samples (about 50 µL) were collected in the morning of study day 14 from non-fasted animals by puncturing the vena facialis under isoflurane anesthesia. After plasma preparation, the samples were transferred to the Analytical Chemistry Laboratory of the test facility and frozen at about -80°C prior to analysis.

11. Sacrifice and pathology

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically following fixation, preparation of tissue sections and hematoxylin and eosin staining.

Pathology:

S	W	H		S	W	H		S	W	H	
✓		#	adrenals	✓		#	jejunum (w. Peyer's patches)	✓		#	rectum
✓			aorta	✓	✓	✓	kidneys	✓			salivary glands*
✓		#	bone marrow§	✓			lachrymal glands%	✓		#	seminal vesicles
✓	✓	#	brain	✓			larynx	✓			skin
✓		#	caecum	✓	✓	✓	liver	✓		#	spinal cord (3 levels)@
✓		#	colon	✓		#	lung	✓	✓	#	spleen
✓		#	duodenum	✓		#	lymph nodes#	✓		#	sternum w. marrow
✓	✓	#	epididymides	✓			mammary gland (♂+♀)	✓		#	stomach (fore- & glandular)
✓			esophagus	✓		#	muscle, skeletal	✓	✓	#	testes
✓		#	eyes (with optic nerve)	✓			nerve, peripheral (sciatic n.)	✓		#	thymus
✓			femur (with joint)	✓			nose/nasal cavity‡	✓		#	thyroid/parathyroid
✓			gall bladder	✓	✓	✓	ovaries and oviduct**	✓		#	trachea
✓		✓	gross lesions	✓			pancreas	✓		#	urinary bladder
✓			Harderian gland	✓			pharynx	✓	✓	✓	uterus (weight incl. cervix)
✓	✓	#	heart	✓		#	pituitary	✓		✓	vagina
✓		#	ileum	✓		#	prostate&				

S: sampled; **W**: weighed; **H**: histopathologically examined; ✓: all groups, #: control and top dose.

§ from femur; # axillary and mesenteric; @ cervical, thoracic, lumbar; *mandibular and sublingual, ** oviduct not weighed; % extraorbital, ‡ histopathology at level III, &with coagulating gland

The immunorelevant organs and tissues were evaluated according to the following parameters:

Thymus:

- Increased/decreased grade of cortico-medullar ratio (related only to area)
- Increase of starry sky cells
- Changes of cellular density in the cortex
- Changes of cellular density in the medulla

Spleen:

- Changes of the cellularity of PALS, lymphoid follicles, marginal zone, red pulp
- Altered cellular composition of follicles
- Altered number of germinal centers

Lymph nodes (mesenteric and axillary lymph nodes):

- Changes in the cellularity of follicles, interfollicular area, paracortical area, medulla
- Altered cellular composition of paracortex
- Altered number of germinal centers
- Hyperplasia of high endothelial venules

Peyer's patches (of the jejunum):

- Changes of the cellularity of follicles (including mantle zone and germinal centers)
- Changes of the cellularity of interfollicular area

Bone marrow:

- Changes of the cellularity
- Changes of the myeloid/erythroid ratio

Whenever the histopathologic evaluation of the immunorelevant organs and tissues did not reveal a morphologic alteration of these items and/or whenever no other pathologic finding was noted, these organs were diagnosed as "no abnormalities detected".

Special attention was given for the synchrony of the morphology of the estrous cycle in ovaries, uterus, cervix, and vagina.

A correlation between gross lesions and histopathological findings was attempted.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above

B. OBSERVATIONS

1. Mortality

Male animal # 5 and female animal # 25 of test group 0 (0 ppm) died prematurely during the blood sampling procedure on study day 14.

2. Clinical signs of toxicity

No test substance-related effects were observed in male and female animals.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Mean body weights were significantly lower in female animals of test group 3 (2500 ppm) from study days 7 to 28 (max. of -23% on study day 21).

Mean body weight change values were lower in male animals of test group 3 (2500 ppm) from study days 7 to 28, significantly on study days 7 and 21 showing a body weight loss and the maximum deviation of -130% on study day 7. These animals recovered and gained weight from study day 14 onwards. In female animals of test group 3 (2500 ppm) body weight change values were significantly lower from study days 7 to 28 and a continuous body weight loss was observed between study days 0 to 21 (max. decrease of -161% on study day 21). The changes observed in male and female animals of test group 3 (2500 ppm) were regarded to be related to treatment and adverse.

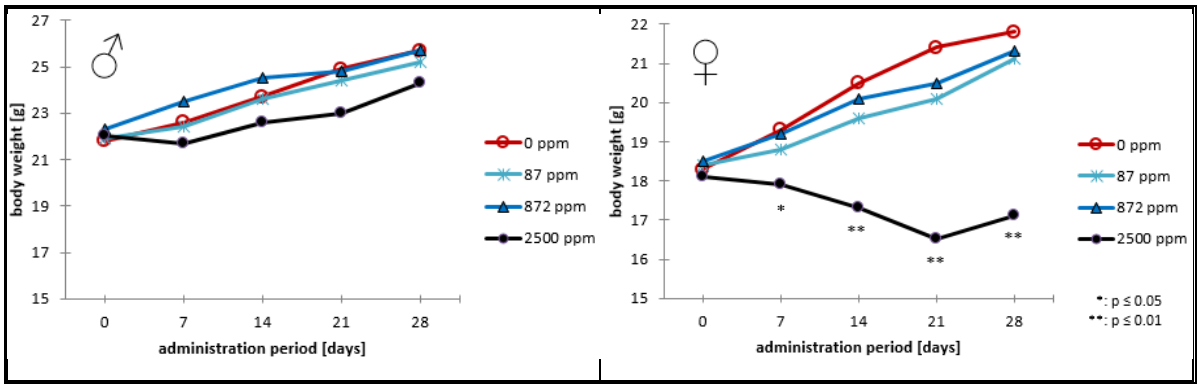
No changes of toxicological relevance were observed in male and female animals of test groups 1 and 2 (87 and 872 ppm). All recorded values were within the biological range typical for this strain of mice.

Table 5.8.1-12: Body weight and body weight change

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
Body weight [g]								
- Day 0	21.8	21.9	22.3	22.0	18.3	18.4	18.5	18.1
- Day 28	25.7	25.2	25.7	24.3	21.8	21.1	21.3	**17.1****
Δ% (compared to control#)		-1.9	±0.0	-5.2		-2.9	-2.0	**-21.4**
Body weight change (g)								
- Day 0-7	0.9	0.5	1.2	**-0.3***	1.0	0.4	0.7	**-0.2****
Δ% (compared to control#)		-43.2	+36.4	**-130**		-56.0	-34.0	**-120**
- Day 0-14	1.9	1.7	2.2	0.6	2.1	1.3	1.6	**-0.8****
Δ% (compared to control#)		-13.5	+13.5	-68.7		-40.6	-23.6	**-136**
- Day 0-21	3.3	2.5	2.5	**1.0***	2.7	1.8	1.9	**-1.6****
Δ% (compared to control#)		-24.8	-24.2	**-68.5**		-34.2	-27.5	**-161**
- Day 0-28	4.0	3.2	3.4	2.3	3.0	2.7	2.8	**-1.0****
Δ% (compared to control#)		-19.0	-15.5	-41.5		-8.7	-6.7	**-133**

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided);# values are rounded

Figure 5.8.1-4: Body weight development



D. FOOD CONSUMPTION, WATER CONSUMPTION AND TEST SUBSTANCE INTAKE

1. Food consumption

Food consumption was significantly decreased in female animals of test group 3 (2500 ppm) on study day 7 (-88%). This change was regarded to be related to treatment and adverse.

All other recorded values including those of male animals of the test group 3 (2500 ppm) were within the biological range typical for this strain of mice.

Table 5.8.1-13: Food consumption per animal and day

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
Food consumption [g]								
- Days 4-7	5.0	5.9	5.1	5.4	5.8	6.1	5.1	0.7
Δ% (compared to control[#]		+17.2	+2.0	+8.8		+5.2	-12.1	-87.9
- Days 11-14	4.5	4.8	4.7	4.9	5.5	10.7	5.0	6.2
Δ% (compared to control[#]		+6.7	+5.8	+10.1		+93.6	-8.2	+11.8
- Days 18-21	5.0	5.1	5.3	5.1	6.2	5.3	6.0	5.9
Δ% (compared to control[#]		+2.2	+6.7	+2.6		-15.2	-4.0	-5.6
- Days 25-28	6.0	5.7	5.2	6.1	6.2	7.4	7.0	7.2
Δ% (compared to control[#]		-4.9	-13.3	+3.4		+20.2	+13.7	+16.9

Statistical evaluation (males): * $p \leq 0.05$; ** $p \leq 0.01$; Dunnett test (two-sided);# values are rounded

2. Water consumption

No test substance-related changes in volume were observed during the daily visual inspection of the water bottles.

3. Test substance intake

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following table:

Table 5.8.1-14: Test substance intake

	Males			Females		
Dose level [ppm]	**87**	**872**	**2500**	**87**	**872**	**2500**
Reg.No 6011210 (mg/kg bw/d)	20	180	587	32	249	718

E. BLOOD ANALYSES

1. Hematology

No treatment-related changes among hematological parameters were observed. At the end of the administration period, in females of test group 2 (872 ppm) platelet counts were higher compared to controls, but the change was not dose-dependent. Therefore, the finding was regarded to be incidental.

Table 5.8.1-15: Hematology parameters (selected) – 28-day mouse study (Day 29)

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
Platelets [giga/l]	1437	1394	1670	1339	1120	1303	**1422***	1136

Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$; Kruskal-Wallis + Wilcoxion (two-sided)

2. Clinical chemistry

The following statistically significant changes of clinical-chemistry parameters were considered treatment-related (see Table 5.8.1-16):

Males

- ↑ Alkaline phosphatase (ALP) at 2500 ppm (adverse)
- ↑ Alanine aminotransferase (ALT) at 2500 ppm (adverse)
- ↓ Cholesterol at 2500 ppm (adverse)
- ↓ Triglycerides at 872 ppm and 2500 ppm (adverse)
- ↓ Total protein and albumin at 2500 ppm (adverse)
- ↓ Cholesterol at 2500 ppm (adverse)
- ↓ Total bilirubin at 87, 872 and 2500 ppm (considered to reflect increased conjugation and excretion via liver enzyme induction; adaptive / non-adverse)

Females

- ↑ Alkaline phosphatase at 2500 ppm (adverse)

The following statistically significant changes of clinical-chemistry parameters were assessed to be incidental, i.e. not considered treatment-related:

Males

- ↑ Aspartate aminotransferase (AST) at 2500 ppm (within historical control range)
- ↓ Alkaline phosphatase (ALP) at 87 ppm (no dose-response)
- ↓ Globulin at 872 and 2500 ppm (within historical control range)
- ↓ Chloride at 2500 ppm (within historical control range)

Females

🡻 Aspartate aminotransferase (AST) at 87 and 872 ppm (no dose-response)

🡹 Cholesterol at 872 and 2500 ppm (within historical control range)

🡻 Total bilirubin at 87 and 872 ppm (not dose-dependent)

Table 5.8.1-16: Clinical chemistry parameters – 28-day mouse study

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
ALT	0.78	0.69	0.81	**1.34**	1.03	0.71	0.96	1.55
AST [µkat/l]	3.38	3.53	3.48	4.98*	4.94	3.52*	3.08*	3.82
	Historical control range: 2.81 - 8.71							
ALP [µkat/l]	2.12	1.77*	2.10	**3.95***	2.38	2.26	2.31	**4.76***
Cholesterol [mmol/l]	2.61	2.22	2.53	**2.00***	1.76	1.74	2.22*	2.23
					Historical control range: 1.46 - 2.37			
Triglycerides [mmol/l]	0.78	0.61	**0.54***	**0.29***	0.75	0.59	0.31	0.37
Total protein [g/l]	50.73	49.19	49.46	**45.47***	46.74	48.19	46.28	46.73
Albumin [g/l]	31.02	30.85	31.10	**28.54***	30.08	31.22	29.97	29.88
Globulin [g/l]	19.71	18.35	18.37*	16.93*	16.66	16.96	16.31	16.85
	Historical control range: 15.64 - 20.21							
Chloride [mmol/l]	113.6	113.6	114.9	117.8*	114.3	113.4	113.6	112.5
	Historical control range: 110.2 – 119.6							
Total bilirubin [µmol/l]	1.56	1.01*	0.63*	0.65*	1.59	1.21*	0.80*	1.23

Statistical significance - * = p≤0.05; ** = p≤0.01 (Kruskal-Wallis / Kruskal-Wallis+ Wilcoxon, 2-sided)

3. Test substance concentration in blood plasma

Based on the analytical results it can be concluded that Reg.No. 6011210 was bioavailable after oral administration. Under the sampling conditions applied, measured plasma concentrations of parent compound were underproportional over concentrations of Reg.No. 6011210 in the diet.

Table 5.8.1-17: Reg.No. 6011210 plasma concentration

		Males		Females	
BAS 750 F Diet concentration [ppm]	**Sampling on Study Day**	**Samples evaluated [N]**	**Mean ± SD [ng/mL]**	**Samples evaluated [N]**	**Mean ± SD [ng/mL]**
0	14	5	*4 samples below LOQ*[#]	5	*all below LOQ*
87	14	5	149 ± 81	5	180 ± 57
872	14	5	326 ± 144	5	304 ± 105
2500	14	5	261 ± 124	5	641 ± 1027

[#] One control male sample with measured 10 ng/mL

F. NECROSCOPY AND PATHOLOGY

1. Organ weight

Terminal body weight and organ weight data are summarized in Table 5.8.1-18.

The terminal body weight (TBW) was significantly decreased in female animals at 2500 ppm (-16% compared to control). This reduction in body weight resulted in a decreased absolute spleen weight and in an increased relative brain weight.

Table 5.8.1-18: Organ weights

Sex		Males				Females			
Organ weight	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #	Absolute weight	Δ%	Relative weight [% of bw]	Δ% #
Terminal weight [g]	0	21.35				17.875			
	87	20.88	(-2)			17.14	(-4)		
	872	20.74	(-3)			17.78	(-1)		
	2500	19.44	(-9)			**15.08**	**(-16)**		
Brain (mg)	0	458.25		2.158		465.25		2.605	
	87	462.0	(+1)	2.214	(+3)	457.6	(-2)	2.674	(+3)
	872	458.4	(±0)	2.212	(+3)	471.0	(+1)	2.657	(+2)
	2500	467.2	(+2)	**2.405***	**(+11)**	443.6	(-5)	**2.949***	**(13)**
Epididymides (mg)	0	52.75		0.248		13.0		0.073	
/	87	53.4	(+1)	0.256	(+3)	13.2	(+2)	0.077	(+6)
Ovaries (mg)	872	55.8	(+6)	0.27	(+9)	14.4	(+11)	0.081	(+12)
	2500	53.0	(±0)	0.273	(+10)	10.4	(-20)	0.069	(-5)
Heart (mg)	0	126.25		0.59		114.75		0.641	
	87	120.4	(-5)	0.577	(-2)	113.6	(-1)	0.662	(+3)
	872	129.2	(+2)	0.62	(+5)	126.8	(+11)	0.717	(+12)
	2500	111.0	(-12)	0.571	(-3)	94.2	(-18)	0.621	(-3)
Kidneys (mg)	0	319.0		1.496		251.5		1.406	
	87	288.0	(-10)	1.377	(-8)	233.4	(-7)	1.362	(-3)
	872	286.6	(-10)	1.381	(-8)	261.2	(+4)	1.470	(+5)
	2500	**264.8***	**(-17)**	1.362	(-9)	226.4	(-10)	1.494	(+6)
Liver (mg)	0	973.0		4.559		838.0		4.682	
	87	916.2	(-6)	4.386	(-4)	770.4	(-8)	4.497	(-4)
	872	1033.6	(+6)	**4.989***	**(+9)**	**966.2***	**(+15)**	**5.44***	**(+16)**
	2500	**1347.8***	**(+39)**	**6.939***	**(+52)**	**1155.8***	**(+38)**	**7.666***	**(+64)**
Historical ctrl data (liver wt)		*854 - 1040*		*3.716 – 4.664*		685.0 – 886.6		4.172 – 5.447	
Spleen (mg)	0	41.5		0.195		51.75		0.29	
	87	40.8	(-2)	0.195	(±0	46.6	(-10)	0.271	(-6)
	872	37.4	(-10)	0.181	(-7)	49.4	(-5)	0.279	(-4)
	2500	**32.0***	**(-23)**	0.165	(-15)	**33.0***	**(-36)**	0.215	(-26)
Testes (mg)	0	194.75		0.914		84.25		0.473	
/	87	171.6	(-12)	0.822	(-10)	80.0	(-5)	0.466	(-1)
Uterus (mg)	872	178.6	(-8)	0.863	(-6)	107.4	(+27)	0.598	(+26)
	2500	176.6	(-9)	0.909	(-1)	72.8	(-14)	0.470	(±0)

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test, two sided)
# Values may not calculate exactly due to rounding of figures

In high-dose group males the decreased absolute kidney and spleen weights as well as the increased relative brain weight was related to the slightly but not significantly decreased terminal body weight (-9%). In addition, no histopathological correlates were observed that explained the weight changes.

The increased absolute and relative liver weights (statistically significant changes printed in bold) were in both sexes above or near the maximal value of the historical control data in at 872 ppm (males: 1033.6 mg, **4.989%**; females: **966.2 mg**, **5.44%**) and clearly above the historical control data at 2500 ppm (males: **1347.8 mg**, **6.939%**; females: **1155.8 mg**, **7.666%**). The changes were considered to be treatment-related.

The marked liver weight increase in females at 2500 ppm was likely to have masked an even more pronounced reduction of the terminal body weight (TBW) in this group compared to controls. Therefore, an additional assessment was performed. In Table 5.8.1-19, the terminal body weight of the individual females from the high-dose group were compared to the mean TBW (17.875 g) of control females. The individual values varied between -9.9% and -25.6%. Considering the high liver weights in test group 3, the TBW without the liver weight was calculated and compared to the mean control TBW without the liver weight (17.037 g, for calculation see Table 5.8.1-20). With this method, the decrease of the individual TBW in test group 3 females was even more prominent and varied between 15.026 g (-11.8%) and 12.325 g (-27.66%).

Table 5.8.1-19: High-dose group females: effect of liver weight increase on terminal body weight

Animal no.	**Terminal body weight**		**Liver wt**	**Terminal body weight minus liver wt****	
	(g)	Δ% control *	**(mg)**	(g)	Δ% control ***
36	15.0	-16.1%	1205	13.795	-19.03%
37	15.0	-16.1%	1232	13.768	-19.19%
38	13.3	-25.6%	975	12.325	-27.66%
39	16.1	-9.9%	1074	15.026	-11.80%
40	16.0	-10.5%	1293	14.707	-13.68%
Mean value	15.08		1155.8	13.924	-18.27%

* TBW of test group 3 females compared to mean TBW of female control group (17.875 g)
** TBW after subtraction of the liver wt
*** Comparison to corresponding mean control TBW after subtraction of liver weight (17.037 g)

Table 5.8.1-20: Body and liver weight data for control group females

Animal no.	Terminal body weight (g)	Liver wt (mg)	Terminal body weight minus liver wt (g)
21	18.3	865	17.435
22	17.8	806	16.994
23	17.0	754	16.246
24	18.4	927	17.473
25	*Animal died prematurely*		
Mean value	**17.875**	**838**	**17.037**

2. Gross pathology

There were two decedents in the study. The control male animal # 5 showed a severe dilation of the right kidney. For the control female # 25, no macroscopic findings were observed. Two female animals of test group 2 (872 ppm) as well as all males and 4 females of test group 3 (2500 ppm) showed a dark brown discoloration of the liver.

All other findings were single observations. They were considered to be spontaneous in origin and without any relation to treatment.

3. Histopathology

Treatment-related findings were noted in the liver of male and female animals and in the kidney in males.

Kidney

In males, the incidence of basophilic tubules was increased at 2500 ppm, but the severity was minimal. Only very few tubules were affected. This finding did not explain the decreased absolute kidney weight in males of this group. In females, the number of affected animals was higher in controls than in treated animals. Basophilic tubules of a minimal grade can occur as a frequent background finding even in controls. A treatment-related effect in males was assumed but assessed to be non-adverse as only very few tubules were affected.

Table 5.8.1-21: Kidney histopathology

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
No. of animals	5	5	5	5	5	5	5	5
KIDNEYS								
examined	5	5	5	5	5	5	5	5
Tubules, basophilic Grade 1	1	1	2	5	3		1	2

Liver

Hepatocellular hypertrophy was observed in the liver of all males and females at 872 ppm and 2500 ppm. At 2500 ppm, the hypertrophy showed a diffuse distribution pattern. The hepatocytes were slightly enlarged and showed a fine granular eosinophilic cytoplasm. The cytoplasmic change might correlate to the macroscopically observed dark brown discoloration. In males and females at 872 ppm the hypertrophy (without clearly visible cytoplasmic eosinophilic granularity) occurred centrilobular. The hypertrophy resulted in increased liver weights in male and female animals in these test groups.

Most of the males and females in the control group and at the low-dose (87 ppm) showed a diffuse hepatocellular fatty change, whereas no fatty change was noted in males and females of the mid- and high-dose groups (872 and 2500 ppm).

(Multi)focal necroses were seen in 2 males at 872 ppm and in 4 males as well as one female at 2500 ppm. One of the affected males of the high-dose group showed a few necroses of small size and a few necroses of larger size (grade 3). In all other animals affected, there were single or very few, very small necroses.

Table 5.8.1-22: Liver histopathology

	Males				Females			
Dose level [ppm]	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
No. of animals	5	5	5	5	5	5	5	5
LIVER								
examined	5	5	5	5	5	5	5	5
Hypertrophy, centrilobular Gr. 2			5				5	
Hypertrophy, diffuse Gr. 2				5				5
Necrosis, (multi)focal			2	4				1
Grade 1			2	3				1
Grade 3				1				
Fatty change, diffuse	4	5			4	5		
Grade 1	2	3			2	4		
Grade 2	2	2			2	1		
Fatty change, (multi)focal			2					

Ovaries, uterus, cervix and vagina

In the evaluated ovarian sections, no corpora lutea were present in 3 (out of 5) females of the high-dose group. The uterus in these 3 females was diffusely atrophic. The size of the uterus was decreased, especially the myometrium showed smooth muscle cells reduced in size. In 2 of these females this effect was slight. In the third affected female, the atrophy of the uterus was moderate and, in addition, this female showed a slight hypertrophy with mucification in cervix and vagina.

These findings were considered to be related to the reduced terminal body weight (TBW) in this test group. The mean TBW in control females was 17.875 g and varied between 17.0 and 18.4 g (see Table 5.8.1-20). In all high dose-group females, the TBW was clearly below the mean control value. Both females of test group 3 without findings in the genital tract showed a reduction of the TBW of only about 10% (16 g and 16.1 g), whereas the individual TBW of the 3 females with findings in the genital tract were reduced by about -16% (15 g) in two of these females and, in particular, by -25.6% (13.3 g) in female animal No. 38 showing moderate uterus atrophy, and slight hypertrophy with mucification in cervix and vagina.

Considering the high liver weights in test group 3 females, the TBW minus liver weights were calculated and compared to the mean control terminal body weight minus liver weight. With this method, the decrease of the individual TBW in test group 3 females was even more prominent and varied between -11.8% and -13.68% in the two females without findings in the genital tract, about -19% for the two females with slight findings and -27.66% in the female with the most severe findings (Table 5.8.1-19).

Therefore, all findings in the female genital tract were considered to be secondary to dramatically reduced TBW in these animals.

Decedents

The macroscopically diagnosed severe dilation in the right kidney in control male # 5 correlated histopathologically with a unilaterally extremely dilated renal pelvis. The control female # 25 did not show histopathological findings that might explain the premature death.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be spontaneous in origin and without any relation to treatment.

III. DISCUSSION

Reg.No. 6011210 was administered via the diet to groups of 5 male and 5 female C57BL/6 J Rj mice over a period of 4 weeks at concentrations of 0 (vehicle control; test group 0), 87 (test group 1), 872 (test group 2) and 2500 ppm (test group 3).

With regard to clinical examinations, signs of general systemic toxicity were observed in mice of test group 3 (2500 ppm) indicated by reduced food consumption, decreased mean body weight parameters in female animals and decreased body weight change values in both sexes. The changed parameters were assessed to be related to treatment and adverse. No signs of toxicological relevance were observed for male and female animals in test groups 1 and 2 (87 and 872 ppm).

Regarding clinical pathology, in mice of both sexes of test group 3 (2500 ppm) higher alkaline phosphatase (ALP) activities and in males of the same test group lower total protein, albumin, cholesterol and triglyceride levels indicated a changed liver cell metabolism probably due to liver enzyme induction. Slightly higher alanine aminotransferase (ALT) activities in males of the mentioned test group reflected a leakage of this enzyme through the liver cell membrane. Taken together and in combination with increased liver weights and liver histopathology, these clinical pathology changes were considered adverse.

Regarding pathology, target organs were the liver in males and females and the kidneys in males.

The **liver** weights (absolute/relative) were significantly increased in males and females at 872 ppm (males: relative +9%; females: +15%/+16%) and at 2500 ppm (males: +39%/+52%; females: +38%/+64%). The increased liver weights correlated with a slight hepatocellular hypertrophy that was observed in the liver of all males and females at and above 872 ppm. At 2500 ppm, the hypertrophy was diffuse. Additionally, the hepatocytes showed a fine granular eosinophilic cytoplasm. In males and females at 872 ppm the hypertrophy occurred centrilobular.

The granular eosinophilic cytoplasm might explain the dark brown discoloration of the liver that was macroscopically seen in two female animals at 872 ppm as well as in all males and in 4 females at 2500 ppm. Single or few, very small focal necroses were observed in two males 872 ppm and in 4 males and in one female at 2500 ppm. One of the affected males of the high-dose group showed in addition few focal necroses of larger size. All these findings were regarded to be treatment-related. Because the liver weight increase was quite high and clearly above the historical control data in males and females at 2500 ppm and because focal necroses were seen in males at 872 ppm and in male and female animals at 2500 ppm, the hepatocellular hypertrophy in males at and above 872 ppm as well as in females at 2500 ppm was regarded to be adverse. The hepatocellular hypertrophy led to a decrease of fat storage in hepatocytes. Whilst most of male and female animals in the control group and at 87 ppm showed a diffuse hepatocellular fatty change, no fat storage was seen in males and females at 872 ppm and 2500 ppm.

The decreased fat storage in hepatocytes was regarded to be secondary due to the hypertrophy and was assessed as non-adverse.

All males of the high-dose group showed very few basophilic tubules in the **kidneys**, whereas in the control group only one male (out of 5) was affected. In female animals, the highest incidence occurred in controls. The finding was assumed to be treatment-related, but the effect was considered to be non-adverse as only very few tubules were affected.

In the **ovaries**, no corpora lutea were present in the evaluated ovarian sections in 3 (out of 5) females of the high dose group (2500 ppm). The **uterus** in these 3 females was diffusely atrophic. In two of these females this effect was slight. In the third affected female the atrophy of the uterus was moderate and, in addition, this female showed a slight hypertrophy with mucification in **cervix** and **vagina**. The severity of these findings in the genital tract correlated with the degree of the body weight reduction in individual females. Therefore, these findings were considered secondary to the significantly reduced terminal body weight in females at 2500 ppm. Decreases in body weight of rodents can be associated with altered estrus cycle or complete cessation of the cycle as well as with a decrease in corpora lutea as described in the literature (e.g. Terry et al., 2005).

All other histopathological findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be spontaneous in origin and without any relation to treatment.

IV. CONCLUSION

Dietary administration of Reg.No 6011210 to C57BL/6 J Rj mice for 4 weeks caused test-substance related adverse signs of systemic toxicity (reductions in body weight gain and feed intake, evidence of liver toxicity from clinical chemistry and pathology findings) in males at concentrations of 872 ppm (128 mg/kg bw/d) and 2500 ppm (587 mg/kg bw/d) and in females at 2500 ppm (718 mg/kg bw/d). Under the study conditions, a NOAEL of 87 ppm (20 mg/kg bw/d) was established in male mice and a NOAEL of 872 ppm (249 mg/kg bw/d) was derived in female mice.

CA 5.8.2 Supplementary studies on the active substance

Mechanistic investigations of liver enzyme induction and cell proliferation

Studies of liver enzyme and cell proliferation induction were carried out in Wistar rats and in C57BL/6J mice (both wild-type and Pxr KO / Car KO strains) after dietary exposure to dose levels used in the carcinogenicity studies. In addition, *in vitro* investigations in primary hepatocyte cultures from human donors and from wild-type / knockout mice were carried out.
In the rat BAS 750 F administered for treatment periods of 3-28 days at a dose of 3600 ppm (top-dose of the combined chronic toxicity and carcinogenicity study) caused only weak responses – no induction of cell proliferation and very little impact on CYP enzyme activities. In wild-type mice, a marked induction of mainly Cyp2b10 was found, and a dose-dependently increased induction of liver cell proliferation (in male mice at all dose levels tested (20 – 200 ppm) and in females at 50 and 250 ppm. The findings were associated with increased serum ALT, liver weight increases and hypertrophy, but there was no evidence for degenerative changes. None of these findings, except for slight liver weight increases, were observable in Car /Pxr double knockout mice. In vitro studies with primary hepatocyte cultures from human donors and from wild-type and Car /Pxr double knockout mice provided further evidence that the main nuclear hormone receptor in BAS 750 F-mediated liver activation is Car, while Pxr did not seem to be involved. In human male and female hepatocytes, neither BAS 750 F nor the reference compound phenobarbital had an impact on replicative DNA synthesis. The assessment of CYP3A4 and CYP 2B6 revealed a small increase in BROD activity in male human hepatocytes by BAS 750 F, which however was not confirmed by increased CYP2B6 mRNA levels. Phenobarbital induced CYP2B6 and CYP3A4 at enzyme activity and mRNA level in both sexes.
Overall, the mechanistic data indicated that liver effects in C57BL/6J mice comprising increased serum ALT levels, increased liver weight, hypertrophy and liver cell proliferation are Car-mediated and therefore of limited relevance for human risk assessment.

Assessment of the immunotoxicity potential

A complete and comprehensive toxicology study database exists for BAS 750 F. The standard subchronic and chronic toxicity studies conducted with this active ingredient have assessed its potential impact on a number of potential immune-related endpoints, including hematological parameters like white blood cell count; spleen and thymus weights; and histopathology of the spleen, thymus, lymph nodes, or bone marrow. There were no treatment-related changes in white blood cell (WBC) count, select differential blood cell counts (lymphocytes, neutrophils, basophils, monocytes), or histology of the spleen, thymus, lymph node or bone marrow in any study. There was no evidence of a specific immunotoxic effect on any immune-related parameter. Not surprisingly, sporadic effects on single parameters were observed in some studies, but none supported a specific immunotoxic effect.

Studies of liver enzyme and cell proliferation induction in C57BL/6J mice

Report: CA 5.8.2/1
[redacted] 2015 a
A 28 day dietary study with BAS 750 F in male and female C57BL mice: Elucidation of hepatic mode of action and time course
2014/1170760
Guidelines: <none>
GLP: no
Dates of work: 22-Apr-2014 (start of in-life period) to 06-Jun-2014 (necropsy last sub-group)]
Report date 27-Nov-2015

Report: CA 5.8.2/2
[redacted] 2016 a
BAS 750 F - S-phase response study in livers (histopathological examination) of mice for the study: A 28 day dietary study with BAS 750 F in male and female C57BL mice: Elucidation of hepatic mode of action and time course
2014/1170771
Guidelines: <none>
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report: CA 5.8.2/3
[redacted] 2015 c
A 7 day dietary study with BAS 750 F in male and female C57BL/6 wild-type and Pxr KO/Car KO mice
2015/1037704
Guidelines: <none>
GLP: no

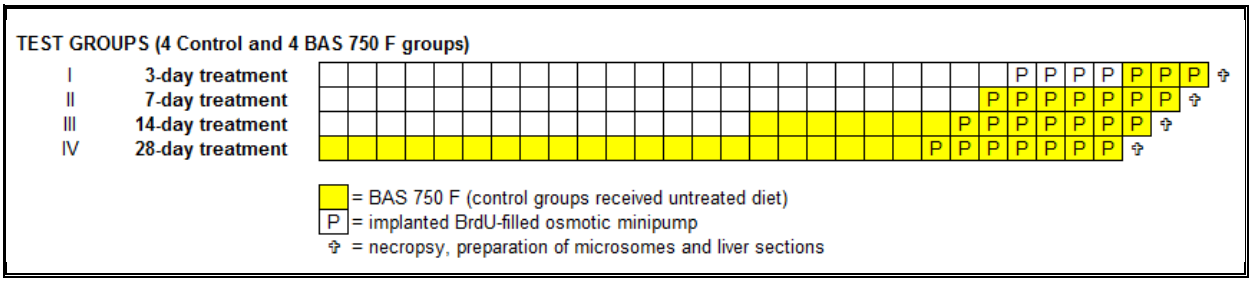
Report: CA 5.8.2/4
[redacted] 2016 b
BAS 750 F - S-phase response study in livers (histopathological examination) of mice for the study: A 7 day dietary study with BAS 750 F in male and female C57BL/6 wild-type and Pxr KO/Car KO mice
2015/1040901
Guidelines: <none>
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Two studies were carried out in C57BL mice to characterize the liver enzyme induction profile and the extent of liver cell proliferation following dietary exposure to BAS 750 F (Batch no. COD-001740, purity: 98.8%).

The first dietary exposure study ("peak study") was designed to determine the time course and the peak of liver cell proliferation at the top dose level administered in the mouse carcinogenicity study. For this, groups of 10 mice were administered BAS 750 F at either 0 ppm (untreated diet), 200 ppm (males) or 250 ppm (females) for durations of 3, 7, 14, or 28 days. Seven days before necropsy all animals were implanted osmotic minipumps filled with bromodeoxyuridine (BrdU) solution for immunohistochemical detection and quantification of zonal-specific cell proliferation. Effects on body weight, food consumption were assessed over the in-life period. On the day of necropsy, blood was sampled for determination of ALT, AST and ALP serum activities; terminal body weight and liver weights were recorded and livers were processed for (immuno)histopathological examination.

Figure 5.8.2-1: Experimental design (peak study)



The second dietary study ("main study") investigated the dose-response relationship of the cell proliferation and enzyme induction response produced by BAS 750 F after 7-day treatment (i.e. at the treatment duration that had produced the highest extent of cell proliferation in the peak study). Groups of 10 male C57BL mice were administered dose levels of 0, 20, 50 and 200 ppm (males) or 250 ppm (females). In addition, the top dose level was administered also to male and female Car/PxR knockout mice for comparison of the enzyme induction and cell proliferation response. As in the peak study, blood was sampled for determination of ALT, AST and ALP serum activities on the day of necroscopy; terminal body weight and liver weights were recorded and livers were processed for (immuno)histopathological examination.

In both studies, the enzyme induction profile was assessed on the level of liver microsomal enzyme activity (EROD. PROD, BROD, BQ and LAH), protein levels (Western blotting for Cyp1a1, Cyp1a2, Cyp2b10, cyp3a11 and Cyp4a) and gene expression via Taqman analysis (Cyp1a1, Cyp1a2, Cyp2b10, Cyp 3a11 and Cyp4a10). In addition, the activity of glucuronidation of ^{125}I-radiolabelled thyroxine (T_4) was assessed in the main study in both wild-type and Car/Pxr knockout mice.

Results of peak study

Treatment of males with 200 ppm BAS 750 F or females with 250 ppm BAS 750 F did not cause treatment related clinical signs or obvious changes in food intake. The dietary concentrations corresponded to intakes of about 30 mg/kg bw/d in males and 45 mg/kg bw/d in females. Treatment had no effect on body weight in males; in females, body weights were marginally higher in groups treated for 3 days (+8%**) or 7 days (+6%*). Small but statistically significant increases in serum ALT levels were seen in male mice after 3, 7, and 14 day treatment (up to 2-fold). ALP levels were marginally increased after 3-day treatment. AST levels were not significantly changed in males at any time point. In female mice ALT levels were increased at all four time points (max. 3-fold on day 7), ALP levels were marginally increased after 28-day treatment. Again, there were no significant effects on AST at any time point. Liver weight increases were observed at all time points in both sexes (up to 1.5-fold in males – day 28 and up to 1.6-fold in females –day 7).

Significant increases especially in Cyp2b10 and, to a lesser extent, Cyp3a11 levels were observed taking into consideration the assessment of mRNA levels, protein levels and enzyme activities. The peak hepatic Cyp2b10 enzyme induction appeared to occur following 14 days of BAS 750 F administration. Overall, the assessment of liver enzyme induction showed consistent changes at the level of enzyme activity, protein and gene expression to allow the conclusion that the main nuclear hormone receptor in BAS 750 F-mediated liver activation is CAR in both male and female mice, although a role of PXR cannot be ruled out. There was no evidence to suggest that AhR or PPARalpha were involved in the BAS 750 F-mediated response.

BAS 750 F induced hepatocellular hypertrophy at all time points with increasing severity over time. In males, the hypertrophy showed a centrilobular and in females a diffuse pattern. Females additionally showed a decreased vacuolation of centrilobular hepatocytes at all time points. There were no degenerative liver changes in this study.

In males, BAS 750 F at 200 ppm induced statistically significant increases in liver cell proliferation of comparable magnitude after 3 and 7 day treatment. In females, BAS 750 F at 250 ppm induced an increased S-Phase response after 3- and 7-day treatment, with the highest proliferation observed on day 7. The S-phase response was not increased after 14-day or 28-day treatment in either male or female groups administered BAS 750 F.

In conclusion, the results of the first study indicated that a 7-day duration of dietary exposure to BAS 750 F leads to a peak in liver cell proliferation response in male and female C57BL/6 mice. Additionally, the study showed that BAS 750 F is an inducer of murine Cyp2b and Cyp3a indicating it may activate the constitutive androstane receptor (Car) and possibly the pregnane X receptor (Pxr).

Results of main study

7-day dietary treatment of male and female C57BL/6 wild type and C57BL/6 Pxr KO/Car KO mice had no obvious effect on feed intake up to the highest dose level tested (200 ppm in males, and 250 ppm in females), compared to control group mice. The calculated mean intakes were 3.05, 6.39, 36.28 mg/kg bw/d in male WT mice given 20, 50, or 200 ppm, respectively; in female WT mice, the dietary concentrations of 20, 50 and 250 ppm corresponded to intakes of 4.90, 11.21 and 58.58 mg/kg bw/d. Male and female Pxr KO/Car KO mice that were exposed to the high-dose concentrations of 200 and 250 ppm, had mean intakes of 31.47 and 49.72 mg/kg bw/d. In the male WT mice there was a statistically significant increase in ALT levels only at the top-dose level of 200 ppm (+46%***); AST and ALP values were similar to controls. In male Pxr KO /Car KO mice, mean serum activities of the liver enzymes ALT, AST and ALP were marginally lower than the respective control group values, but were not statistically significantly changed. In female WT mice, ALT values were increased by 3.4-fold at 250 ppm; AST and ALT mean values were similar to controls. In the female Car Pxr / Car KO mice given 250 ppm BAS 750 F showed a slight but statistically significant decrease in ALP compared to controls (ca. -26%). Treatment with BAS 750 F had no statistically significant effect on terminal body weights. In WT mice, liver weights at 50 ppm were slightly increased in males and marginally increased in females at 20 and 50 ppm. Liver weight increases of about +40% were seen at the top-dose levels of 200 ppm (males) and 250 ppm (females). In the KO mice, liver weights were marginally increased in males at 200 ppm (+8%) and slightly increased in females at 250 ppm (+14%).

In male wild-type mice, BAS 750 F significantly induced Cyp2b10 across all markers assessed (mRNA, protein and enzyme activity) and at all dose levels evaluated. BAS 750 F caused only small increases in Cyp1a (across mRNA, protein and enzyme activity levels) and Cyp3a (at the mRNA and protein level only). However, the induction of all three enzymes was completely abolished in the male Pxr KO/Car KO mice, when the top dose of BAS 750 F was administered. The male Pxr KO/Car KO mice showed elevated Cyp4a mRNA levels, protein levels and 12-OH LAH production following BAS 750 F administration, while the wild-type mice did not show these changes at any dose level. The female WT mice showed a similar pattern to the male WT mice, with strong Cyp2b10 induction and only minor Cyp1a (enzyme activity only) and Cyp3a (mRNA only) induction. Again these responses were completely absent in the Pxr KO/Car KO mouse model following BAS 750 F administration at 250 ppm. The Cyp4a induction pattern was also similar in the female mice with only small increases in protein and activity in the WT mice, but stronger increases in mRNA, protein and enzyme activity in the Pxr KO/Car KO mice. There was only a small decrease in UDPGA thyroxine-glucuronosyl transferase (T_4-GT) activity in the female mice following administration of BAS 750 F at the top dose level; T_4-GT levels were unchanged in the male WT and in all Pxr/Car KO mice. Therefore, there were no compound related increases in thyroxine glucuronidation.

In wild-type mice, BAS 750 F induced increased hepatocellular proliferation and centrilobular hepatocellular hypertrophy in male animals at all dose levels tested. In WT females, BAS 750 F induced increased proliferation at dose levels of 50 ppm and 250 ppm, and diffuse hepatocellular hypertrophy with decreased centrilobular vacuolation at 250 ppm. Decreased centrilobular vacuolation is assumed to be a reflection of higher metabolic demand on these cell which results in less fat/glycogen storage. In Pxr KO/Car KO mice, hepatocellular hypertrophy and cell proliferation were not observed at the tested dose levels of BAS 750 F (200 ppm in males and 250 ppm in females).

Taken together, these data strongly suggest that the main nuclear hormone receptor in BAS 750 F-mediated liver activation is Car due to the effect on Cyp2b10 mRNA levels, protein levels and PROD/BROD enzyme activities in WT mice and the absence of any effect in the Pxr KO/Car KO mouse model. The small increases in Cyp3a mRNA levels (in both males and females) and protein levels (in the males only) are also likely to be mediated by the Car receptor and not the Pxr receptor as demonstrated by Scheer et al., 2008 for Phenobarbital. From the enzyme induction results generated within this study, there was no evidence to suggest that Ahr or Pparα were involved in the BAS 750 F-mediated WT mouse response and although genes normally regulated (Cyp4a) by the latter nuclear hormone receptor were induced in the Pxr KO/Car KO mice, this observation has been reported for Phenobarbital, a prototypical Car activator in this mouse receptor KO model (Tamasi et al, 2009).

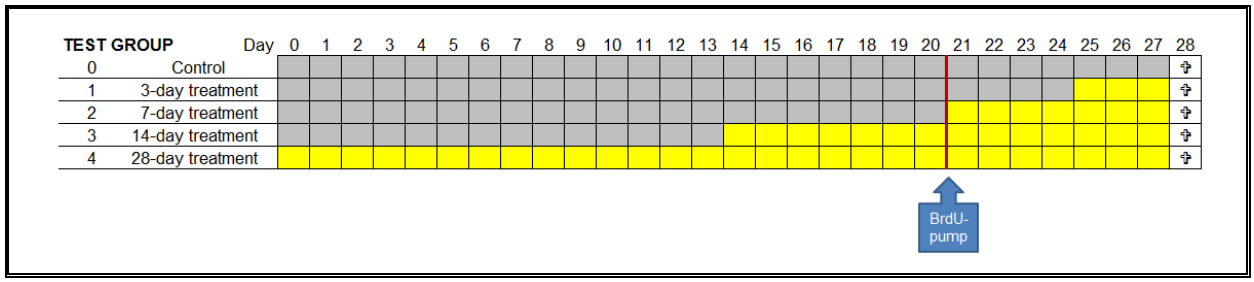
Studies of liver enzyme and cell proliferation induction in Wistar rats

Report:	CA 5.8.2/5 [redacted] 2015d BAS 750 F- S-Phase response study in Wistar rats - Administration via the diet for 3, 7, 14 and 28 days 2014/1170772
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Dates of work:	08-Jul-2014 to 17-Feb-2015 [in life phase: 14-Jul-2014 (start of treatment) to 12-Aug-2014 (last necropsy)]
Report date	12-Oct-2015
Report:	CA 5.8.2/6 [redacted] 2015 b Ex vivo analysis of liver samples taken at termination of a 3, 7, 14 and 28 day dietary study administering BAS 750 F in the diet to male and female Wistar rats 2014/1170773
Guidelines:	<none>
GLP:	no
Report date	27-Nov-2015

EXECUTIVE SUMMARY

The liver enzyme profile of BAS 750 F and the potential to induce liver cell proliferation was investigated in Wistar rats. BAS 750 F (batch COD-001740; purity: 98.8%) was administered to groups of 10 male and 10 female Wistar rats via the diet at 3600 ppm. The tested dietary concentration of 3600 ppm corresponded to the top dose level investigated in the rat combined chronic toxicity / carcinogenicity study with BAS 750 F. Four treatment durations (3, 7, 14, or 28 days) were assessed to determine the time point of maximum induction of S-phase response; the vehicle control group received rodent feed for 28 days. Seven days prior to necropsy, osmotic minipumps containing bromodeoxyuridine (BrdU) were implanted subcutaneously.

Figure 5.8.2-2: Experimental design



The scope of investigations comprised clinical signs during treatment, food consumption and body weights (determined weekly), liver weights, liver histopathology, quantification of BrdU-labelled hepatocytes in immunohistochemically stained liver sections. The liver enzyme profile was assessed by Taqman determination of cytochrome P450 mRNA levels (CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP4A1), Western blotting for characterization of protein levels and measurement of enzyme activities (EROD, BROD, PROD, BQ and LAH).

BAS 750 F treatment with 3600 ppm caused significant reductions in body weight gain in male rats at all treatment durations and in females only following 28-day treatment.

There were no obvious effects on food consumption. Mean test substance intakes after 3, 7, 14, or 28 days of treatment were 260, 279. 239, and 228 mg/kg bw/d in the respective male groups and 223, 228, 197 and 302 mg/kg bw/d for the respective female groups. At necroscopy, body weights were significantly decreased in males after 7, 14, and 28-day treatment (by 4, 5, and 9%, respectively) and in females after 28-day treatment (-6%). Absolute liver weights were not changed in males at any time point and were slightly increased in females fed BAS 750 F for 14 or 28 days (by 9% and 14%, corresponding to relative weight increases of +11% and +14%, respectively). In males, relative liver weights were slightly increased at all time points (by 6-11% percent, maximum after 28 days). There was no histopathological correlate to the slight liver weight increases in any of the male or female test groups.

BAS 750 F treatment of male and female rats had very little impact on the range of cytochrome P450 enzyme activities assessed during this study.

CYP2B mRNA levels were increased (between 23- and 104-fold) with associated increases of protein levels, while CYP1A1 and CYP3A mRNA and protein levels were only slightly induced. There was no relevant induction of CYP1A2 or CYP4A1.

Assessment of S-phase response did not reveal evidence for a treatment-related increase of liver cell proliferation in in any of the male or female test groups receiving 3600 ppm BAS 750 F via the diet for durations of 3 -28 days.

(DocID 2014/1170772)

Liver enzyme and cell proliferation induction in primary hepatocyte cultures

Report: CA 5.8.2/7
Elcombe B. et al., 2016 a
BAS 750 F - Enzyme and DNA-Synthesis induction in cultured male and female wild-type and Pxr KO/Car KO mouse hepatocytes
2015/1037705
Guidelines: <none>
GLP: no

EXECUTIVE SUMMARY

The potential for BAS 750 F (batch: COD-001740, purity: 98.8%) to activate Car and/or Pxr receptors and stimulate cell proliferation was investigated by comparison of the responses in isolated male and female C56BL/6 wild-type (WT) and Pxr KO/Car knockout (KO) mouse hepatocytes. Primary monolayer hepatocytes were prepared from approx. 8-wk old male and female mice obtained from Harlan UK Ltd, Blackthorn, UK (WT mice) or from Taconic Farms, Germantown, U.S.A. (KO mice). In pre-tests, administration of 30 µM or higher concentrations of BAS 750 F caused substantial cytotoxicity (endpoint: ATP depletion) in both male and female wild-type and Pxr KO/Car KO mouse hepatocyte cultures. Therefore, 10 µM was the top BAS 750 F concentration assessed during the main study; further test concentrations were 0.3, 1 and 3 µM, as well as the vehicle control DMSO (0.1% v/v) and phenobarbital (100 and 1000 µM). Cell proliferation was determined by BrdU incorporation in hepatocyte nuclei over the last 3 days of 4-day culture, followed by fixation, immunostaining and quantification. Epidermal growth factor (EGF, 25 ng/mL) was assayed as positive cell proliferation control item in parallel. Enzyme activities of PROD (Cyp 2b), BROD (Cyp2b and Cyp3a) and BQ (Cyp3a11) were assessed, and gene expression of Cyp2b10 and Cyp3a11 were assayed via Taqman® mRNA analysis, using mouse ß-actin as internal standard.
In both male and female WT mouse hepatocytes, Cyp2b10 mRNA levels and activity (as demonstrated by increased PROD and BROD activities) were induced by BAS 750 F, demonstrating Car-activation. S-phase was also induced in both male and female WT hepatocytes by BAS 750 F.

In the Pxr KO/Car KO mouse hepatocytes BAS 750 F failed to cause any increase in the CYP enzyme activities, mRNA levels or replicative DNA synthesis again suggesting that the increases in enzyme activities and hyperplasic responses to BAS 750 F were Car-mediated taking into consideration the results generated from the WT mouse hepatocytes. The top two concentrations of BAS 750 F decreased enzyme activities and mRNA levels, which again may be indicative of early cytotoxicity not yet detected by changes in ATP levels.

Report: CA 5.8.2/8
Elcombe B., 2016 a
BAS 750 F - Enzyme and DNA-Synthesis induction in cultured male and female human hepatocytes
2015/1037703
Guidelines: <none>
GLP: no

EXECUTIVE SUMMARY

In close analogy to studies performed in cultured mouse hepatocytes, BAS 750 F (batch: COD-001740, purity: 98.8%) was also tested in human liver cells for its potential to activate nuclear hormone receptors CAR and/or PXR receptors (therefore inducing CYP2B and CYP3A liver enzyme activity) and to stimulate cell proliferation [via replicative DNA synthesis (S-phase response)] in isolated male and female human hepatocytes. The liver cells from a single male and a single female donor were supplied cryopreserved and plateable from Invitrogen™ (Life Technologies), Warrington, UK. Documented quality checks of the donor cells from the supplier indicated a cell viability of 93-95% and a robust enzyme induction profile.
An initial cytotoxicity assay (endpoint: ATP depletion) indicated cytotoxicity at concentrations of 3 µM and above, which was therefore selected at top concentration in the main investigations; further test concentrations were 0.1, 0.3, and 1 µM, as well as the vehicle control DMSO (0.1% v/v) and phenobarbital (100 and 1000 µM). Cell proliferation was determined by BrdU incorporation in hepatocyte nuclei over the last 3 days of 4-day culture, followed by fixation, immunostaining and quantification. Epidermal growth factor (EGF, 25 ng/mL) was assayed as positive cell proliferation control item in parallel. Enzyme activities of PROD (CYP2B6), BROD (CYP2B6 and CYP3A4) and BQ (CYP3A4) were assessed, and gene expression of CYP2B6 and CYP3A4 were assayed via Taqman® mRNA analysis, using human ß-actin as internal standard.

The results of enzyme induction investigations demonstrated that following treatment of male human hepatocytes with BAS 750 F, the CYP2B6 and CYP3A4 mRNA levels and enzyme activities were essentially unchanged, with only a small increase in BROD enzyme activity being measured. In the female human hepatocytes, there were no increases in either CYP2B6 or CYP3A4 enzyme activities or mRNA levels following BAS 750 F administration.

In both the male and female human hepatocytes, PB induced CYP2B6 and CYP3A4, as measured by Taqman® mRNA analysis and PROD, BROD and BQ activities.

Neither BAS 750 F nor PB had any impact on replicative DNA synthesis in the male and female human hepatocytes, although EGF produced robust responses in both sets of hepatocytes.

Immunotoxicological potential of BAS 750 F

A complete and comprehensive toxicology study database exists for BAS 750 F. The standard subchronic and chronic toxicity studies conducted with this active ingredient have assessed its potential impact on a number of potential immune-related endpoints, including hematological parameters like white blood cell count; spleen and thymus weights; and histopathology of the spleen, thymus, lymph nodes, or bone marrow. There were no treatment-related changes in white blood cell (WBC) count, select differential blood cell counts (lymphocytes, neutrophils, basophils, monocytes), or histology of the spleen, thymus, lymph node or bone marrow in any study. There was no evidence of a specific immunotoxic effect on any immune-related parameter. Not surprisingly, sporadic effects on single parameters were observed in some studies, but none supported a specific immunotoxic effect.

No effects were observed in either the 28-day or 90-day toxicity studies in the rat. In the long-term toxicity/carcinogenicity study in the rat, terminal body weight (toxicity phase) was significantly decreased in top-dose females (3600 ppm) after 12 months resulting in increased mean relative weights of the spleen and other organs. In the carcinogenicity phase, terminal body weights were also significantly decreased in top-dose group females (3600 ppm) after 24 months. These decreases resulted in decreased absolute weight of the spleen, as well as other organs. Given the general systemic toxicity observed at the high dose as well as the absence of histopathological findings in the spleen, this high-dose finding does not point to a specific immunosuppressant effect (US EPA Office of Pesticide Programs guidance document on Part 158 toxicology data requirements, May 1, 2013).

An increase in thymus weight was observed in females at the highest dose tested (1000 ppm) in the 28-day mouse study. The change was considered adverse by the study authors; however, given the absence of any histopathological finding in the thymus, as well as the significant systemic toxicity (reductions in body weight gain and feed intake, evidence of liver toxicity from clinical chemistry and pathology findings) observed at this dose, the finding does not indicate a specific, immunotoxic effect. Decreases in the relative eosinophil count (females) and the albumin/globulin ratio (males and females) were observed in the 90-day mouse study at the highest dose tested (750 ppm). The change in albumin/globulin ratio was likely due to a change in function of the liver (site of synthesis) seen at that dose. Therefore, given the significant general systemic toxicity (especially liver) that was observed at the high dose, because WBC and differential neutrophil and monocyte counts were unaffected by treatment, and since there was no evidence of bone marrow pathology, the observations do not point to a specific immunotoxic effect. In the 18-month oral carcinogenicity study in mice, the blood smears evaluated after 12-month or 18-month treatment did not point to a shift in the blood cell composition by treatment with BAS 750 F. Spleen and thymus weights were also unaffected in that study, and there were no histopathological findings in the thymus, spleen, or bone marrow after 18 months of treatment.

A treatment-related decrease in thymus weight was observed in males at 1000/250 mg/kg bw/day and in females at ≥300/125 mg/kg bw/day in the 28-day dog study; however, these findings were interpreted to be secondary to the reduced terminal body weight in these animals (even after significant dose de-escalation in both sexes) and to possess high inter-individual variability. In the absence of any histopathological findings in the thymus, they do not indicate specific suppression of the immune system. No treatment-related changes were observed in thymus weights or histopathology in either sex in the 90-day dog study up to the highest dose tested (180 mg/kg bw/day).

In the 2-generation reproduction study in rat, spleen and thymus weights were slightly reduced in F_1 and F_2 pups at the highest dose tested (200 mg/kg bw/day); however, as in F_0 parental animals, these changes were clearly related to decreased body weights, and there were no anomalous findings in these organs at gross necropsy. Finally, globulin levels were decreased in pregnant rabbit does prior to sacrifice (GD 29) at the highest dose tested (25 mg/kg bw/day) in the prenatal developmental toxicity study in rabbits. The observation was not considered adverse by the study authors and does not point to a specific immunotoxic effect in the absence of changes in any other immune-related parameter, including leukocyte count and differential WBC counts. It was likely related to changes in liver (site of synthesis) function in these animals.

In summary, sporadic or isolated effects on single parameters were observed in routine subchronic, chronic, or developmental toxicity studies with BAS 750 F. Other than in the rabbit developmental study, no change was observed in any parameter at doses below those at which frank or general systemic toxicity, including body weight decrease, was reported. Decreased globulin levels at the highest dose tested in pregnant does was not considered adverse by the study authors of the rabbit study and was likely the outcome of slightly impaired function of the liver, the site of synthesis of plasma proteins. In the absence of changes in any other immune-related parameter including leukocyte count and differential WBC counts, the finding does not point to a specific immunotoxic effect. Histopathological correlates were not observed for any reported changes in immune system organ weights across the data base. Some organs, like the thymus, are known to be sensitive to stress or general systemic toxicity and can reflect such non-treatment-related changes [Dewhurst I. et al. (2015): “Retrospective analysis of the immunotoxic effects of plant protection products as reported in the Draft Assessment Reports for their peer review at EU level”, External Scientific Report, EFSA Supporting Publication 2015:EN-782]. Based on the weight and strength of the evidence, a specific immunotoxic potential for BAS 750 F is not supported.

CA 5.8.3 Endocrine disrupting properties

This chapter includes a summary of an in vitro aromatase inhibition assay with BAS 750 F, its enantiomers and with the metabolite Reg.No. 6011210 (Section A) as well as an assessment of the endocrine disruption potential of BAS 750 F performed on the basis of the current interim criteria of Regulation 1107/2009 (Section B).

Summary of in vitro aromatase inhibitor study

BAS 750 F (Pure active ingredient, 50:50 R:S racemate), the R-Enantiomer Reg.No. 5934591, the S-Enantiomer Reg.No. 5934588 and the livestock metabolite Reg.No. 6011210 were tested *in vitro* for their effect on human aromatase activity (CYP 19) using human CYP19 supersomes. The non-radioactive test method corresponded to OCSPP (EPA) Guideline 890.1200 and included negative and positive control substances. In a separate validation study, additional compounds with known aromatase inhibiting potential were assessed using this test method, which produced similar half-maximum inhibition (IC_{50}) values as the radioactive method described in the EPA Guideline. Under the study conditions, the S-enantiomer of BAS 750 F (Reg.No. 5934588) was found with the lowest IC_{50} concentration of 0.58 µM, followed by BAS 750 F (the 50:50 R,S-racemate) with an IC_{50} of 0.92 µM and the R-enantiomer Reg.No. 5934591 (IC_{50} of 2.97 µM). This graduated response of aromatase inhibition for BAS 750 F and its enantiomers was reproducibly found in all individual test runs. The BAS 750 F metabolite Reg.No. 6011210 (= M750F022) had a very weak inhibitory effect on aromatase activity; at the highest test concentration of 316 µM the remaining aromatase activity of Reg.No. 6011210 was still 46% of the maximum activity.

In view of the different aromatase inhibiting potential that were derived for the two BAS 750 F enantiomers, it is of interest to note that the enantiomers also are different in terms of metabolisation. As shown in the rat metabolism study with BAS 750 F (see MCA 5.1), the relative amounts of the enantiomers were approx. 1:1 in the application formulation and remained highly similar in the methanol extracts of faeces. In the methanol extracts of liver and kidney as well as in plasma, however, the ratio between S- and R-enantiomer shifted towards a higher relative amount of the R-enantiomer.

A. MECHANISTIC STUDIES

Report: CA 5.8.3/1
Mentzel T., 2016 c
Reg.No. 5834378 (BAS 750 F, R,S-racemate), Reg.No. 5934588 (S-enantiomer), Reg.No. 5934591 (R-enantiomer), Reg.No. 6011210 (metaboite) - Human recombinant aromatase assay
2015/1261377
Guidelines: EPA 890.1200
GLP: no

Report: CA 5.8.3/2
Mentzel T., 2016 d
Letrozole – Econazole nitrate – 4-OH ASDN – Fenarimol – Epoxiconazole – Nitrofen – Atrazine – Bis(2-ehtylhexyl)phthalate – Human recombinant aromatase assay
2016/1001905
Guidelines: EPA 890.1200
GLP: no

EXECUTIVE SUMMARY

BAS 750 F (Reg.No. 5834378, pure active ingredient, 99.4% pure, batch: L85-12), the S enantiomer Reg.No. 5934588 (99.5% pure, batch: L84-256) the R enantiomer Reg.No. 5934591 (98.9% pure, batch: L84-254) plus BAS 750 F metabolite Reg.No. 6011210 (99% pure, batch: L85-116; *syn* M750F022) were tested *in vitro* for their effect on human aromatase activity (CYP 19). Human CYP19 supersomes (aromatase + reductase) were exposed to the test substances, or to the positive reference substance 4-OH-ASDN at concentrations ranging from 10^{-10} to 3.16 x 10^{-4} M as well as to the solvent DMSO. Enzyme activity was determined fluorometrically using dibenzylfluorescein as a model substrate. Resulting activity values were fitted using a 4-parameter regression model, which yielded sigmoidal inhibition curves and allowed for calculation of IC_{50} values.
BAS 750 F and both enantiomers had a measurable effect on aromatase (CYP19) activity. The small confidence interval and high R^2 obtained underline the high reproducibility of the individual measurements and allowed for an accurate comparison of the different compounds. Under the study conditions, the S-enantiomer of BAS 750 F (Reg.No. 5934588) was found with the lowest half-maximum inhibition (IC_{50}) concentration of 0.58 µM, followed by BAS 750 F (the 50:50 R,S-racemate) with an IC_{50} of 0.92 µM and the R-enantiomer Reg.No. 5934591 (IC_{50} of 2.97 µM). This graduated response of aromatase inhibition for BAS 750 F and its enantiomers was reproducibly found in all individual test runs. Overall, the IC_{50} values obtained for the racemate and the two enantiomers were all within the same order of magnitude (difference less than a factor of 10).

The metabolite Reg.No. 6011210 (= M750F022) had a very weak effect on aromatase activity. The calculated IC_{50} value of 715 µM estimated on the basis of the modeled dose-response curve was not considered to be a sufficiently precise estimate. Even at the solubility limit concentration of 316 µM, the remaining enzyme activity was still 46% of the maximum value. Therefore, according to criteria of the OCSPP Guideline 890.1200, Reg.No. 6011210 cannot be securely identified as an inhibitor of CYP19 enzyme activity.

The reference compound 4-hydroxy-androstenedione (4-OH ASDN) had a comparable effect as in other studies using this assay system. The obtained IC_{50} value for 4-OH-ASDN of about 0.02 µM is lower by a factor of approx. 3.6 compared to IC_{50} data derived using radiolabeled C19 androgens as substrates (OCSPP Guideline 890.1200). Further, a separate validation for this protocol demonstrated that inhibitor substances and non-inhibitors are identified as such and that IC_{50} values fall in a similar range as reported for assays using radiolabeled C19 androgens as substrates. (DocID 2015/1261377)

I. MATERIAL AND METHODS

The analysis of aromatase (CYP19) enzyme activity can be used to identify chemicals that can inhibit the catalytic activity of aromatase through an interaction with the substrate binding site on the enzyme. The standard method to detect aromatase inhibitors uses placental tissue as a source of aromatase enzyme and radiolabeled C19 androgens as substrates (OCSPP Guideline 890.1200). In the present study a modified method published by Stresser D.M.et al. (Analyt. Biochem. 284, 427-430, 2000) was applied, which uses recombinant aromatase and the fluorometric substrate O-benzyl fluorescein benzyl ester (DBF). The test method has recently been validated in separate comparative assays of inhibitor and non-inhibitor compounds that were recommended for validation purposes by OCSPP Guideline 890.1200.

The aim of the study was to determine the IC_{50} of Reg.No. 5834378 (BAS 750 F pure active ingredient), Reg.No. 5934588 (S-enantiomer), Reg.No. 5934591 (R-enantiomer) and Reg.No. 6011210 (metabolite) to recombinant human CYP19 (aromatase) and to compare the effect of these compounds towards human aromatase enzyme activity.

A. MATERIALS

1. Test Material:

Test item 1	Reg.No. 5834378 (BAS 750 F pure active ingredient)
Description:	solid powder
Batch #:	L85-12
Purity / content:	99.4% (racemate, R/S ratio: 50/50)
Test item 2	Reg.No. 5934588
Description:	solid powder
Batch #:	L84-256
Purity / content:	99.5% (S-Enantiomer of BAS 750 F)
Test item 3	Reg.No. 5934591
Description:	solid powder
Batch #:	L84-254
Purity / content:	98.9% (R-Enantiomer of BAS 750 F)
Test item 4	Reg.No. 6011210
Description:	solid powder
Batch #:	L85-116
Purity / content:	99.0%

2. Vehicle: DMSO (final concentration: 1%)

3. Reference substance: 4-Hydroxy-androstenedione (4-OH ASDN)

Description: solid powder
Batch #: 081k2133V (Sigma-Aldrich F25525)
Purity / content: 99.6%

4. Test substances assessed in validation study

Test item 1 (reference): 4-OH-ASDN (CAS-No. 566-48-3)
Description: solid powder
Batch #: 081k2133V (Sigma-Aldrich F25525)
Purity / content: 99.6%

Test item 2: Atrazine (CAS-No. 1912-24-9)
Description: solid powder
Batch #: SZBD158XV (Sigma-Aldrich #45330)
Purity / content: 99.1%

Test item 3: Bis(2-ethylhexyl)phthalate (CAS-No. 117-81-7)
Description: solid powder
Batch #: SZBB167XV (Fluka #36735)
Purity / content: 99.7%

Test item 4: Nitrofen (CAS-No. 1836-75-7)
Description: solid powder
Batch #: SZBA307XV (Sigma-Aldrich #33374)
Purity / content: 99.6%

Test item 5: Fenarimol (CAS-No. 60168-88-9)
Description: solid powder
Batch #: SZBD071XV (Fluka #45484)
Purity / content: 99.9%

Test item 6: Econazole nitrate (CAS-No. 24169-02-6)
Description: solid powder
Batch #: BCBL5063V (Sigma Aldrich #E4632)
Purity / content: 98%

Test item 7: Epoxiconazole (CAS-No. 133855-98-8)
Description: solid powder
Batch #: SZBD099XV (Fluka #36848)
Purity / content: 99%

Test item 8: Letrozole (CAS-No. 112809-51-5)
Description: solid powder
Batch #: 104M4759V (Sigma-Aldrich #L6545)
Purity / content: 98%

B. STUDY DESIGN AND METHODS

1. Dates of work: 02-Sep-2015 to 22-Sep-2015 (main study)
[20-Aug-2015 to 22-Oct-2015 (validation study)]

2. Test method

The assay was conducted in a 96-well microplate utilizing the recombinant human aromatase (Corning Supersomes Human CYP19 (aromatase + reductase) expressed in baculovirus/insect cells and the fluorometric artificial substrate dibenzylfluorescein (DBF). Test compounds were dissolved in DMSO (final DMSO concentration 1%). After addition of the test compounds including dilutions and all cofactors (1.3 mM $NADP^+$, 0.4 mU Glucose-6-phosphat-dehydrogenase, 3.3 mM Glucose-6-phosphate and 3.3 mM $MgCl_2$), the enzymatic reaction was started with 100 µl of enzyme/substrate mix (4 pmol/ml enzyme, 0.4 µM DBF). Plates were incubated for 30 minutes at 37°C. The aromatase-mediated oxidation of DBF was stopped by addition of 75 µl 2 M NaOH, which also results in a cleavage of the oxidized DBF to the fluorescent product fluorescein. To develop an adequate signal to noise ratio, plates were incubated for another 2 hours at 37°C. Measurement was conducted at 490 nm excitation wavelength, 530 nm emission wavelength and 515 nm cut-off.

In this study, the experimental design described by Stresser et al. was adapted to correspond to OCSPP Guideline 890.1200, including 4 individual test runs and four replicates per concentration and substance in each test run. 12 wells per 96-well test plate without enzyme were used as control to determine background fluorescence. In addition, the maximum enzyme activity under the study conditions ("full activity") was determined in vehicle-only samples using DMSO only in 12 wells per plate. Pre-tests were carried out to determine the solubility limit and to select the appropriate test concentrations test compound. 12 concentrations were tested, spaced by half order of magnitude, and ranging from concentration eliciting no inhibition up to either the solubility limit or maximum inhibition. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, all tests were performed by the same technician.

Validation study

For the comparison of the method with the radiolabeled methodology described in OCSPP Guideline 890.1200, the half maximal inhibition of human aromatase enzyme was determined in two series of tests with reference substances, including proficiency chemicals recommended by OCSCPP Guideline 890.1200. In the first series, four inhibitor substances (econazole nitrate, 4-OH ASDN, fenarimol and nitrofen), as well as the fungicide epoxiconazole were analyzed. The analysis was repeated in five independent experiments. In a second series, the two non-inhibitor compounds atrazine and bis(2-ethylhexyl)phthalate as well as another inhibitor compound, letrozole, was analyzed in four independent test runs. Again the aromatase inhibitor 4-OH ASDN was included as reference substance. Slopes and IC_{50} values from individual runs were compared.

3. Data interpretation

The method used in this study is measuring the generation of a fluorescent product for the analysis of human aromatase activity. Absolute fluorescence values were corrected by subtraction of mean background control of each individual plate and normalized to the full activity control to achieve % activity values.

Values of background and full activity controls were determined and ratios of full activity / background activity were calculated. Average ratios that are below 15% of mean full activity were considered to represent an ideal activity range for activity measurements.

5. Statistical analysis

Dose Response analyses were made using a log-logistic 4-parameter model. Assumptions, such as normality and homogeneity of residuals, were checked for each model calculated in plotting the sample vs theoretical quantiles of a normal distribution and the model residuals vs. the fitted values. Parameter estimates made by the model such as IC_{50}, Slope, as well as minimum and maximum response were back-transformed and were reported on the scale experimental readings were taken on. A global model was calculated for each substance across test runs and replicates. To avoid pseudo-replication, average values (mean) per concentration across replicates were calculated. In addition, for a comparison between runs a four-parameter log-logistic model was fitted for each of the 4 test runs with 4 replicates per run. Also parameter estimates for all models were compared for each test run and replicate. Comparisons were made calculating the ratio and its standard error between test runs or replicates. Significant deviations from 1 (parameters between test runs or replicates are identical) were detected using a t-test.

Model fit was assed using both the adjusted R^2 and a lack-of-fit-test, comparing a standard linear model (one-way ANOVA) to the four-parameter log logistic model. All calculations were made using R 3.2.1 (R Core Team 2015). Dose-response models were calculated using the “drc” package version 2.5-12 (Ritz & Strebig 2015).

II. RESULTS

A. Human Aromatase Inhibition

1. Main study:

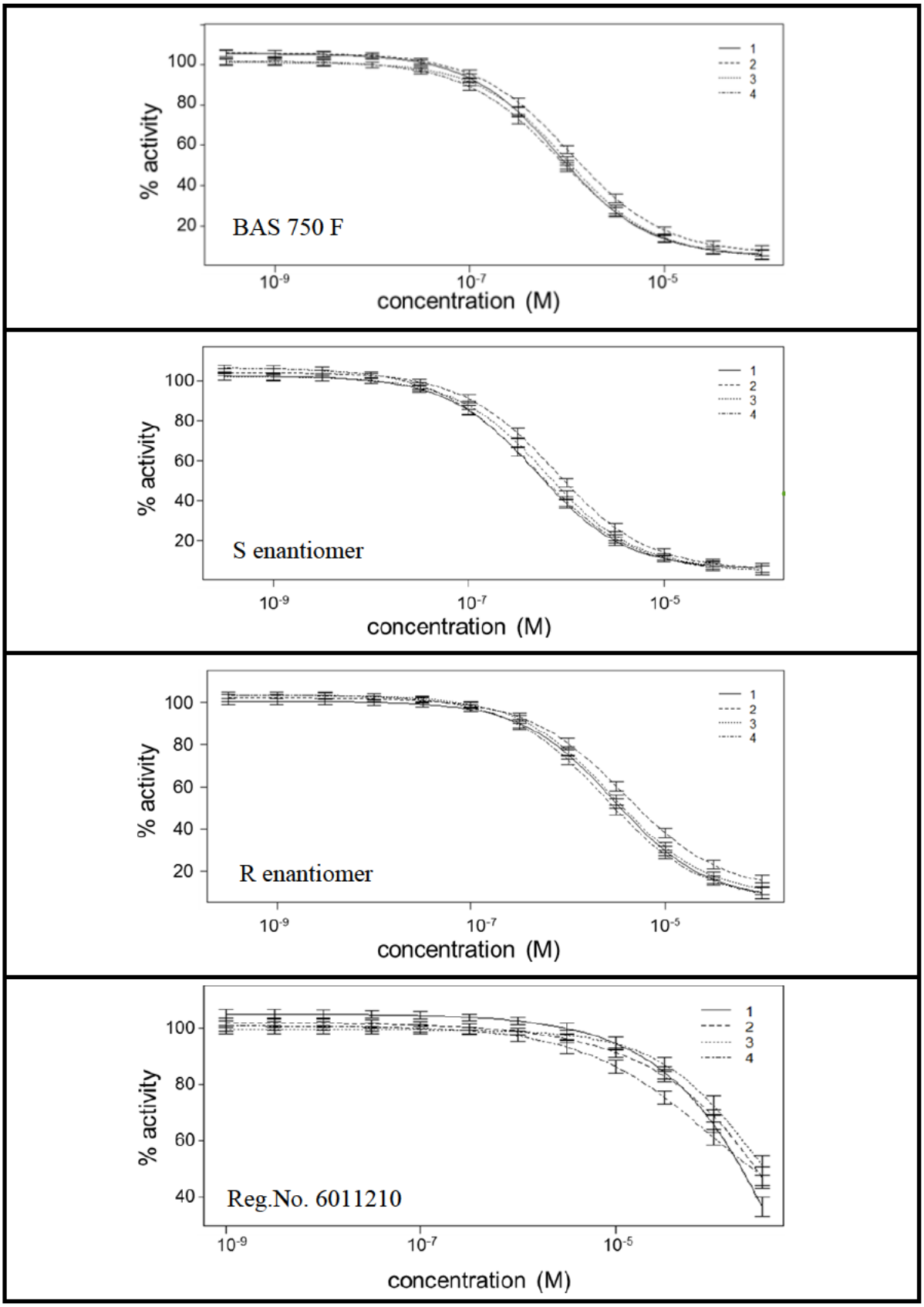
BAS 750 F showed concentration-dependent inhibition of human aromatase activity with an IC_{50} of 0.92 µM. By comparison, the S-enantiomer was found with an IC_{50} of 0.58 µM (slightly more potent), and the R-enantiomer with an IC_{50} of 2.97 µM (less potent). For all three test compounds, complete inhibition of aromatase activity was achieved at their solubility limit of 100 µM, while at the concentration of 10^{-9} M no significant aromatase inhibition was observable.

Table 5.8.3-1: Effect of test compounds on aromatase enzyme activity

	Human aromatase IC_{50} [µM]		
	Mean	**SE**	**95% confidence interval**
Test substance			
BAS 750 F	0.92	0.05	0.82 – 1.03
Reg.No. 5934588 (S enantiomer)	0.58	0.03	0.51 – 0.65
Reg.No. 5934591 (R enantiomer)	2.97	0.02	2.52 – 3.14
Reg.No. 6011210 (metabolite)	715	1432	–
4-OH ASDN (positive control)	0.0196	0.00169	0.0162 – 0.0230

For Reg.No. 6011210 (*syn.* M750F022), no complete inhibition of aromatase could be achieved. Even at the solubility limit concentration of 316 µM a mean activity of 46% was still measured. At a compound concentration of approximately 3.2 µM no aromatase inhibition was found. A dose-response curve was generated using a 4-parameter log-logistic-model. However, according to statistical analysis, the calculated IC_{50} of 715 µM was not different from zero. Accordingly, a precise IC_{50} value cannot be provided for Reg.No. 6011210. OCSPP Guideline 890.1200 recommends a classification as specific inhibitor only for compounds which have >50% inhibition. With appreciation for the error in measurement, the BAS 750 F metabolite cannot be securely identified as aromatase inhibitor.

Figure 5.8.3-1: Comparison of BAS 750 F with S enantiomer Reg.No. 5934588, R enantiomer Reg.No. 5934591 and metabolite Reg.No. 6011210 induced inhibition of human aromatase



2. Validation study:

Half maximal inhibition values determined within the validation study were compared with published aromatase inhibition data derived from studies that used radiolabeled C19 androgens as substrates. According to literature, selected compounds span a broad effect range from a half maximal inhibition of 6.5 µM for nitrofen to an IC_{50} as low as 0.9 nM for letrozole. The aromatase inhibition values determined for the same test compounds with the non-radioactive test design were within the same range. Although absolute IC_{50} values were, in some cases, slightly different from specific published values, they were generally very similar. Ranking of these test compounds according to their potency was identical for both the non-radioactive and the radioactive assays, which is further underlined by a good correlation of effects measures. Overall, the method using fluorometric substrate O-benzyl fluorescein benzyl ester, was shown to be suitable for identification of substances that may inhibit aromatase activity. Further it allows a precise comparison of different compounds for their effect on aromatase activity.

Table 5.8.3-2: Validation study: Effect of test compounds on aromatase activity

	Human aromatase inhibition *in vitro*				
	Study data (non-radiolabeled method)			**Published data (radiolabeled method)**	
	IC_{50} [µM]		**-log (IC_{50})**	**-log (IC_{50})**	**Reference**
	Mean	**SE**	**Mean**	**Mean**	
Test series 1 (20.08. – 28.08.2015)					
Econazole nitrate	0.0039	0.0002	8.41	8.665	Bubert et al. (2008)
4-OH-ASDN (Series No. 1)	0.0151	0.0013	7.82	7.15	EPA (2007)
Epoxiconazole	0.0525	0.0026	7.28	–	–
Fenarimol	1.55	0.1000	5.81	5.24	EPA (2007)
Nitrofen	18.6	2.5704	4.73	5.19	EPA (2007)
Test series 2 (11.10. – 22.10.2015)					
Letrozole	0.0033	0.0001	8.48	9.05	EPA (2007)
4-OH-ASDN (Series No. 2)	0.0224	0.0020	7.65	7.15	EPA (2007)
Atrazine *	–	–	–	–	–
Bis(2-ethylhexyl)phthalate **	–	–	–	–	–

* Atrazine: IC_{50} could not be determined – max. inhibition was 36% at highest possible test concentration. According to OCSPP Guideline 890.1200 (2011), atrazine was classified as “non-inhibitor”

** bis(2-ethylhexylphthalate: no significant inhibition up to 100 µM, therefore no IC_{50} could be calculated. Accordingly, the substance was classified as “non-inhibitor”

Bubert C. ,Woo L.W.L, and Sutcliffe O.B. (2008): “Synthesis of Aromatase Inhibitors and Dual Aromatase Steroid Sulfatase Inhibitors by Linking an Arylsulfamate Motif to 4-(4H-1,2,4-triazol-4-ylamino)benzonitrile: SAR, Crystal Structures, in vitro and in vivo Activities”, ChemMedChem 3 (11), 1708-1730

EPA (2007): “Integrated summary report on aromatase”, 11 Dec 2007, http://www.epa.gov/endo/pubs/aromatase_isr.pdf

III. DISCUSSION

For this aromatase inhibition study a protocol was used, which is based on a non-radioactive substrate for the determination of effects on aromatase enzyme activity. The reference compound 4-hydroxy-androstenedione (4-OH-ASDN) has a comparable effect as in other studies using this assay system. The obtained IC_{50} value for 4-OH-ASDN is lower by a factor of 3.6 compared to IC_{50} data derived using radiolabeled C19 androgens as substrates (OCSPP Guideline 890.1200). Further, a separate validation for this protocol demonstrated that inhibitor substances and non-inhibitors are identified as such, and that IC_{50} values fall in a similar range as reported for assays using radiolabeled C19 androgens as substrates.

IV. CONCLUSION

Under the conditions of the human aromatase inhibition study, the S-enantiomer of BAS 750 F (Reg.No. 5934588) was found with the lowest half-maximum inhibition (IC_{50}) concentration of 0.58 µM, followed by BAS 750 F (the 50:50 R,S-racemate) with an IC_{50} of 0.92 µM and the R-enantiomer Reg.No. 5934591 (IC_{50} of 2.97 µM).

The metabolite Reg.No. 6011210 (= M750F022) had a very weak inhibitory effect on aromatase activity; at the top test concentration of 316 µM the remaining enzyme activity was still 46% of the maximum.

B. ASSESSMENT OF THE ENDOCRINE DISRUPTING POTENTIAL

The most widely used definition of an endocrine disruptor is based on the WHO/IPCS (2002):

> *'An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations'.*

This definition is based on hazard identification, looking at whether the effects reported are regarded to be ED-related and supported by mechanistic information.

According to Regulation (EC) No 1107/2009, Annex II, Point 3.6.5 'an active substance shall only be approved if, (…), it is not considered to have endocrine disrupting properties that may cause adverse effect in humans.'

Nevertheless, there is no regulatory guidance available yet on how to address endocrine disruption (ED) and no final criteria are established.

Pending the adoption of the final scientific criteria for the determination of ED properties, currently the so called **interim criteria** are applied. There were two Interim criteria defined within Regulation (EC) No 1107/2009, Annex II, Point 3.6.5:

1) *'(…) substances that are or have to be classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as carcinogenic category 2 and toxic for reproduction category 2, shall be considered to have endocrine disrupting properties.'*
2) *'Substances such as those that are or have to be classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as toxic for reproduction category 2 and which have toxic effects on the endocrine organs, may be considered to have such endocrine disrupting properties.'*

BAS 750 F belongs to the triazole class of fungicide compounds acting by blockage of sterol biosynthesis, i.e. inhibition of lanosterol 14α-demethylase(CYP51A1), in fungal cell membranes. The inhibition of mammalian aromatase (CYP19) is a known "off-target" effect of azole-class fungicides. The difference in potency to inhibit fungal CYP51 and mammalian CYP19 is substance-specific. Therefore, substance-specific factors (mainly potency for aromatase inhibition and toxicokinetics/metabolism) will determine whether adverse effects occur as a result of endocrine disruption.

No classification is proposed for BAS 750 F for carcinogenicity or for reproductive toxicity (see MCA 5.5 and MCA 5.6), applying classification criteria as stipulated in Regulation (EC) No. 1272/2008. Thus, the conditions of the Interim Criterion 1 are not met for BAS 750 F.

For evaluation of the second criterion, it has to be determined whether toxic effects are observed on endocrine organs. Thus, in this section the evaluation of effects on reproductive organs, pituitary, thyroid, and adrenals from subchronic, chronic, and reproduction studies are compiled and the adversity of observed effects is assessed. In addition, effect endpoints which might be linked to an endocrine-mediated mechanism are compiled from the reproduction toxicity studies and assessed.

Assessment of endocrine disruption potential of BAS 750 F

A complete and comprehensive toxicology study database exists for BAS 750 F. The studies conducted with this active ingredient have assessed its potential impact on a plethora of both endocrine-related and non-endocrine endpoints. Out of all studies in the database, endocrine-related endpoints were assessed primarily in the 2-generation reproduction study in rats and the developmental toxicity studies in rats and rabbits, but also in the subchronic toxicity studies in rats, mice and dogs, the combined chronic toxicity / carcinogenicity study in rats and the carcinogenicity study in mice. A summary of this assessment is given below; details are provided in data compilations presented in Annex I (reproduction toxicity endpoints) and Annex II (findings in endocrine organs) at the end of this section.

In the rat 2-gen study, BAS 750 F was administered via the diet to groups of 25 male and female Wistar rats at dose levels of 0, 25, 75 or 200 mg/kg bw/d. Treatment-related effects were not observed on any potential endocrine-related parameter in any parental or offspring generation, including anogenital distance; anogenital index; time to vaginal opening or balanopreputial separation; sex ratio; nipple development; estrous cycle duration; male or female mating and fertility indices; gestation index; sperm count, motility, or morphology; differential ovarian follicle count; number of implantation sites; and post-implantation loss. There were no treatment-related histopathological changes in the reproductive tract of any parental animal. Changes in organ weights at the high-dose were sporadic, bore no relationship to dose, or were attributable to the decrease in body weight observed in parental animals. At necropsy, dilated renal pelves were found in 23 high-dose F_2 pups (6 males, 17 females) compared to 3 affected female pups in the control. The finding was considered by the study authors to be treatment-related but secondary to the general delay in development of the high-dose pups (decreased body weight gain up to 19%), largely reversible and therefore non-adverse in this study. In addition, a treatment-related increase in renal pelvic dilation was *not* observed at any dose in F_1 pups. Dilated renal pelves can be a common finding in developmental toxicity studies in rodents (Haschek and Rousseaux 1998), and was observed in both controls and test groups at an incidence of up to 7.6% of fetuses (across 21% of litters) in the developmental toxicity study in rats. The incidence of this frequently occurring variation was not statistically significantly increased in the treated groups and was well within the historical control range in that study; therefore, it was not considered a specific developmental or endocrine-related effect in either study. Slight reductions in the number of surviving F_1 and F_2 pups from PND 1-4 were noted at the high-dose level (200 mg/kg/day) only, but the resulting pup viability indices were not statistically significantly changed compared to control values and were within the historical control range. The findings were significantly influenced by total litter losses in one F_0 and one F_1 dam that showed markedly reduced feed intake during lactation with associated clinical signs of insufficient maternal care, leading to a slight increase in pup mortality and decreased body weights in surviving pups. Pup weight gains of this high-dose group were reduced to a comparable degree as the body weight changes of the parent animals and were a secondary effect of maternal systemic toxicity. Therefore, the slight decreases in pup survival and body weight at the highest dose tested were the result of maternal systemic toxicity and are not considered specific, reproductive or endocrine-related effects.

Administration of BAS 750 F to pregnant Wistar rats from GD 6-19 did not lead to treatment-related developmental toxicity of any kind when tested at doses of up to 400 mg/kg bw/d. Mean placenta weights were slightly but statistically significantly increased at the high dose. Although well within the historical control range, this change was considered to be potentially treatment-related, but non-adverse. However, a specific, endocrine-related effect on placental weight cannot be inferred given the significant maternal systemic toxicity observed at the top dose level, which included statistically significant reductions in feed intake, body weight gain (17%), carcass weight and corrected body weight gain (34%). Similarly, when BAS 750 F was administered daily to New Zealand White rabbits by stomach tube from GD 6-28, no developmental toxicity was observed at doses up to and including the highest dose of 25 mg/kg bw. The high dose was selected based on severe toxicity including mortality that was observed at 50 mg/kg bw/d and above in a range-finding study in non-pregnant rabbits. There was, thus, no evidence of any developmental or endocrine-related effect in the developmental toxicity studies conducted in rats and rabbits.

There was no evidence of a specific endocrine-related effect in any of the short-term or long-term toxicity studies with BAS 750 F, including each lifetime carcinogenicity bioassay conducted in rats and mice. A treatment-related increase in follicular cell hyperplasia in the thyroid glands of males treated at 200 ppm and possibly also in females treated at 250 ppm (but the finding was not statistically significantly different from control females) was observed in the mouse carcinogenicity study. The increase at the top-dose level occurred in the face of a relatively high background incidence of follicular cell hyperplasia in the male and female control groups. Since the thyroid was not identified as target organ in the mouse 90-day study despite administration of dose levels exceeding the MTD, and the thyroid was also not affected in other species, the weight-of-evidence points to an exacerbation of a mouse-specific, age-related background lesion. Thus, while considered to be treatment-related in male and potentially treatment-related in female mice at the top-dose level of the mouse cancer study, the observed increased incidence of follicular cell hyperplasia was not considered adverse, in the absence of any signs of degenerative change in the thyroid. The incidence of follicular cell adenoma in the thyroid was slightly higher in the 200/250 ppm treated animals. However, the incidence was not statistically significantly changed compared to the control group and was comparable to the background incidences in this strain of mice. This indicates that the follicular cell adenomas were incidental and not related to treatment. Given its lack of adversity, the evidence of systemic toxicity (decreased body weight gain) as well as significant liver toxicity observed at the high dose, follicular cell hyperplasia is not considered a specific endocrine-related effect. Sporadic changes in reproductive organ weights were observed in the 90-day dog study. However, the changes were either not treatment-related or attributable to the sexual immaturity of the animals. Inter-individual variation in the age of sexual maturity is known to occur in dogs and is a frequent observation of the test facility.

BAS 750 F was tested in an in vitro, human recombinant aromatase assay conducted according to USEPA guideline OPPTS 890.1200. The IC_{50} for racemic BAS 750 F was 0.92 µM in the study, indicating the potential to interact with the aromatase enzyme. However, it is important to keep in mind that inhibition of the enzyme in vitro will not necessarily translate into an adverse effect on the endocrine organs. The in vitro aromatase assay (OPPTS 890.1200) was established by the USEPA as a single, first-tier screening tool used to estimate the *potential* for interaction with the estrogen synthesis pathway. A second tier of in vivo assays was also designed to be the definitive assays to test for adverse effects on the endocrine system, including any relationship to dose.

The 2-generation reproduction study in rats is the definitive assay to assess ED in mammals ("Endocrine Disruptor Screening Program; Proposed Statement of Policy," 63 Federal Register 248 (28 December 1998), pp. 71542-71568). As summarized above, BAS 750 F caused no specific endocrine-related anomaly in that study.

In summary, based on the results of in vivo tests conducted with BAS 750 F, there is no evidence of a specific effect of this compound on the endocrine system or in any endocrine organ. BAS 750 F has been shown to inhibit the aromatase enzyme in vitro like other members of the azole class of fungicides. However, in vitro activity did not translate into any specific endocrine-related effect in vivo. This observation is supported by the lack of carcinogenic effects in two lifetime cancer bioassays conducted in rats and mice, as well as the absence of specific reproductive or developmental toxicity in a 2-generation reproduction study and two developmental toxicity studies. There is, therefore, no justification for classifying BAS 750 F as an endocrine disruptor.

Final conclusion on ED assessment

Based on the classification proposal for BAS 750 F and the fact that no adverse effects on endocrine organs were identified, BAS 750 F is not an endocrine disruptor and does not meet the currently applied Interim Endocrine Disruptor Definition Criteria of Regulation (EC) No 1107/2009.

Annex I: Assessment of potentially ED-relevant effect endpoints in reproduction toxicity studies

Rat 2-gen study

Anogenital distance and anogenital index: Anogenital distance and index of all male and all female F_1 and F_2 pups of all test groups were comparable to the concurrent control group. The following statistically significant changes to control groups were considered to be spontaneous/incidental findings: In F_1 male pups of the low-dose group (25 mg/kg bw/d) increased anogenital distance In F_1 females of the low dose group decreased index and in females of the high dose group (200 mg/kg bw/d) increased index. In F_2 male pups of the high-dose group increased index (considered to be the consequence of the lower body weight). In F_2 female pups anogenital distance was increased in all treatment groups (LD: +2%, MD: +3%, HD: +4%). However, as the anogenital index was unchanged and quite close to the control in all treated F_2 female pups, these minimal differences were considered to be incidental and not related to treatment.

Areola / nipple retention: The apparent number and percentage of male F_1 and F_2 pups having areolae was not influenced by the test substance when examined on PND 12. During the re-examination on PND 20 no areolae were detected at all in any of the male pups regardless of the test group.

Differential ovarian follicle count: There were no significant differences between the control and high dose group of F1 dams with regard to numbers of primordial and growing follicles or total incidence of primordial plus growing follicles.

Estrous cycle length / duration: Examinations revealed regular estrous cycles in the F_0 females of all test groups including the control (with mean durations between 4.1-4.2 days). Similarly, in F_1 females, of the control, LD and MD groups, similar mean estrous cycle durations were obtained (4.0 – 4.2 day). In F_1 females of the high-dose group, the mean estrous cycle duration was increased (4.6* vs. 4.1 days), accordingly resulting in a reduced number of cycles within the observation period. However, as the apparent mean cycle prolongation is only half a day and the value is well within the historical control range, the statistically significant observation is not considered to be related to treatment.

Gestation length: The mean duration of gestation was similar in all test groups (i.e. in F_0 dams between 22.1 and 22.4* [$p \leq 0.05$] days and in F_1 dams between 22.0 and 22.2 days). The statistical significant difference for the high-dose F0 group is not considered to be an effect of the test substance. The average difference is less than half a day and all values are well within the historical control range of the test facility.

Parturition difficulties: There was one high-dose F_1 female (No. 379) which had a completely stillborn litter. This female showed distinct signs of impaired well-being such as piloerection and a distinctly poor general state before its litter was born on GD 23/24. Litters with only stillborn pups occur at rare intervals also in control animals. Historically, in the last two years 3 such completely stillborn control litters were noted at the test facility. Neither in the entire F_0 generation nor in the other dams of the F_1 generation high-dose group findings indicative of a general delay of parturition or any parturition complications were observed, nor were there any indications of prenatal developmental toxicity in late gestation.

Taking all this together, an association of this finding to the treatment seems rather unlikely. Worst case it might have been the result of the impaired well-being of this rat before parturition but not an independent impact of the test compound on reproductive performance.

Post-implantation loss: In F_0 dams, there were no indications for test substance-induced intrauterine embryo-/fetolethality, since the postimplantation loss did not show any significant differences between the groups (3.9% / 5.5% / 1.3% / 5.2% loss/dam). In F_1 dams, apparently higher rates of postimplantation loss were observed in treatment groups compared to controls (2.4% / 5.0% / 7.1%** ($p \leq 0.01$) and 8.9% loss/dam in control / 25 / 75 / 200 mg/kg bw/d) test groups. However, these rates are well covered by the historical control range of the test facility (0.9 – 17.7%). while the concurrent F_1 control rate was quite low. Also, the high value at the top dose of 200 mg/kg bw/d (without showing a statistically significant difference to control) is owing to female #388 which had only one resorbed implant, resulting in a postimplantation loss of 100% for this animal. Thus this animal contributed immoderately to the group mean which would have been 4.76% without this animal.

Pup sexual development (vaginal opening / preputial separation): A statistically significant difference in the mean time point of vaginal opening indicating a delay was obtained in the low- and high-dose groups when compared to the concurrent control group. However, the data of these dose groups (30.9 and 31.8 days) were well within the historical control range (29.5-31.9 days), while the mean value of the concurrent control group (30.0 days) was found to be at the low end of the historical control data range. Therefore, any apparent delay of vaginal opening seen in this study is not considered to be the result of a specific mechanism of toxicity. There was no indication for an effect of treatment with regard to preputial separation in male F_1 pups, the mean number of days to reach the criterion in the control and 25, 75 and 200 mg/kg bw/d test groups was 43.1, 42.7, 43.2, and 43.5 days. A slightly lower body weight of the high-dose offspring had no impact on the puberty age of the animals.

Pup weight and pup weight development: Decreased pup weights at 200 mg/kg bw/d on PND 1 (F_1: -9%, F2: -7%) until weaning on PND 21 (F_1: -10%, F_2: -14%) Reduced pup weight gain during lactation (F_1: up to -15%, F_2: up to -19%). No treatment-related effects were concluded for the low- and mid-dose group pups.

Pup necropsy findings: Findings in F_1 pups occurred without relation to dosing (4 – 5 – 8 – 5 in control – 25 – 75 – 200 mg/kg bw/d groups) and could be found at comparable or even higher incidences in the historical control data base. In F2 pups, dilated renal pelves were found in 23 high-dose F_2 pups (6 males, 17 females) compared to 3 affected female pups in the control. The finding was considered by the study authors to be treatment-related but secondary to the general delay in development of the high-dose pups (decreased body weight gain up to 19%), largely reversible and therefore non-adverse in this study. In addition, a treatment-related increase in renal pelvic dilation was *not* observed at any dose in F_1 pups. Dilated renal pelves can be a common finding in developmental toxicity studies in rodents (Haschek and Rousseaux 1998), and was observed in both controls and test groups at an incidence of up to 7.6% of fetuses (across 21% of litters) in the developmental toxicity study in rats. The incidence of this frequently occurring variation was not statistically significantly increased in the treated groups and was well within the historical control range in that study up to the highest tested dose level of 400 mg/kg bw/d; therefore, it was not considered a specific developmental or endocrine-related effect in either study.

Reproductive organs: No treatment-related findings were noted in reproductive or hormone-sensitive organs examined. For further details, see Annex II.

Sex ratio: The sex distribution and sex ratios of live F_1 or F_2 pups on the day of birth and on PND 21 did not show substantial differences between controls and treated groups. All differences observed were regarded to be spontaneous in nature.

Sperm parameters: No treatment-related effects were observed in F_0 and in F_1 male parental animals (motility of sperms and incidence of abnormal sperms in cauda epididymis, sperm head counts in the testis and in the in cauda epididymis).

Time-to-mating: The mean duration until sperm was detected (GD 0) did not any test-substance related effect. In F_0 matings, the duration varied between 2.4 and 3.0 days, in F_1 matings between 2.5 and 3.0, in both cases without any relation to dosing.

Oral prenatal developmental toxicity study in rats

Post-implantation loss: No test-substance related and/or biologically relevant differences between control and test groups up to maternally toxic dose levels (400 mg/kg bw/d, HDT). The mean number of live fetuses/litter in control group and high-dose group was identical.

Fetal weights: Mean females fetal weights were significantly reduced at 400 mg/kg bw/d (3.4* vs. 3.6 g). However, the mean weight value of 3.4 g corresponded exactly to the mean fetal weight of the historical control data. Male fetal weights were not significantly changed. Overall, a relationship to treatment is not assumed.

Placenta effects: Mean placenta weights were slightly increased at the top-dose level 400 mg/kg bw/d (+13.6%**). The placenta effect did not influence the development of the fetuses at all, therefore was not associated with an adverse outcome.

Gender-specific findings: The sex distribution of the fetuses in treatment and control groups was comparable.

Evidence of teratogenicity: There was no evidence of teratogenicity up to the highest dose level tested.

Oral prenatal developmental toxicity study in rabbits

Post-implantation loss: No test-substance related and/or biologically relevant differences between control and test groups up to 25 mg/kg bw/d, (HDT), which had a lower extent of post-implantation loss than the control group. The mean number of live fetuses/litter in control group and high-dose group was identical.

Fetal weights: The mean fetal weights of test groups were not influenced by the test substance and did not show any biologically relevant differences in comparison to the control group.

Placenta effects: Mean placenta weights were not significantly changed by treatment (control: 5.5 g, 25 mg/kg bw/d: 5.3 g).

Gender-specific findings: The sex distribution of the fetuses in treatment and control groups was comparable.

Evidence of teratogenicity: There was no evidence of teratogenicity up to the highest dose level tested.

Annex II: Compilation of findings in endocrine organs from BAS 750 F studies

Adrenal gland

Rat 90-day study: decreased abs. weight at 3600 ppm (males: -20%*, females: -22%**) associated with decreased terminal body weights (males: -7%^{ns}, females: -8%**). No statistically significant change in relative adrenal weights and no histopathological findings in cortex or medulla at 3600 ppm. Therefore considered to be not treatment-related up to 3600 ppm (HDT).

Rat 2-gen study: decreased abs. weight in males at 200 mg/kg bw/d (F0: -12%**, F1: -12%**), were considered to be secondary to decreased terminal body weights (F0: -11%*, F1: -12%**). No treatment-related findings were noted upon histopathology.

Rat 2-year study, 52-wk satellite groups: decreased abs. weight in females at 3600 ppm (-13%*) considered to be secondary to decreased terminal body weight (-15%**)

Rat 2-year study, 104-wk main groups: In males increased abs. weight and rel. weight at 100 ppm in males (+7%*) and decreased abs. weight at 3600 ppm (-8%**). In females increased rel. weight at 600 ppm (+11%), decreased abs. weight and increased rel. weight at 3600 ppm (-8%**, +18%**) were considered to be secondary to decreased terminal body weight in males at 3600 ppm (-12%**) and in females at 600 ppm (-8%**) and 3600 ppm (-23%**). Because there was no dose-response relationship, the increased absolute adrenal weight in males at 100 ppm and the increased relative adrenal weights in males at 600 and 3600 ppm were regarded to be incidental.

At gross necropsy, the number of macroscopic foci in the adrenal cortex were increased in female rats (4–12–9–11 at 0–100–600–3600 ppm). However, the foci corresponded to a variety of different histopathological findings (adenoma, hyperplasia, accessory cortical tissue, cystic degeneration, fatty change or multifocal hypertrophy) there was no consistent histopathology correlate to these macroscopic foci, therefore they were not regarded to be treatment related.

Mouse 90-day study: increased abs. and rel. weights in males at 750 ppm (abs.: +46%* [6.0±1.4 mg vs. 4.1±1.5 mg], rel: +51%**) were without any histopathological correlate and were therefore considered to be not toxicological relevant. There were no significant organ weight changes and no histopathological changes in females (incl. no eosinophilic change of cytoplasm in adrenal cortex, as observed at 250 ppm in the mouse cancer study, see below).

Mouse 18-month carcinogenicity: at 200 ppm weights were increased in male mice at 200 ppm (abs. +27%**, rel.: +39%**) but were not associated with any histopathological change. In females at 250 ppm, the relative adrenal weight was significantly increased (+22%*), which was however associated with reduced body weight (-13%**); absolute adrenal weights were not significantly changed in females. The only histopathological change was a more eosinophilic aspect of the adrenocortical cell cytoplasm together with a minimal to slight size increase of individual eosinophilic cells in female mice of the high-dose group (20/50** vs 2/50 in control mice). The incidence of cortical hypertrophy without cytoplasmic changes in treated female animals was low and comparable to control incidences. The changes in the adrenal gland were considered to be treatment-related but not adverse.

Dog 90-day study: no significant weight changes, no gross necropsy or treatment-related histopathology findings were observable in male and female dogs up to 180 mg/kg bw/d (HDT).

Cauda epididymis

Rat 90-day study: see epididymides.

Rat 2-gen study: decreased abs. weights at 200 mg/kg bw/d (F0: -7%*, F1: -9%**), considered to be secondary to decreased terminal body weights (F0: -11%*, F1: -12%**). The observed decrease at 25 mg/kg bw/d in F1 males (abs. and rel. weights -6%*) is considered to be incidental, due to the lack of a dose-response relationship (no significant change at 75 mg/kg bw/d).

Rat 2-year study, 52-wk satellite groups: see epididymides

Rat 2-year study, 104-wk main groups: see epididymides

Mouse 90-day study: see epididymides

Mouse 18-month carcinogenicity: see epididymides

Dog 90-day study: see epididymides.

Epididymides

Rat 90-day study: no significant weight changes, no gross necropsy or treatment-related histopathology findings were observable in male and female rats up to 3600 ppm (HDT).

Rat 2-gen study: decreased abs. weights at 200 mg/kg bw/d (F0: -6%**, F1: -8%**) and increased rel. weights at 200 mg/kg bw/d in F0 (+6%**), were considered to be secondary to decreased terminal body weights (F0: -11%*, F1: -12%**). Increased rel weight at 75 mg/kg bw/d in F1 (+4%*) were also considered secondary to decreased terminal body weight (-3%^{ns}). Decreased abs. weight at 25 mg/kg bw/d in F1 (-5%*) was considered to be incidental, due to the lack of a dose-response relationship. No treatment-related gross or histopathological findings

Rat 2-year study, 52-wk satellite groups: no significant change of organ weights, no treatment-related gross or histopathology findings up to 3600 ppm (HDT)

Rat 2-year study, 104-wk main groups: Rel. weight was significantly increased at 3600 ppm (+2%**) which considered to be secondary to reduced body weight (-12%). No treatment-related gross or histopathology findings up to 3600 ppm (HDT)

Mouse 90-day study: No treatment related organ weight change and no histopathological findings up to 750 ppm (HDT).

Mouse 18-month carcinogenicity: No treatment related organ weight change and no histopathological findings up to 200 ppm (HDT).

Dog 90-day study: no significant weight changes, no gross necropsy or treatment-related histopathology findings were observable in male and female dogs up to 180 mg/kg bw/d (HDT).

Ovaries

Rat 90-day study: no significant weight changes, no gross necropsy or treatment-related histopathology findings were observable in female rats up to 3600 ppm (HDT).

Rat 2-gen study: decreased abs. ovary weights at 200 mg/kg bw/d (F0: -9%**), considered to be secondary to decreased terminal body weights (F0: -6%**). The Differential Ovarian Follicle Count (DOFC) did not indicate any treatment-related effects. No treatment-related histopathology findings.

Rat 2-year study, 52-wk satellite groups: No treatment-related changed organ weight. No gross or histopathology findings up to 3600 ppm (HDT) that would suggest a treatment-related effect

Rat 2-year study, 104-wk main groups: No treatment-related changed organ weight. No treatment-related gross or histopathology findings up to 3600 ppm (HDT)

Mouse 90-day study: No treatment related organ weight change and no histopathological findings up to 750 ppm (HDT).

Mouse 18-month carcinogenicity: No treatment related organ weight and histopathological changes up to 250 ppm (HDT)

Dog 90-day study: reduced ovary weights at 90 and 180 mg/kg bw/d (not statistically significant). No treatment-related histopathology findings. Female Beagle dogs reach their first estrus between 8 and 14 months of age (Rehm et al., 2007). In the current study, the absolute and relative uterus and ovaries weight in females of all test groups showed varying weights. Histopathological examination showed that only single animals of control or treated females had already reached the first estrus (presence of functional or old degraded corpora lutea). Furthermore, mammary glands were immature in most animals (characterized by undifferentiated ducts in the dermis of the nipples). In spite of this, a great range of variability in sexual organ development was noted when examining ovaries, vagina, uterus and mammary gland of each female animal. Therefore, the weight changes in uterus and ovaries in females of all test groups was attributed to sexual immaturity which was ascribed to the age-related variations in reaching the first estrus and sexual maturity.

Pituitary gland

Rat 90-day study: Pituitary gland weights were not determined. no gross necropsy or histopathology findings were observable in male and female rats at 3600 ppm (HDT).

Rat 2-gen study: In F1 males relative weights were increased at 200 mg/kg bw/d (+14%*). In F1 males at 25 mg/kg bw/d abs. and rel. weights were increased (abs.: +13%*, rel.: +14%*). These weight changes were considered to be incidental, due to the lack of a dose-response relationship. In females, decreased abs. weights at 200 mg/kg (F0: -14%**, F1: -13%**), were considered to be secondary to decreased terminal body weights (F0: -6%**, F1: -11%**). No treatment-related histopathology findings.

Rat 2-year study, 52-wk satellite groups: In 3 females of test group 13, a focal hyperplasia was observed in the pars distalis of the pituitary gland. No control female showed this finding. Because the number of adenomas (23/22) and of focal hyperplasia (12/13) in the pars distalis of the pituitary gland in main group females was comparable between control females and females of high dose group, this was regarded as incidental.

Rat 2-year study, 104-wk main groups: No weight changes, gross necropsy or histopathology findings that could indicate treatment-related effects up to 3600 ppm (HDT)

Mouse 90-day study: Organ weights were not determined. No treatment related histopathological findings in males or females up to 750 ppm (HDT)

Mouse 18-month carcinogenicity: Organ weights were not determined. No treatment related histopathological findings in males or females up to 200 ppm in males and 250 ppm in females (HDT).

Dog 90-day study: no significant or treatment-related weight changes, no gross necropsy or treatment-related histopathology findings were observable in male and female dogs up to 180 mg/kg bw/d (HDT).

Prostate

Rat 90-day study: Prostate weights were not determined. No gross necropsy or histopathology findings were observable at 3600 ppm (HDT).

Rat 2-gen study: no treatment-related organ weight changes or histopathological changes up to dose levels of 200 mg/kg bw/day (HDT)

Rat 2-year study, 52-wk satellite groups: Prostate weights were not determined. No gross or histopathology findings up to 3600 ppm (HDT) that would suggest a treatment-related effect

Rat 2-year study, 104-wk main groups: Prostate weights were not determined. No treatment-related gross or histopathology findings up to 3600 ppm (HDT)

Mouse 90-day study: No treatment related organ weight change and no gross or histopathological findings up to 750 ppm (HDT).

Mouse 18-month carcinogenicity: Organ weights were not determined. No treatment related gross or histopathological findings.

Dog 90-day study: reduced prostate weights at 90 and 180 mg/kg bw/d (not statistically significant); corresponded to reduced organ size of 1 dog at 90 mg/kg bw/d and two dogs at 180 mg/kg bw/d.

Sexual maturity in male Beagle dogs has been estimated to occur between 7 and 8 months of age (Goedken et al., 2008; James and Heywood, 1979) which was also the age of the animals at the start of the study. However, inter-individual variations with regard to the age of reaching maturity are known to occur and are also an occasional observation at our test facility. This seems to be also valid for the weight changes in the testes and prostate glands. Weight deviations were consistent with histopathological patterns of sexual immaturity (small acini and abundant interstitial stroma). Comparable low weights have been observed in controls of one 90-day study from the historical control data base of the test facility. In the absence of a histopathological findings in the prostate indicating a degenerative process (e.g. atrophy), these weight deviations and macroscopic findings were regarded as age-related and within the range of inter-individual variability in reaching full maturity. Thus the reduced prostate weights at the mid- and high-dose were considered to be not treatment-related.

Seminal vesicle

Rat 90-day study: Seminal vesicle weights were not determined. No gross necropsy or histopathology findings were observable at 3600 ppm (HDT).

Rat 2-gen study: increased rel. weight at 25 (+10%*) and 200 mg/kg bw/d (+12%**) in F0 males. The abs. seminal vesicle weights were not significantly changed. The increased rel. weights in F0 at 200 mg/kg bw/d may be secondary to reduced body weights (-12%**), the finding at 25 mg/kg bw/d was considered incidental. No significant weight changes were noted in F0 males at 75 mg/kg bw/d or in F1 males at any dose level. There were no histopathological findings. Overall, the slight weight increases in F0 males of the low- and high-dose level are considered to be incidental. There were no histopathological findings observed.

Rat 2-year study, 52-wk satellite groups: No gross or histopathology findings up to 3600 ppm (HDT) that would suggest a treatment-related effect

Rat 2-year study, 104-wk main groups: No treatment-related gross or histopathology findings up to 3600 ppm (HDT)

Mouse 90-day study: Organ weights were not determined. No treatment related histopathological findings up to 750 ppm (HDT).

Mouse 18-month carcinogenicity: Organ weights were not determined. No treatment related histopathological findings.

Dog 90-day study: Dogs do not have seminal vesicles.

Testes

Rat 90-day study: No significant weight changes, no gross or treatment-related histopathology findings up to 3600 ppm (HDT)
Rat 2-gen study: decreased abs. testes weights at 200 mg/kg bw/d (F0: -5%**, F1: -7%**), considered to be secondary to decreased terminal body weights (F0: -11%*, F1: -12%**). Decreased testes weight at 25 mg/kg bw/d in F1 males (abs./rel. -6%*) is considered to be incidental, due to lack of a dose-response relationship (no significant change at 75 mg/kg bw/d). No treatment-related findings were noted upon histopathology.
Rat 2-year study, 52-wk satellite groups: No significant weight changes, no gross or histopathology findings up to 3600 ppm (HDT)
Rat 2-year study, 104-wk main groups: Rel. testes weights were increased at 3600 ppm (+9%*), which was considered to be secondary to reduced body weights at that dose level (-12%**). In all treatment groups the number of macroscopic foci in the testes were increased (0–5–4–10 at 0–100–600–3600 ppm). The macroscopically diagnosed foci in the testes mostly represented Leydig cell adenomas, Leydig cell hyperplasia, multifocal tubular degeneration, or tubular mineralization, histopathologically. None of these findings showed significant differences between control and high-dose animals. Since there was no consistent histopathological correlate to these macroscopic foci, they were not regarded to be treatment related.
Mouse 90-day study: No treatment related organ weight change and no histopathological findings up to 750 ppm (HDT).
Mouse 18-month carcinogenicity: No treatment related organ weight changes or histopathological findings.

Dog 90-day study: increased relative testes weights at 180 mg/kg bw/d (not statistically significant). Relative testes weight increases did not have a histopathologic correlate and the absolute testes weights were within historical control values: the finding was therefore regarded to be incidental.

Thyroid

Rat 90-day study: Rel. thyroid weight was significantly increased in females at the top-dose level of 3600 ppm (+26%*). There was no significant increase in abs. thyroid weights, terminal body weights were decreased at 3600 ppm (-8%**). There were no findings at gross necropsy. The weight increase was not accompanied by histopathological findings at 3600 ppm in females (the only finding was altered colloid in 1 of 10 females at the mid-dose level). In males, single occurrences of follicular cell hypertrophy/hyperplasia with altered colloid occurred at the low-dose and at the top-dose level, were regarded as incidental.

Rat 2-gen study: no statistically significant or biologically relevant organ weight changes. Histopathology of the thyroid was not performed in the rat 2-gen study

Rat 2-year study, 52-wk satellite groups: Thyroid weights were not affected by treatment. No gross necropsy or histopathology findings suggesting a relationship to treatment were observed up to 3600 ppm (HDT).

Rat 2-year study, 104-wk main groups: Thyroid weights were not determined after 2-yr treatment. Number of males with altered colloid of the thyroid was significantly increased at the low- and high-dose level. Because there was no dose-response relationship, the occurrence of this finding was regarded to be incidental.

Mouse 90-day study: Organ weights were not determined. No treatment related histopathological findings up to 750 ppm (HDT). One control female was found with a follicular cell adenoma.

Mouse 18-month carcinogenicity: Thyroid weights were not determined. Findings at gross necropsy affected only single animals and were therefore not considered to be treatment-related. Thyroid follicular cell hyperplasia was significantly increased in males of the high-dose group at 200 ppm (42% – 32% – 34% – 74%**); a non-statistically significant increase was also seen in females at 250 ppm compare to control females (52% vs. 42%). Follicular cell adenoma were not significantly increased in treatment groups compared to concurrent controls, and the incidences were comparable to the available historical control data. In view of the high background incidences in controls, and in the absence of degenerative changes, the observed increased incidences of follicular cell hyperplasia at 200 ppm in males and potentially at 250 ppm in females were considered to be treatment related but not adverse.

Dog 90-day study: no statistically significant or biologically relevant organ weight changes, no treatment-related histopathological changes up to dose levels of 180 mg/kg bw/day (HDT)

Uterus

Rat 90-day study: no statistically significant or biologically relevant organ weight changes, no treatment-related histopathological changes up to dose levels of 3600 ppm (HDT)

Rat 2-gen study: No statistically significant or biologically relevant organ weight changes, no histopathology findings.

Rat 2-year study, 52-wk satellite groups: Uterus weights were not significantly changed by treatment. There were no gross necropsy or histopathology findings that would suggest a relationship to treatment

Rat 2-year study, 104-wk main groups: Uterus weights were not significantly changed by treatment. There were no gross necropsy findings. 5 females with adenocarcinoma were found at 3600 ppm (vs 1 control group female with adenocarcinoma). The incidence of adenocarcinoma (10%) was well within the historical control range of the test facility (2-30%). Therefore, the occurrence of adenocarcinoma was regarded to be incidental.

Mouse 90-day study: No treatment related organ weight change and no histopathological findings up to 750 ppm (HDT).

Mouse 18-month carcinogenicity: Mean absolute uterus weights were not significantly changed but the rel uterus weight was increased at 250 ppm (+32%*), the. The increase in rel. uterus weights was considered secondary to reduced body weight (-13%**). There were no treatment-related histopathological findings.

Dog 90-day study: increased uterus weights at 15 mg/kg bw, reduced uterus weights at 90 and 180 mg/kg bw/d (not statistically significant)

Female Beagle dogs reach their first estrus between 8 and 14 months of age (Rehm et al., 2007). In the current study, the absolute and relative uterus and ovaries weight in females of all test groups showed varying weights. Histopathological examination showed that only single animals of control or treated females had already reached the first estrus (presence of functional or old degraded corpora lutea). Furthermore, mammary glands were immature in most animals (characterized by undifferentiated ducts in the dermis of the nipples). In spite of this, a great range of variability in sexual organ development was noted when examining ovaries, vagina, uterus and mammary gland of each female animal. Therefore, the weight changes in uterus and ovaries in females of all test groups was attributed to sexual immaturity which was ascribed to the age-related variations in reaching the first estrus and sexual maturity.

Vagina

90-day rat study: no histopathological changes up to dose levels of 3600 ppm (HDT)

Rat 2-gen study: No treatment-related histopathology findings.

Rat 2-year study, 52-wk satellite groups: There were no gross necropsy or histopathology findings that would suggest a relationship to treatment

Rat 2-year study, 104-wk main groups: There were no gross necropsy or histopathology findings that would suggest a relationship to treatment

Mouse 90-day study: Organ weights were not determined. No treatment related histopathological findings. No evidence of treatment-related effect on estrous cycle.

Mouse 18-month carcinogenicity: Organ weights were not determined. No treatment related histopathological findings.

90-day dog study: no treatment-related gross necropsy or histopathological changes up to dose levels of 180 mg/kg bw/day (HDT)

CA 5.9 Medical Data

BAS 750 F is a new fungicide active ingredient. BAS 750 F has not yet been sold commercially and aside from pilot scale preparations, has been handled by only a limited number of employees or contract scientists involved in regulatory and field biological testing. Therefore, human data is limited at this time.

1. A search in the databases listed below - restricted to “pps=human” and “ct d human” - has been performed on January 15th, 2016 via DIMDI-host for the following terms:

BAS 750*
CAS 1417782-03-6
Mefentrifluconazole
5834378

ME66	MEDLINE	NLM
ME0A	MEDLINE Alert	NLM
EM74	EMBASE	2005 Elsevier B.V.
EA08	EMBASE Alert	2005 Elsevier B.V.
CL63	CancerLit	NCI
CCTR93	Cochrane Library - Central	Cochrane

2. Crosscheck via ChemIDplus (http://chem2.sis.nlm.nih.gov/chemidplus/chemidlite.jsp)

3. Crosscheck via PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez)

4. GUA-internal literature database “FAUST”

5. Regarding the databases HSDB (NLM) and GESTIS (BGIA)

6. Register of the internal medical ward

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

All persons handling crop protection products are surveyed by regular medical examinations. There are no specific parameters available for effect monitoring of BAS 750 F. Thus, the medical monitoring program is designed as a general health check-up, with special interest in the primary target organs presumed to be relevant by analogy from animal experiments.

The surveillance program includes a general physical examination including neurological status, red and white blood cell counts, liver enzymes. Adverse health effects suspected to be related to BAS 750 F exposure have not been observed.

CA 5.9.2 Data collected on humans

No reports of adverse effects were identified during routine monitoring of production plant workers and among personnel involved in the experimental biological testing or field trials with BAS 750 F or BAS 750 F containing products. There is no evidence or data available to support any findings in relation to poisoning with BAS 750 F.

CA 5.9.3 Direct observations

BAS 750 F has not yet been sold commercially and aside from pilot scale preparations, has been handled by only a limited number of employees or contract scientists involved in regulatory and field biological testing.

No human cases of intoxication or poisoning deriving from BAS 750 F are known to BASF SE.

CA 5.9.4 Epidemiological studies

Neither data on exposure of the general public nor epidemiologic studies are available for BASF SE, nor is BASF SE aware on any epidemiologic studies performed by third parties.

As such, no observations regarding health effects after exposure of the general public are known to us.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

An analytical method for determination of BAS 750 F in blood plasma of rats and mice is available. Clinical tests are not known. No specific symptoms of poisoning are seen or have been identified in animal studies (see below).

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

See safety data sheet / precautions; symptomatic and supportive treatment, no specific antidote known.

CA 5.9.7 Expected effects of poisoning

Specific clinical signs of poisoning are not expected, based on results or acute and subacute studies in animals. In these studies, clinical observations consisted of unspecific signs like impaired body weight development and food consumption. In dogs, vomiting was occasionally observed at dose levels greater than 90 mg/kg body weight. In an acute neurotoxicity study in rats treatment-related neurobehavioral changes (unsteady gait, decreased grip strength, increase of landing foot splay, and impaired motor activity) were observed at a dose level of 2000 mg/kg. These observations were transient as they were only noted at the day of application.

We create chemistry

BAS 750 F

Document M-CA, Section 6

RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED AND PLANT METABOLISM

Compiled by:

[redacted]

Telephone: [redacted]
E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

CA 6 RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED AND PLANT METABOLISM

CA 6.1 Storage stability of residues

Report: CA 6.1/1
Guedez-Orozco A.-A.,Eilers B., 2015 a
Storage stability of BAS 750 F in plant matrices
2015/1106709
Guidelines: OECD 506, EPA 860.1380, EEC 7032/VI/95 rev. 5
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

The study is planned over a period of two years. This report gives the results after about 18 months.

EXECUTIVE SUMMARY

A freezer storage stability study was performed investigating plant samples spiked with BAS 750 F at 0.1 mg/kg. The samples were analysed for BAS 750 F with BASF method No. L0076/09, which determine the analyte by means of LC-MS/MS. The study is planned over a period of two years.
The data included in this interim report indicate that residues of BAS 750 F are stable for about 18 months in tomato fruit, apple fruit, grapes fruit, lemon fruit, wheat grain, dried bean seed, dried peas seed, soybean seed, rape seed, wheat whole plant no roots, wheat straw, potato tuber.

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 750 F
   Description:
   Lot/Batch #: L85-12
   Purity: 99.4 %
   CAS#: 1417782-03-6
   Spiking levels: 0.1 mg/kg

2. **Test Commodity:**
   Crop: tomato fruit, apple fruit, grapes fruit, lemon fruit, wheat grain, dried bean seed, dried peas seed, soybean seed, rape seed, wheat whole plant no roots, wheat straw, potato tuber
   Sample size: 5 g

B. STUDY DESIGN

1. Test procedure

A storage stability study at ≤-18°C was carried out with BAS 750 F in plant matrices. The test compound was added to untreated sample matrices at a level of 0.1 mg/kg. The samples were kept in PE-containers at ≤-18°C for up to 551 days. After time intervals of 0, 30, 90, 180, 360 and 550 days, samples were removed from storage and have been analyzed for BAS 750 F using LC-MS/MS detection.

2. Description of analytical procedures

BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane. The final determination was performed by LC-MS/MS. The analytical method used was BASF L0076/09, which was validated in the study [see KCA 4.1.2/21 2015/3001681]. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The recoveries of BAS 750 F from plant matrices after the various storage periods are summarized in Table 6.1-1 to Table 6.1-3.

All compounds remained stable over the whole storage period of about 18 months (550 days) in every matrix examined. The analytical method was validated at the level used for fortification with every series of analysis. The average of all recoveries for BAS 750 F was between 70 and 110% for all samples. The procedural recoveries (freshly spiked samples at 0.01 mg/kg and 0.1 mg/kg) were in a range of 70 and 110%, except for one samples of the matrix dried bean seed with 132%.

Table 6.1-1: Storage stability of BAS 750 F in wheat whole plant, straw and grain and soybean seed

Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B
Wheat whole plant				**Wheat straw**				**Wheat grain**				**Soybean seed**			
0	90.3	(102)	88.8	0	83.3	(96.8)	86.0	0	94.8	(97.2)	97.6	0	90.3	(94.7)	95.3
31	93.3	(100)	93.3	29	91.8	(99.2)	92.5	30	94.5	(92.9)	102	30	90.3	(92.6)	97.5
85	87.8	(97.3)	90.3	86	90.8	(106)	85.5	85	97.0	(105)	92.6	85	85.8	(106)	81.2
177	92.1	(95.9)	96.0	184	92.3	(102)	90.8	182	91.5	(89.7)	102	182	91.5	(104)	88.0
369	83.3	(91.5)	91.0	363	87.8	(102)	85.8	361	101	(99.8)	101	361	90.5	(95.5)	94.8
550	95.5	(101)	94.5	551	99.5	(106)	93.5	547	93.0	(92.3)	101	550	88.0	(94.1)	93.5

A: mean in stored samples, % of nominal
B: mean procedural, in freshly spiked samples
(values in brackets are corrected for procedural recoveries)

Table 6.1-2: Storage stability of BAS 750 F in rape seed, potato tuber, apple fruit and lemon fruit

Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B
Rape, seed				**Potato, tuber**				**Apple, fruit**				**Lemon, fruit**			
0	90.3	(110)	81.8	0	91.3	(98.9)	92.3	0	84.8	(91.6)	92.6	0	90.0	(98.9)	91.0
31	85.8	(101)	85.3	29	84.8	(90.6)	93.5	30	89.0	(94.4)	94.3	29	94.0	(96.4)	97.5
85	91.5	(97.9)	93.5	83	89.5	(89.7)	99.8	85	91.5	(111)	82.7	83	92.5	(95.1)	97.3
177	86.0	(93.0)	92.5	184	60.8	(78.9)	77.0	182	81.8	(83.0)	98.5	182	95.5	(96.0)	99.5
369	76.3	(87.1)	87.5	369	76.0	(81.9)	92.8	358	80.8	(84.3)	95.8	358	93.4	(101)	92.6
*378	93.5	(103)	90.8	*378	91.5	(91.3)	100								
550	94.3	(101)	93.3	551	76.3	(86.4)	88.3	547	78.0	(83.9)	93.0	547	98.3	(101)	97.0

A: mean in stored samples, % of nominal
B: mean procedural, in freshly spiked samples
(values in brackets are corrected for procedural recoveries) / * reserve samples

Table 6.1-3: Storage stability of BAS 750 F in dried bean, grape fruit, tomato fruit and dried peas seed

Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B	Storage Interval (Days)	A		B
Dried bean, seed				**Grape, fruit**				**Tomato, fruit**				**Dried peas, seed**			
0	93.1	(98.7)	94.3	0	89.3	(97.8)	91.3	0	89.8	(99.2)	90.5	0	89.5	(97.5)	91.8
30	94.0	(95.2)	98.8	31	93.1	(95.2)	97.8	31	93.8	(97.0)	96.8	29	94.8	(98.4)	96.3
85	94.8	(105)	90.3	85	85.3	(90.5)	94.3	85	87.3	(95.4)	91.5	83	91.3	(93.1)	98.0
182	97.3	(97.0)	100	177	87.3	(124)	70.5	177	74.9	(92.0)	81.5	182	101	(95.5)	106
358	88.3	(66.9)	132	358	86.1	(94.5)	91.1	358	76.6	(82.3)	93.1	361	96.5	(101)	95.3
547	102	(104)	97.8	547	91.0	(95.3)	95.5	546	77.0	(84.6)	91.0	550	98.3	(103)	95.0

A: mean in stored samples, % of nominal
B: mean procedural, in freshly spiked samples
(values in brackets are corrected for procedural recoveries)

CONCLUSION

It can be concluded that BAS 750 F is stable in plant matrices for at least 550 days when stored under deep frozen conditions. The study is planned for covering two year storage and according to the results obtained it is expected that BAS 750 F remains stable during the whole period of two years.

Report:	CA 6.1/2 Heger N., Taraschewski I., 2015 a Storage stability of Reg.No. 6011210 in animal matrices 2015/1106710
Guidelines:	OECD 506 (Oct. 2007), EPA 860.1380, EEC 7032/VI/95 rev. 5
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

A freezer storage stability study was performed investigating animal matrices (cow liver, kidney, muscle, fat, milk and cream; hen egg) spiked with M750F022 (Reg. No. 6011210) at 0.1 mg/kg. The samples were analysed for M750F022 with BASF method No. L0309/01, which determine the analyte by means of GC/MS.
The data indicate that residues of M750F022 are stable for six month in animal matrices and that the samples can be stored at least for 6 months prior to analytical measurement.

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** M750F022
   Description:
   Lot/Batch #: L85-106
   Purity: 98.3 %
   CAS#: -
   Spiking levels: 0.1 mg/kg

2. **Test Commodity:**
   Crop: cow liver, kidney, muscle, fat, milk and cream; hen egg
   Sample size: 5 g

B. STUDY DESIGN

1. Test procedure

A storage stability study at ≤-18°C was carried out with M750F022 in bovine milk, cream and tissues and hen egg. The test compound was added to untreated liver, kidney, muscle, fat, milk, cream and egg samples at a level of 0.1 mg/kg. The samples were kept in PE-containers at ≤-18°C for up to 183 days. After time intervals of 0, 30, 90, 120 and 180 days, samples were removed from storage and have been analyzed for M750F022 using GC-MS detection.

2. Description of analytical procedures

The animal commodities were analyzed for residues of M750F022 using method L0309/01.
A 5 g sample aliquot is extracted for fat contained matrices (milk, fat, cream) by shaking or macerating with 50 mL acetonitrile and 20 mL iso-hexane. 20 mL of the acetonitrile extract is shaken again with 20 mL iso-hexane. An aliquot of the acetonitrile phase is dried and dissolved in MeOH/H2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS.

For protein contained matrices (egg, muscle, liver and kidney) a 5 g sample aliquot is extracted by macerating with 50 mL MeOH/H2O/2N HCl (70/25/5). 20 mL of the extract is shaken with 20 mL 0.2N NaOH and 100 mL cyclohexane (muscle and liver) or dichloromethane (egg and kidney). An aliquot of the cyclohexane or dichloromethane phase is dried and dissolve in MeOH/H2O (50/50). Then, a SPE clean-up step is carried out for the preparation of the samples by GC/MS.
The method has a limit of quantitation of 0.01 mg/kg in each matrix for the analyte.

II. RESULTS AND DISCUSSION

The recoveries of M750F022 from cow milk, cream and tissues after the various storage periods are summarized Table 6.1-4.
All compounds remained stable over the whole storage period of about 180 days in every matrix examined. The analytical method was validated at the level used for fortification with every series of analysis. The average of all recoveries for M750F022 was between 70 and 110% for all samples. The procedural recoveries (freshly spiked samples at 0.01 mg/kg and 0.1 mg/kg) were in a range of 70 and 120%, except for two samples of the matrix cow muscle with 67.2% and 67.4%.

Table 6.1-4: Storage stability of M750F022 in bovine milk, cream and tissue

Recovery [%] Reg. No. 6011210									
Day	**A**		B	**A**		B	**A**		B
	Cow Liver			**Cow Kidney**			**Cow Muscle**		
0	82.2	(108)	76.0	66.5	(91.5)	72.7	70.1	(96.0)	73.1
	-	-	-	83.5	(108)	77.4	-	-	-
28	70.0	(78.8)	88.9	-	-	-	-	-	-
29	-	-	-	-	-	-	83.7	(125)	67.2
31	-	-	-	76.8	(105)	73.1	-	-	-
86	85.7	(101)	85.2	-	-	-	-	-	-
90	-	-	-	80.3	(95.1)	84.4	-	-	-
91	-	-	-	-	-	-	77.2	(97.2)	79.4
114	82.9	(102)	81.2	102	(127)	80.7	-	-	-
115	-	-	-	-	-	-	95.3	(141)	67.4
178	-	-	-	93.4	(84.6)	110	-	-	-
181	-	-	-	-	-	-	106	(149)	71.5
183	84.4	(87.0)	97.0	-	-	-	-	-	-
Day	**A**		B	**A**		B	**A**		B
	Cow Fat			**Cow Milk**			**Cow Cream**		
0	101	(93.7)	107	84.3	(103)	81.5	85.0	(81.7)	104
27	-	-	-	-	-	-	96.3	(88.3)	109
28	87.0	(81.1)	107	81.8	(99.0)	82.6	-	-	-
84	-	-	-	77.5	(88.3)	87.8	100	(89.5)	112
87	98.5	(87.4)	113	-	-	-	-	-	-
113	-	-	-	78.3	(87.6)	89.3	-	-	-
115	92.0	(85.8)	107	-	-	-	93.8	(89.3)	105
179	-	-	-	80.0	(94.5)	84.7	93.0	(101)	91.9
180	88.8	(105)	84.6	-	-	-	-	-	-

Day	**A**		B
	Hen Egg		
0	89.6	(112)	79.8
28	76.3	(90.1)	84.6
85	90.3	(96.0)	94.1
113	78.4	(112)	70.3
178	79.9	(102)	78.4

A: mean in stored samples, % of nominal
B: mean procedural, in freshly spiked samples
(values in brackets are corrected for procedural recoveries)

CONCLUSION

It can be concluded that M750F022 is stable in animal matrices for at least 180 days when stored under deep frozen conditions.

Report:	CA 6.1/3 Heger N.,Guedez-Orozco A.-A., 2015 b Storage stability of BAS 750 F in animal matrices 2015/1106711
Guidelines:	EEC 7032/VI/95 rev. 5, EEC 91/414 (1607/IV/97 Rev. 2), EEC 91/414 Annex II (Part A Section 6), EEC 91/414 Annex III (Part A Section 8), EPA 860.1380, OECD 506
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

A freezer storage stability study was performed investigating animal matrices (cow liver, kidney, muscle, fat, milk and cream; hen egg) spiked with BAS 750 F at 0.1 mg/kg. The samples were analysed for BAS 750 F with BASF method No. L0272/01, which determine the analyte by means of LC/MS/MS.
The data indicate that residues of BAS 750 F are stable for six month in animal matrices and that the samples can be stored at least for 6 months prior to analytical measurement.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F
Description:
Lot/Batch #: L85-12
Purity: 99.4 %
CAS#: 1417782-03-6
Spiking levels: 0.1 mg/kg

2. Test Commodity:
Crop: cow liver, kidney, muscle, fat, milk and cream; hen egg
Sample size: 5 g

B. STUDY DESIGN

1. Test procedure

A storage stability study at ≤-18°C was carried out with BAS 750 F in bovine milk, cream and tissues and hen egg. The test compound was added to untreated liver, kidney, muscle, fat, milk, cream and egg samples at a level of 0.1 mg/kg. The samples were kept at ≤-18°C for up to 182 days. After time intervals of 0, 30, 90, 120 and 180 days, samples were removed from storage and have been analyzed for BAS 750 F using LC-MS/MS detection.

2. Description of analytical procedures

The animal commodities were analyzed for residues of BAS 750 F using method L0272/01.
A 5 g sample aliquot is extracted for fat contained matrices (milk, fat, cream) with a mixture of acetonitrile and iso-hexane. An aliquot of the extract is centrifuged and two times partitioned against iso-hexane.
For protein contained matrices (egg, muscle, liver and kidney) a 5 g sample aliquot is extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract is centrifuged and two times partitioned at alkaline conditions against cyclohexane.
The analysis of BAS 750 F is performed by LC-MS/MS.
The method has a limit of quantitation of 0.01 mg/kg in each matrix for the analyte.

II. RESULTS AND DISCUSSION

The recoveries of BAS 750 F from cow milk, cream and tissues after the various storage periods are summarized Table 6.1-5.

All compounds remained stable over the whole storage period of about 180 days in every matrix examined. The analytical method was validated at the level used for fortification with every series of analysis (at 0.01 mg/kg and 0.1 mg/kg). The average of all recoveries for BAS 750 F was between 70 and 110% for all samples.

Table 6.1-5: Storage stability of BAS 750 F in bovine milk, cream and tissues

Mean Recovery [%] Reg.No.5834378									
Day	**A**		B	**A**		B	**A**		B
	Cow Liver			**Cow Kidney**			**Cow Muscle**		
0	104	(108)*	96.3	104	(91.8)	113	99.5	(94.5)	105
28	92.0	(92.9)	99.0	-	-	-	-	-	-
29	-	-	-	90.5	(91.6)	98.8	-	-	-
30	-	-	-	-	-	-	91.3	(93.6)	97.5
89	-	-	-	-	-	-	99.2	(93.5)	106
90	-	-	-	96.7	(93.8)	103	-	-	-
120	90.0	(97.5)	98.0	88.0	(92.1)	95.5	87.3	(86.4)	101
177	105	(112)	93.7	-	-	-	-	-	-
182	-	-	-	99.5	(96.6)	103	94.3	(84.0)	112
Day	**A**		B	**A**		B	**A**		B
	Cow Fat			**Cow Milk**			**Cow Cream**		
0	90.8	(97.4)	93.2	97.6	(103)	94.8	96.9	(98.4)	98.5
29	-	-	-	83.8	(93.5)	89.6	-	-	-
32	83.7	(96.2)	87	-	-	-	88.9	(94.2)	94.4
83	-	-	-	-	-	-	100	(101)	98.5
84	-	-	-	98.2	(101)	96.9	-	-	-
85	93.0	(101)	92.3	-	-	-	-	-	-
116	-	-	-	83.5	(93.0)	89.8	-	-	-
117	84.8	(94.9)	89.4	-	-	-	-	-	-
118	-	-	-	-	-	-	89.9	(93.3)	96.4
177	-	-	-	-	-	-	91.5	(90.8)	101
178	-	-	-	96.0	(95.1)	101	-	-	-
180	87.5	(102)	86.2	-	-	-	-	-	-

Day	**A**		B
	Hen Egg		
0	107	(98.1)	109
28	88.0	(102)	86.5
83	105	(102)	103
118	87.8	(88.6)	99.0
180	105	(94.7)	111

A: mean in stored samples, % of nominal
B: mean procedural, in freshly spiked samples
(values in brackets are corrected for procedural recoveries)

CONCLUSION

It can be concluded that BAS 750 F is stable in animal matrices for at least 180 days when stored under deep frozen conditions.

Triazole Derivative Metabolites (TDMs)

The following paragraph is an exact copy of the relevant dossier section submitted to UK CRD in August 2014. It summarizes the results of in total 10 studies which were performed by the TDMG member companies.

The stability of residues of 1,2,4-triazole , triazole alanine, triazole acetic acid and triazole lactic acid was investigated in a range of frozen crop and animal commodities.

1,2,4-triazole was demonstrated to be stable for the full duration of studies (12 - 54 months) in crops representative of the high starch, high oil and high water crop groups according to the OECD guideline 506. There were, however, some commodities in which 1,2,4-triazole was not stable for the full duration of the study; these were wheat grain from one study (12 months), wheat straw (40 months), turnip root (40 months), radish root (12 months), soybean seed (12 months) and tomato fruit (40 months). For a few other matrices the data were inconclusive since a quick degradation occurred at the very beginning of the study while thereafter 1,2,4-triazole seemed to remain fairly stable.

Triazole alanine was demonstrated to be stable for the full duration of the studies (12 - 54 months) in most crops representative of the high starch, high oil, high water and high protein crop groups according to the OECD guideline 506. Inconclusive results were obtained for oilseed rape seed and oilseed rape oil. Triazole alanine was also demonstrated to be stable in milk and eggs for the full duration of the studies (12 months).

Triazole acetic acid was demonstrated to be stable in most crop and animal commodities tested for the full duration of the studies (12 - 54 months). The crop commodities are representative of the high starch, high oil, high water and high protein crop groups according to the OECD guideline 506 and the animal commodities were milk and eggs. There were, however, some commodities in which triazole acetic acid was not stable for the full duration of the study; these were wheat straw (40 months), wheat bran (40 months) and radish tops (12 months). In one study the results were inconclusive for wheat grain but two other studies demonstrate that triazole acetic acid is stable in wheat grain for at least 25-26 months upon freezer storage

Triazole lactic acid was demonstrated to be stable in all crop commodities tested for at least 12 months. The crop commodities are representative of the high starch, high oil, high acid, high water and high protein crop groups according to the OECD guideline 506.

A summary of the stability results for all analytes and matrices is presented in the table below.

Table 6.1-6: Stability of Triazole Metabolites in Crop and Animal Commodities Following Freezer Storage

Commodities[1]	Crop	Commodity	Nominal period of stability demonstrated (months)			
			1,2,4-Triazole	Triazole alanine	Triazole acetic acid	Triazole lactic acid
Crops – high starch	Wheat	Grain	≥ 54 -- -- 12 --	≥ 54 -- ≥ 15 ≥ 26 --	NC[3] ≥ 25 -- ≥ 26 --	-- -- -- -- ≥ 12
		Straw[2]	40 -- --	≥ 54 -- ≥ 15	40 ≥ 25 --	-- -- --
		Flour	≥ 54 ≥ 12	≥ 54 ≥ 12	≥ 54 ≥ 12	-- --
		Bran[2]	NC[3]	≥ 54	40	--
	Barley	Grain	-- --	≥ 36 --	-- ≥ 36	-- --
		Straw[2]	-- --	≥ 36 --	-- ≥ 36	-- --
	Turnip	Root	40	≥ 54	≥ 54	--
	Sugar beet	Root	-- --	-- ≥ 15	≥ 25 --	-- --
	Radish	Root	12	≥ 26	≥ 26	--
Crops – high oil	Oilseed rape	Seed	NC[3] -- -- --	NC[3] ≥ 15 -- --	≥ 54 -- ≥ 24 --	-- -- -- ≥ 12
		Oil	≥ 54	NC[3]	≥ 54	--
		Meal[2]	≥ 54	≥ 54	≥ 54	--
	Soybean	Seed	12	≥ 26	≥ 26	--
	Peanut	Butter	≥ 12	≥ 12	≥ 12	--
Crops – high acid	Orange	Fruit	--	--	--	≥ 12
Crops – high water	Wheat	Forage	NC[3]	≥ 54	≥ 54	--
	Mustard	Leaves	NC[3]	≥ 54	≥ 54	--
	Tomato	Fruit	40	≥ 54	≥ 54	--
		Paste	≥ 54	≥ 54	≥ 54	--
	Apple	Fruit	≥ 12	≥ 12	≥ 12	--
	Cabbage	Head	-- --	-- ≥ 15	≥ 24 --	-- --
	Radish	Tops	≥ 26	≥ 26	12	--
	Lettuce	Lettuce	--	--	--	≥ 12
Crops – high protein	Pea	Dry Seed	-- --	-- ≥ 15	≥ 25 --	-- --
	Navy Bean	Dry Bean	--	--	--	≥ 12
Animal	na	Milk	≥ 12 ≥ 18	≥ 12 --	≥ 12 --	-- --
	na	Liver	≥ 12	--	--	--
	na	Muscle	≥ 12	--	--	--
	na	Fat	≥ 12	--	--	--
	na	Eggs	≥ 12	≥ 12	≥ 12	--

1 Crop commodities according to the categories described in OECD guideline 506.
2 Commodities not included in the crop categories described in OECD guideline 506.
3 Not conclusive : for these compounds / commodities the study suggested a noticeable degradation of residues upon storage but it is unclear whether this is attributable to the study design or demotes a real stability issue.

As the storage stability study for triazole lactic acid was submitted to CRD in mid of June 2015 as a preliminary report, the final report is submitted here.

Report:	CA 6.1/4 Perez R., 2015 a Freezer storage stability of Triazolyl lactic acid in plant samples 2015/7005764
Guidelines:	OECD 506 (Oct. 2007), EPA 860.1380
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

A freezer storage stability study was performed investigating plant samples spiked with Triazolyl lactic acid at 0.1 mg/kg. The samples were analysed for Triazolyl lactic acid with BASF method No. D0905, which determine the analyte by means of LC-MS/MS.
The data included in this report, indicate that residues of Triazolyl lactic acid are stable for about 48 months in wheat grain, navy bean, orange, canola seed and lettuce.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Triazolyl lactic acid (TLA)
Description: Not available
Lot/Batch #: L70-135
Purity: 97.7-98.8%
CAS#: Not available
Spiking levels: 0.1 mg/kg

2. Test Commodity:
Crop: Wheat grain, navy bean, orange, canola seed, lettuce
Type: Cereals; legume vegetables; citrus fruit; oilseeds; leafy vegetables
Variety: Not given
Botanical name: *Triticum aestivum; Phaseolus vulgaris; Citrus sinensis; Brassica napus L.; Lactuca sativa*
Crop parts(s) or processed commodity: Wheat grain, navy bean, orange, canola seed, lettuce
Sample size: 5 g

B. STUDY DESIGN

1. Test procedure

The stability of triazolyl lactic acid (TLA) in various plant matrices (wheat grain, navy bean, orange, canola seed, and lettuce) was investigated under the usual storage conditions (< -18°C) for fortified stored samples for up to 48 months. The plant matrices used were untreated control samples obtained from various field study sites.
Wheat grain, navy bean, orange, canola seed, and lettuce samples were spiked with TLA at a concentration of 0.1 mg/kg and analyzed in duplicate after storage intervals of 0, 1, 3, 6, 12, 18, 24, 30, 36, 42 and 48 months.

For frozen storage in wheat grain, navy bean, orange, canola seed and lettuce, TLA was dosed as follows: Exactly 0.1 mL of methanolic fortification solution containing the analyte TLA at a concentration of 5.0 µg/mL was dosed to pre-weighed 5 g plant specimens. Immediately after dosing, the specimens were stored frozen. The fortification standards were assayed to verify their concentration before use.

Additionally, three unfortified control samples were introduced into the analysis set, one was used as a control and two were used as procedural recovery samples, for each storage interval and matrix. Procedural recovery samples were fortified at 0.1 mg/kg on the day of extraction.

2. Description of analytical procedures

Residue analysis was conducted following procedures of BASF Method No D0905. A plant sample of 5 g was extracted by homogenization with acetonitrile/water (70:30 v/v, 60 mL) using a Polytron homogenizer. An aliquot of the extract was diluted with water and evaporated to about 1.4 mL under nitrogen at 40°C to remove acetonitrile. The resulting aqueous phase was brought to volume of 5 mL with 0.1% formic acid in water. The extract was then filtered through a syringe filter into an autosampler vial for LC-MS/MS determination.

The limit of quantitation (LOQ) of the analytical method for TLA was 0.01 mg/kg in all matrix types.

II. RESULTS AND DISCUSSION

The stability results are expressed as average percentage of the nominal fortification. In order to account for possible variations over the time investigated, the mean procedural recovery results are given in addition. Stored recoveries demonstrate TLA was quantitatively recovered within acceptable limits from all plant matrices stored under freezer conditions over a period of 48 months. No significant differences were seen when comparing freshly-fortified samples to recovery samples stored for 48 months.

Following table shows a summary of the stability data.

Table 6.1-7: Storage stability of TLA in wheat grain, navy bean, orange, canola seed and Lettuce

Mean recovery (%)										
Day	A: mean in stored samples, % of nominal						B: mean in procedural, freshly spiked samples			
	A	B	**A**	B	**A**	B	**A**	B	**A**	B
	Wheat grain		**Navy bean**		**Orange**		**Canola seed**		**Lettuce**	
0	**91**	93	**102**	91	**90**	115	**106**	99	**101**	111
47	**80**	93	**100**	91	**108**	115	**126**	99	**116**	111
97	**110**	93	**114**	91	**120**	115	**131**	117	**117**	111
181	**98**	87	**108**	87	**109**	109	**125**	108	**116**	104
362	**103**	113	**111**	110	**120**	108	**120**	117	**112**	110
548	**118**	107	**113**	109	**119**	111	**118**	99	**118**	104
736	**90**	82	**94**	96	**107**	109	**115**	91	**96**	88
915	**95**	90	**119**	102	**110**	104	**99**	104	**116**	95
1097	**81**	82	**84**	97	**114**	114	**97**	90	**108**	98
1310	**81**	83	**108**	114	**94**	102	**98**	108	**98**	98
1461	**86**	89	**97**	93	**107**	97	**98**	108	**102**	91

III. CONCLUSION

The results obtained in the storage stability study indicate that under freezer storage conditions, residues of TLA were stable in all matrices through the 48 month storage interval.

CA 6.2 Metabolism, distribution and expression of residues

The metabolism and distribution of BAS 750 F in plants was investigated using the active substance radiolabelled in the Chlorophenyl ring (C-label) or in the 3(5)-position of the Triazole ring (T-label).
The test item was either a mixture of ^{14}C-BAS 750 F, ^{13}C-BAS 750 F and unlabelled BAS 750 F. The molecular structures and the positions of the labels are shown below:

BAS Code:	**BAS 750 F**
Registry No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC)	

Figure 6.2-1: Structure of non-radiolabeled BAS 750 F

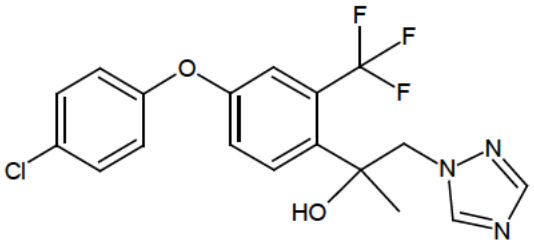


Figure 6.2-2: Structure of [Chlorophenyl-U-^{14}C]-BAS 750 F

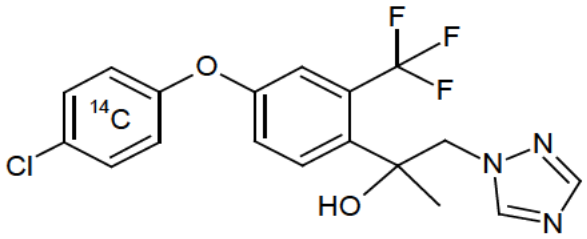


Figure 6.2-3: Structure of [Trifluoromethylphenyl-U-^{14}C]-BAS 750 F

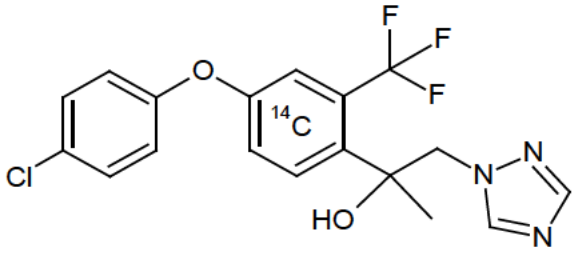


Figure 6.2-4: Structure of [Triazole-3(5)-^{14}C]-BAS 750 F

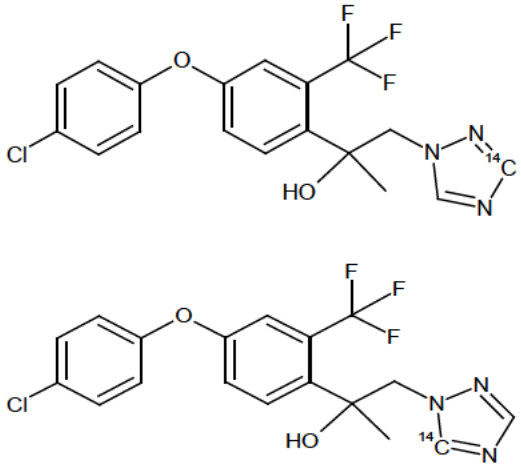


Figure 6.2-5: Structure of [Chlorophenyl-^{13}C]-BAS 750 F

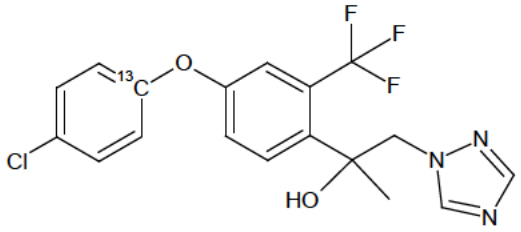


Figure 6.2-6: Structure of [Trifluoromethylphenyl-^{13}C]-BAS 750 F

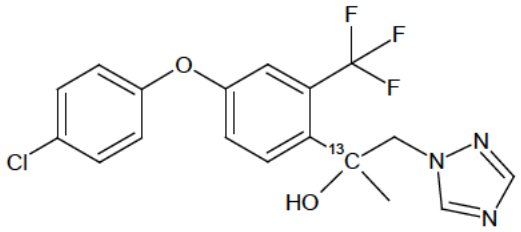
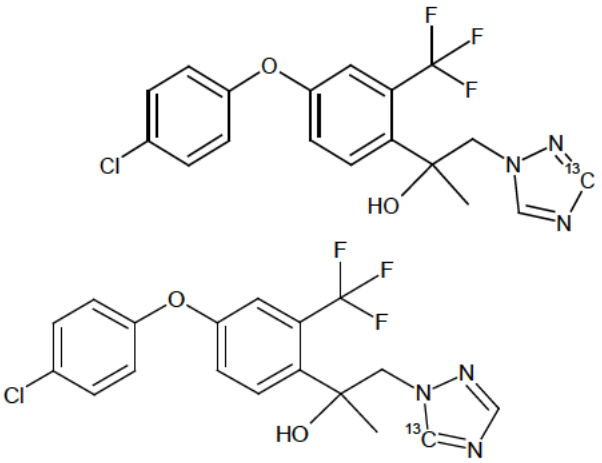


Figure 6.2-7: Structure of [Triazole-3(5)-^{13}C]-BAS 750 F



The BAS 750 F molecule is a three ring structure, it consists of the triazole ring (T-ring) characteristic for the group of azoles, the chlorophenyl-ring (C-ring) at the opposite side of the molecular backbone as well as the trifluoromethylphenyl-ring (TFMP-ring) in between. Based on the structure cleavage of the T-ring is expected, whereas cleavage between the TFMP-ring and the C-ring is less likely.

To account for the expected cleavage in plant between the T-ring and the TFMP-ring, plant metabolism studies (including primary crops wheat, soybean, grape and representative rotational crops) were conducted with first, BAS 750 F labeled in the T-ring, and second BAS 750 F labeled in the other part of the molecule (with radiocarbon placed in the C-ring).

Results of plant metabolism studies including foliar application representing three crop categories and rotational crop studies in representative follow crops, in fact do confirm this labeling strategy: cleavage of the parent backbone occurred at the T-bridge, between the T-ring and the double-ring ether. Cleavage of the ether bridge between the C-ring and the TFMP-ring was observed only in one instance (in wheat straw in low amounts, M750F009, M750F010).

Taken together the data obtained from the plant metabolism studies show that placing the ^{14}C label in either the C-ring or the T-ring enables to quantitatively trace the radioactive residue of BAS 750 F. Based on the conclusion that *in planta* residue components consisting only of the TFMP-ring (non-detectable by either C-label or T-label) are not expected to occur in detectable amounts, an additional experiment with BAS 750 F labeled in the TFMP-ring would not provide additional information, and therefore was not conducted.

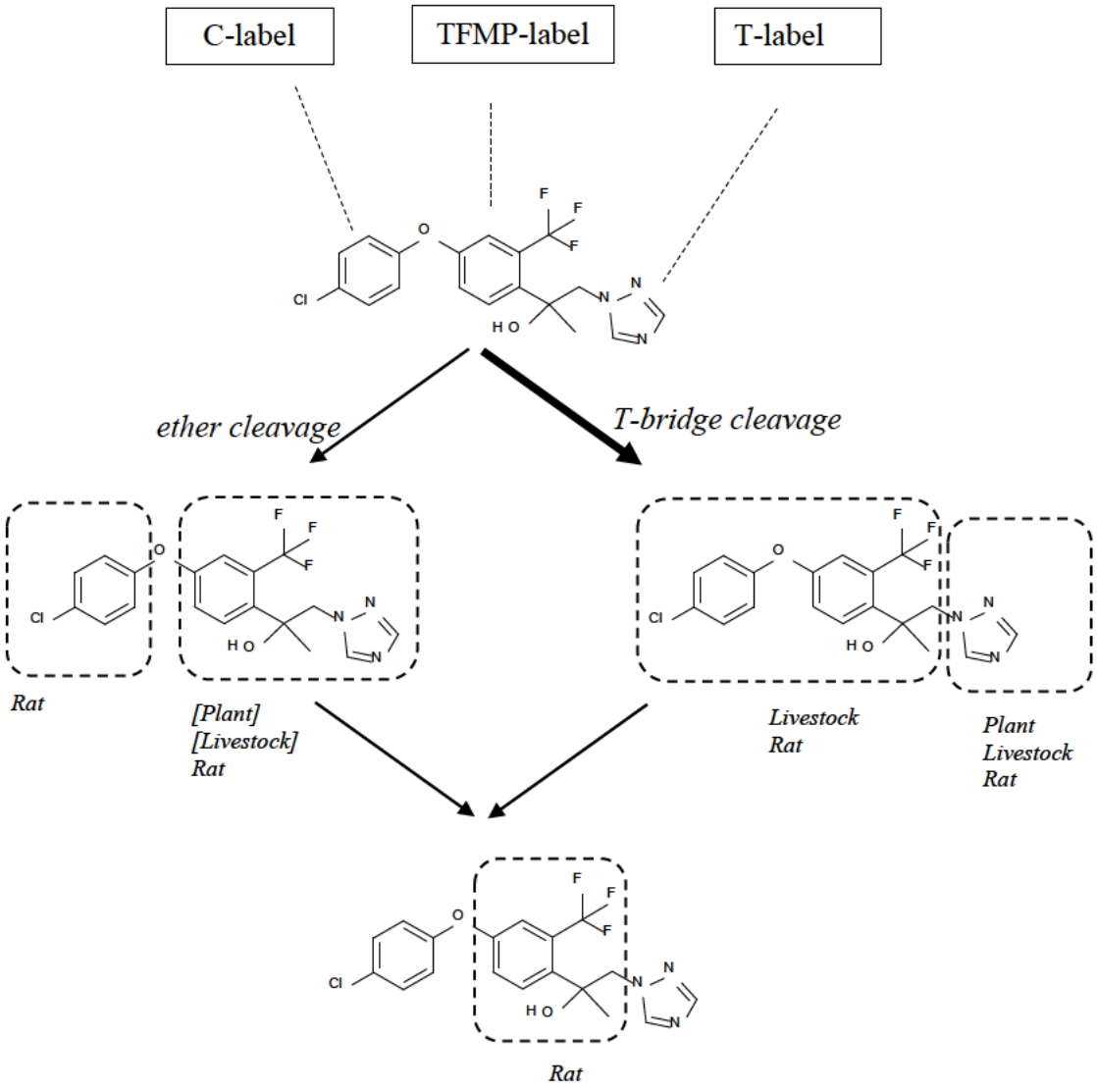
Similarly, to account for the expected cleavage in animal between the T-ring and the TFMP-ring, metabolism studies in rat as well as in livestock (goat, hen, fish) were conducted with first, BAS 750 F labeled in the T-ring, and second BAS 750 F labeled in the other part of the molecule. Results of the rat study indicated that cleavage at the ether bridge between the C-ring and the TFMP-ring does occur in significant amounts in rat. Consequently, with the objective to enable detection of single-ring residue components (containing only the TFMP-ring, thus non-detectable by either C-label or T-label), additional experiments with BAS 750 F labeled in the TFMP-ring were conducted in rat. Investigations with this TFMP-label (rats of both genders, high dose group) confirm that despite the abundance of double-ring cleavage products (M750F054, M750F071), single ring cleavage products (TFMP-ring only) are present only in very minor amounts (one finding of M750F101 at 0.6% of dose in urine of male rats).

To provide comprehensive information of transformation products in livestock, also the metabolism studies in hen and goat were conducted with all three labels. In hen, comparable metabolite profile was obtained for C-label and T-label, indicating absence of ether bridge cleavage in detectable amounts. In goat, with the TFMP-label, cleavage of the ether bridge was detected only in small amounts (M750F003 in kidney at 3% TRR, 0.01 mg/kg), while single ring cleavage products (TFMP-ring only) were not detectable. Investigations in fish with both the C-label and the T-label did show overall absence of ether bridge cleavage, the only cleavage occurred at the T-bridge (1,2,4-triazole), thus no experiment with the TFMP-label was conducted.

Taken together the data obtained from the animal metabolism studies (rat, hen, goat, fish) shows that cleavage at the T-bridge of the BAS 750 F molecule is a key transformation step, while cleavage at the ether bridge is a only a minor transformation step, with quantitative differences in the species investigated: non-detectable in soybean, grape, hen, and fish, occurring in traces in plant (wheat straw only) and in low amounts in ruminant (goat liver) and in detectable amounts in rat (notably in urine).

In summary, the BAS 750 F radiolabels used in the investigation of BAS 750 F metabolism in plant and animal allow to capture comprehensively all transformation products of the BAS 750 F residue.

Figure 6.2-8: Transformation of BAS 750 F by cleavage at T-bridge and/or ether bridge



CA 6.2.1 Metabolism, distribution and expression of residues in plants

Report:	CA 6.2.1/1 Rabe U.,Bogen C., 2015 a Metabolism of 14C LS 5834378 in wheat 2015/1001872
Guidelines:	EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, Test No. 501: Metabolism in crops
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in wheat after foliar application of BAS 750 F labeled either in the chlorophenyl-ring (C-ring) or in the triazole-ring (T-ring). Both labels were applied separately. Foliar spray applications of wheat plants cultivated in indoors (plastic containers located in vegetation hall/greenhouse/pythotron) were carried out two times at a target rate of 150 g a.s./ha. Samples of forage (growth stage BBCH 61) were taken 15 days after the first application (corresponding to 6 days prior to the second application). Samples of grain and straw (BBCH 89) were collected 35 days after the second (last) application. Samples were stored in a freezer at -18°C or below.

Data obtained with C-label and T-label taken together, show a consistent picture of the metabolism in foliar applied wheat. Absence of significant cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in wheat.

For both C- and T-label, similar total radioactivity (TRR) was seen in forage harvested 15 days after the first application (DALA-6) with 2.3 and 2.4 mg/kg, as well as in straw (DALA35) with 14 and 24 mg/kg. In grain, the TRR differed by a factor 10 between C-label (0.06 mg/kg) and T-label (0.63 mg/kg) correlating with a higher concentration of C-labeled metabolites, namely the triazole derived metabolites (TDM). For both labels, solvent extractability (ERR) was high for forage and straw (at least 83% TRR), as well as T-labeled grain (78% TRR). For C-labeled grain, solvent extraction followed by enzyme-mediated solubilization retrieved 85% of TRR reducing the final residue to 0.01 mg/kg (15% TRR). Thus, overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring) which introduces a second hydroxyl group which, alike the hydroxyl group of the parent, is the site for conjugation leading to an array of sugar conjugates. Cleavage of BAS 750 F at the T-bridge leads to 1,2,4-triazole (TA), triazole alanine (TA) and triazole acetic acid (TAA). These metabolites are part of the group of triazole derivative metabolites (TDM) which are common to a range of azole fungicides. Cleavage at the ether bridge does occur, albeit only at minor amounts leading to the metabolites M750F009 and M750F010 (incidental occurrence only in straw, at 1% TRR).

The parent BAS 750 F is present in wheat matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation or translocation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in forage and straw representing at least 80% TRR in forage and at least 50% TRR in straw. A higher proportion of sugar conjugates is observed in straw (BBCH89) with 18-21% TRR compared with forage (BBCH61) with 3-4% TRR. In contrast, unchanged parent is not detected in grain, where TDM account almost exclusively for the radioactive residue (accounting for 77% TRR). Note that due to the cultivation of the wheat plants in plastic boxes with limited drainage, the accessibility of small polar substances might be enhanced (notably radioactive TDMs generated in the soil). Translocation of TDM into grain is reflected by the fact that TRR level in T-labeled grain are significantly exceeding the TRR in C-labeled grain.

In conclusion, the major components of the residue in wheat were identified as unchanged parent BAS 750 F and the common metabolites TDM which together are representing a large proportion of the residue (>80% in forage, >77% in grain, >50% in straw). Other components of the residue were sugar conjugates of parent (unchanged or hydroxylated) individually present <6% TRR as well as minor amounts of two metabolites resulting from ether cleavage (individually present at 1.3% TRR). Overall, metabolism of BAS 750 F in wheat, and by extrapolation, in the *cereal* crop group is considered well-elucidated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

Description:	BAS 750 F **C-label:** Chlorophenyl-U-C14-BAS 750 F, Chlorophenyl-1-C13-BAS 750 F **T-label:** Triazole-3(5)-C14-BAS 750 F, Triazole-3(5)-C13-BAS 750 F
Lot/Batch No:	CFQ41561 (Chlorophenyl-U-C14-BAS 750 F, 7.878 MBq/mg) RS4-2012-173A2 (Chlorophenyl-1-C13-BAS 750 F), 1062-2001 (Triazole-3(5)-C14-BAS 750 F, 5.46 MBq/mg) 1077-1001 (Triazole-3(5)-C13-BAS 750 F)
Purity:	Chlorophenyl-U-C14-BAS 750 F: 99.1% (radiochemical 98.9%) Chlorophenyl-1-C13-BAS 750 F: 97.7% Triazole-3(5)-C14-BAS 750 F: 98.9% (radiochemical 98.8%) Triazole-3(5)-C13-BAS 750 F: 97.1%
CAS No:	1417782-03-6 (chlorophenyl labeled-BAS 750 F, triazole labeled-BAS 750 F)
Development code:	BAS 750 F
Spiking levels:	*not applicable*

2. Test Commodity:

Crop:	Spring wheat	
Type:	cereal	
Variety:	Thassos	
Botanical name:	*Triticum aestivum*	
Crop parts(s) or processed Commodity:	wheat (forage, grain, straw)	
Sample size:	forage	0.55-0.62 kg
	grain	1.58-1.61 kg
	straw	3.53-3.55 kg

3. Soil: sandy loam (for soil physicochemical properties see Table 6.2.1-1).

Table 6.2.1-1: Soil physicochemical properties

soil series	soil type [1)]	pH	OM [2)]	sand (%)	silt (%)	clay (%)	moisture at 1/3 bar (%)	CEC cmol/kg
Bruch West	sandy loam	8.0	2.53	70.4	18.6	11.0	N/A [3)]	9.5

1) USDA scheme, 2) organic matter, corresponds to the total organic carbon (TOC), 3) not applicable

B. STUDY DESIGN AND METHODS

1. Test procedure

The study was conducted at the Agricultural Research Center of BASF in Limburgerhof, Germany (2013-2015). Spring wheat plants were cultivated in containers (size: 0.4 x 0.6 m, total test area = approximately 2.4 m^2) with sandy loam soil. Containers were initially located in a vegetation hall with glass roof and temporarily in a greenhouse. For 14C-label application, they were transferred to climatic chambers (phytotrons). Ten containers each were used per label. The maintenance of the plants was performed in accordance with normal agricultural practice. 15 days after the first treatment (6 days prior to the second application) plants were thinned out and forage (BBCH61) was sampled.

For each test item, BAS 750 F labeled in the chlorophenyl-moiety (C-label) or BAS 750 F labeled in the triazole-moiety (T-label), an application formulation was prepared for application by automatic spray track. Each C14-labeled test item was mixed with C13-labeled test item (1:1 ratio of chlorophenyl-U-C14- and chlorophenyl-1-C13-label, 2:1 ratio of triazole-3(5)-C14- and triazole-3(5)-C13-label). For the first application, tank mixes of the test items (taken up in blank formulation and water) were prepared. The second application formulations were prepared correspondently. Aliquots of the first and second application formulations were taken for confirmation of identity, for purity check (by radio-HPLC) as well as for determination of the C14:C13 isotope ratio (by mass spectrometry).

Both labels were applied separately (see Table 6.2.1-2). Foliar spray applications were carried out two times either with the chlorophenyl-labeled BAS 750 F or the triazole-labeled BAS 750 F at a target rate of 150 g a.s./ha, respectively. Samples of forage (BBCH61) were taken 15 days after the first application (corresponding to 6 days prior to the second, and last, application). Samples of grain and straw (both BBCH 89) were collected 35 days after the last application and then stored in a freezer at -18°C or below.

Table 6.2.1-2: Study design: plant uptake part (wheat)

label	C-label		T-label	
intended use rate [g a.s./ha]	150		150	
application number	2		2	
application interval [days]	21		21	
application growth stages	BBCH49, BBCH69		BBCH49, BBCH69	
sampled matrices	forage, grain, straw		forage, grain, straw	
sampling [DALA] [1)]	forage	-6 (=15 DAT) [2)]	forage	-6 (=15 DAT) [2)]
	grain	35	grain	35
	straw	35	straw	35

[1)] days after last application, [2)] only one application: 15 days after the first application (= 15 DAT) corresponding to 6 days prior to last application (DALA=-6)

2. Description of analytical procedures

Radioanalysis: For TRR determination and the measurement of solid residues following solvent extraction (RRR) or solubilization procedures (final residue), homogenized subsamples were combusted using a sample oxidizer. The resultant ^{14}C-CO_2 was absorbed, mixed with scintillation fluid and radioactivity was determined by liquid scintillation counting (LSC). For liquid samples scintillation fluid was added, and subjected to LSC measurement.
Homogenization/solvent extraction: Plant samples (forage, grain and straw) were homogenized. Forage and straw were extracted with methanol (3x) and water (2x). After each extraction step, solid material was separated from extract by centrifugation and filtration, and the supernatants of methanol and water extracts were each combined.
Grain was extracted three times with a mixture of acetonitrile and isohexane (1:1). After a centrifugation step, the acetonitrile and isohexane phase were separated using a separatory funnel. Obtained acetonitrile and isohexane phases were combined, respectively, and adjusted to a defined volume. Acetonitrile/isohexane extraction was followed by extraction with water (two times). The solid material was separated from the aqueous extract by centrifugation and filtration. The residue after solvent extraction was dried in a fume hood, homogenized and radioassayed.
The aqueous extract was concentrated to dryness, subsequently taken up in water and adjusted to pH 4 with formic acid. For protein precipitation, acetone was added in two steps, the sample incubated in a refrigerator followed by centrifugation to obtain the precipitate and supernatant for further analysis.

Solubilization of the acetone precipitate of the ERR and the RRR:
The acetone precipitate of the aqueous extract obtained from grain was re-suspended and incubated in the presence of protease (at least 2 days). After addition of acetonitrile and water, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume and the residue was subjected to α-amylase, β-amylase and amyloglucosidase treatment.
The residues after solvent extraction (forage, grain and straw of both labels) were re-suspended in 1 % ammonia, incubated overnight, centrifuged and filtered. The solubilizate was adjusted to a defined volume. The residues from forage and straw were subjected to macerozyme treatment, while the residue from grain was processed by incubation with α-amylase/β-amylase/amyloglucosidase.
The resulting residue (forage and straw) was re-suspended and incubated in the presence of macerozyme (at least 2 days). Usually after addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue (forage, grain, straw as well as the residue from protease treatment from aqueous extract of grain) was re-suspended and incubated in the presence of α-amylase, β-amylase and amyloglucosidase (1:1:1 ratio, for approximately 48-72 h), then centrifuged and filtered and adjusted to a defined volume. After addition of acetonitrile, the samples were centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue (forage and straw) was re-suspended and incubated in the presence of β-glucosidase and hesperidinase (approximately 72 h). After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue (forage, straw) was re-suspended. Suitable amounts of laccase and tyrosinase were added and the mixture was incubated for approximately 72 hours. After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.

3. Identification and characterization of the residue

Components of the residue were identified by HPLC-MS as well as by co-chromatography and comparison of retention times. In addition, for the parent BAS 750 F enantiomer-specific HPLC analyses were performed in samples of the application solution, as well as extracts of forage and straw (samples purified by SPE and HPLC fractionation).

II. RESULTS AND DISCUSSION

1. TOTAL RADIOACTIVE RESIDUE (TRR)

Following foliar application of BAS 750 F to wheat at a rate of two times 125 g as/ha, the total ^{14}C residue (TRR) was measured in wheat forage (BBCH61), as well as grain and straw (both BBCH89, see Table 6.2.1-3).

The calculated total radioactive residues (TRR) with the C-label were highest in straw (DALA 35) with 24.38 mg/kg, lower in forage (sampled first and second application) with 2.38 mg/kg, and very low in grain with 0.062 mg/kg.
A similar distribution was seen with the T-label (TRR highest in straw 13.98 mg/kg, lower in forage at 2.31 mg/kg, and grain at 0.62 mg/kg). Notably the TRR in grain was much higher with the T-label compared with the C-label, indicating a difference in composition of the detectable residue (see below, part 3).

Table 6.2.1-3: Total radioactive residue after foliar spray application of BAS 750 F

matrix [BBCH]	DALA [1)]	TRR measured *[mg/kg]*	TRR calculated [2)] *[mg/kg]*
C-label			
forage [61]	-6 (=15 DAT) [3)]	2.472	2.378
grain [89]	35	0.065	0.062
straw [89]	35	24.305	24.380
T-label			
forage [61]	-6 (=15 DAT) [3)]	2.634	2.310
grain [89]	35	0.619	0.620
straw [89]	35	14.339	13.984

[1)] days after last application, [2)] TRR calculated as the sum of ERR and RRR, see Table 6.5/4, and Table 6.5/5
[3)] 15 days after the first application (=15DAT) corresponding to 6 days prior to last application (DALA= -6)

2. EXTRACTABILITY

The extractabilities of 14C residues from wheat forage, straw and grain are summarized in Table 6.2.1-4 and Table 6.2.1-5.

High extractability of ^{14}C residue was seen in forage (>95% TRR for both labels) and straw (>83% both labels). Most of the radioactivity was extracted with methanol (73% TRR or higher) while with subsequent water extraction resulted in additional extraction of 10% TRR or less. Solvent extraction left a RRR in forage of <5% TRR, while the RRR in straw amounted to 17% TRR (4.14 mg/kg, C-label) and 13.6 % TRR (1.9 mg/kg, T-label). The RRR was therefore further investigated by enzyme treatment (see below). Notably, no significant label specific differences were seen for forage and straw.

Extractability in grain was significantly different for both labels, indicative of different composition of the radioactive residue: with C-label 43.9 % TRR of which 24.8% TRR was extracted by water extraction and 17.4% TRR by acetonitrile. In contrast, with the T-label 77.9% TRR was extracted with the majority released by water extraction, acetonitrile had removed only 3.6% TRR.
The RRR after solvent extraction amounted to 56.1% TRR (C-label, 0.035 mg/kg) and 22.1% TRR (T-label, 0.137 mg/kg) and thus was subject to further investigation.

Table 6.2.1-4: Extractability of radioactive residue from forage and straw

matrix	DALA [1)]	TRR [2)]	distribution of radioactive residues							
			methanol extracts [3)]		water extracts [3)]		ERR [2)]		RRR [2)]	
		mg/kg	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
C-label										
forage	-6 (=15DAT)	*2.378*	**94.0**	*2.236*	**1.2**	*0.029*	**95.2**	*2.264*	**4.8**	*0.114*
straw	35	*24.38*	**73.8**	*17.99*	**9.3**	*2.255*	**83.0**	*20.24*	**17.0**	*4.139*
T-label										
forage	-6 (=15DAT)	*2.310*	**94.7**	*2.188*	**1.3**	*0.030*	**96.0**	*2.218*	**4.0**	*0.092*
straw	35	*13.98*	**77.7**	*10.87*	**8.7**	*1.213*	**86.4**	*12.08*	**13.6**	*1.901*

[1)] days after last application, for forage sampling was 15 days after the first application (DAT1=15) corresponding to 6 days prior to last application (DALA=-6). [2)] TRR was calculated as the sum of ERR and RRR with ERR=Extractable Radioactive Residue, RRR=Residual Radioactive Residue (after solvent extraction)
[3)] pool of combined repetitive extracts

Table 6.2.1-5: Extractability of radioactive residue from grain

matrix	DALA[1)]	TRR[2)]	distribution of radioactive residues									
			acetonitrile phases[3)]		isohexane phases [3)]		water extracts [4)]		ERR[2)]		RRR[2)]	
		mg/kg	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
C-label												
grain	35	*0.062*	**17.4**	*0.011*	**1.7**	*0.001*	**24.8**	*0.015*	**43.9**	*0.027*	**56.1**	*0.035*
T-label												
grain	35	*0.620*	**3.6**	*0.022*	**0.2**	*0.001*	**74.2**	*0.460*	**77.9**	*0.483*	**22.1**	*0.137*

[1)] days after last application. [2)] TRR was calculated as the sum of ERR and RRR with. ERR=Extractable Radioactive Residue, RRR=Residual Radioactive Residue (after solvent extraction), [3)] separated phases of acetonitrile/isohexane extracts, [4)] pool of combined repetitive extracts

3. EXTRACTION, CHARACTERISATION AND IDENTIFICATION OF RESIDUES

An overview over the components of the extractable residue is given below in Table 6.2.1-6 and Table 6.2.1-7. An overview over the residue released from RRR by enzyme treatment is given in Table 6.2.1-8 and Table 6.2.1-9.

Table 6.2.1-6: Summary of identified/characterized components in wheat (C-label)

C-labeled radioactive component (min) [4] in ERR & RRR	forage		grain		straw	
	% TRR	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
TRR	**100.0**	*2.378*	**100.0**	*0.062*	**100.0**	*24.380*
BAS 750 F	**84.4**	*2.007*	-	-	**58.6**	*14.297*
M750F018 (20.3)	-	-	-	-	**2.9**	*0.716*
M750F019 (20.9, 21.5, 21.9)	**0.1**	*0.003*	-	-	**5.8**	*1.407*
M750F018 / M750F020 (22.5)	**1.6**	*0.037*	-	-	**6.9**	*1.682*
M750F012 / M750F021 (25.6)	**2.0**	*0.049*	-	-	**4.9**	*1.184*
M750F012 (26.9)	-	-	-	-	**0.6**	*0.158*
ERR	**95.2**	*2.264*	**43.9**	*0.027*	**83.0**	*20.241*
ID [1]	**88.1**	*2.096*	-	-	**75.2**	*18.344*
CHAR [1]	**2.9**	*0.070*	**42.4**	*0.026*	**1.9**	*0.468*
sum of ID/CHAR	**91.1**	*2.166*	**42.4**	*0.026*	**77.2**	*18.812*
RRR	**4.8**	*0.114*	**56.1**	*0.035*	**17.0**	*4.139*
ID [1]	-	-	-	-	**4.5**	*1.101*
CHAR [1]	**2.4**	*0.057*	**41.6**	*0.026*	**3.7**	*0.901*
sum of ID/CHAR	**2.4**	*0.057*	**41.6**	*0.026*	**8.2**	*2.003*
SUM ID/CHAR in ERR/RRR	**93.5**	*2.224*	**83.9**	*0.052*	**85.4**	*20.814*
Final residue [2]	**2.0**	*0.047*	**15.4**	*0.010*	**7.9**	*1.924*
Grand Total [3]	**95.5**	*2.271*	**99.3**	*0.061*	**93.3**	***22.738***

[1] ID=amount identified, CHAR=amount characterized (information on number and quantities of peaks provided in study report), sum ID/CHAR= sum of amounts identified and/or characterized, sum ID/CHAR in ERR/RRR= sum of amounts identified and/or characterized in ERR and in RRR, [2] final residue after solvent extraction and solubilization [3] sum of amounts characterized and identified as well as final residue, [4] Retention times are provided in brackets, M750F019 eluted in three distinct peaks, the peak at 22.5 min and the peak at 25.6 min each did contain two compounds. Note M750F021 is considered an artefact.

Table 6.2.1-7: Summary of identified/characterized components in wheat (T-label)

T-labeled radioactive component (min) [4] in ERR & RRR	forage		grain		straw	
	% TRR	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
TRR	**100.0**	*2.310*	**100.0**	*0.620*	**100.0**	*13.984*
BAS 750 F	**89.3**	*2.062*	-	-	**68.5**	*9.573*
M750F001 (1,2,4-T)	-	-	**1.0**	*0.006*	-	-
M750F029 (TA)	-	-	**45.6**	*0.282*	-	-
M750F030 (TAA)	-	-	**21.4**	*0.133*	-	-
M750F009	-	-	-	-	**1.3**	*0.178*
M750F010	-	-	-	-	**1.3**	*0.180*
M750F018 (20.3)	-	-	-	-	**5.5**	*0.767*
M750F019 (20.9, 20.5, 21.9)	-	-	-	-	**4.8**	*0.671*
M750F018 / M750F020 (22.5)	**1.6**	*0.037*	-	-	**4.3**	*0.603*
M750F012 / M750F021 (25.6)	**1.1**	*0.025*	-	-	**3.4**	*0.471*
M750F012 (26.9)	-	-	-	-	**0.1**	*0.008*
ERR	**96.0**	*2.218*	**77.9**	*0.483*	**86.4**	*12.083*
ID [1]	**92.0**	*2.125*	**67.9**	*0.421*	**83.5**	*11.677*
CHAR [1]	**0.8**	*0.018*	**5.4**	*0.033*	**1.1**	*0.151*
sum of ID/CHAR[1]	**92.8**	*2.142*	**73.3**	*0.454*	**84.6**	*11.828*
RRR	**4.0**	*0.092*	**22.1**	*0.137*	**13.6**	*1.901*
ID [1]	-	-	-	-	**5.5**	*0.772*
CHAR [1]	**1.9**	*0.045*	**19.8**	*0.123*	**1.7**	*0.235*
sum of ID/CHAR[1]	**1.9**	*0.045*	**19.8**	*0.123*	**7.2**	*1.007*
SUM ID/CHAR in ERR/RRR[1]	**94.7**	*2.187*	**93.1**	*0.577*	**91.8**	*12.835*
Final residue [2]	**1.9**	*0.043*	**1.9**	*0.012*	**5.8**	*0.804*
Grand Total [3]	**96.5**	***2.230***	**95.0**	***0.589***	**97.5**	***13.639***

[1] ID=amount identified, CHAR=amount characterized (information on number and quantities of peaks provided in study report), sum ID/CHAR= sum of amounts identified and/or characterized, sum ID/CHAR in ERR/RRR= sum of amounts identified and/or characterized in ERR and in RRR, [2] final residue after solvent extraction and solubilization [3] sum of amounts characterized and identified as well as final residue, [4] Retention times are provided in brackets, M750F019 eluted in three distinct peaks, the peak at 22.5 min and the peak at 25.6 min each did contain two compounds. Note M750F021 is considered an artefact.

Table 6.2.1-8: Characterization of RRR by enzyme treatment (C-label)

C-labeled radioactive component (min) [4]		forage		grain		straw	
		%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
RRR		**4.8**	*0.114*	**56.1**	*0.035*	**17.0**	*4.139*
CHAR [1] by enzyme treatment [5]	AM	**1.0**	*0.023*	**17.1**	*0.011*	**4.2**	*1.026*
	M/C	**0.5**	*0.012*	-	-	**1.7**	*0.421*
	AM/G	**0.3**	*0.008*	**24.5**	*0.015*	**0.8**	*0.198*
	G/H	**0.3**	*0.007*	-	-	**0.7**	*0.167*
	T/L	**0.3**	*0.008*	-	-	**0.8**	*0.191*
	solubilized (sum)	**2.4**	***0.057***	**41.6**	***0.026***	**8.2**	***2.003***
Final residue		**2.0**	*0.047*	**15.4**	*0.010*	**7.9**	*1.924*

RRR		**4.8**	*0.114*	**56.1**	*0.035*	**17.0**	*4.139*
CHAR [1] by HPLC	peak at 31.8 min	-	-	-	-	**0.2**	*0.058*
	other	1.0	*0.023*	-	-	**3.5**	*0.844*
	characterized (sum)	1.0	*0.023*	-	-	**3.7**	*0.901*
ID [1]	BAS 750 F	-	-	-	-	**2.0**	*0.500*
	M750F018 (20.3)	-	-	-	-	**0.4**	*0.099*
	M750F019 (20.9, 21.5, 21.9)	-	-	-	-	**1.4**	*0.330*
	M750F012 / M750F021 (25.6)	-	-	-	-	**0.7**	*0.172*
	identified (sum)	-	-	-	-	**4.5**	*1.101*
	identified/characterized (sum)[3]	**1.0**	***0.023***	-	-	**8.2**	***2.003***
Final residue		**2.0**	*0.047*	**15.4**	*0.010*	**7.9**	*1.924*

[1] ID=amount identified, CHAR=amount characterized (information on number and quantities of peaks provided in study report), [2] final residue after solvent extraction and solubilization, [3] sum of amounts characterized and identified as well as final residue , [4] Retention times are provided in parenthesis, M750F019 eluted in three distinct peaks, the peak at 22.5 min and the peak at 25.6 min each did contain two compounds. Note M750F021 is considered an artefact. [5] Solubilization included sequential treatments with ammonia (AM), with macerozyme/cellulase (M/C), with amylase/amyloglucosidase (AM/G), with glucosidase/hersperinidase (G/H) and finally with tyrosinase/laccase (T/L).

Table 6.2.1-9: Characterization of RRR by enzyme treatment (T-label)

T-labeled radioactive component (min) [4]		forage		grain		straw	
		% TRR	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
RRR		**4.0**	*0.092*	**22.1**	*0.137*	**13.6**	*1.901*
CHAR[1] by enzyme treatment [5]	AM	**0.7**	*0.017*	**15.3**	*0.095*	**3.6**	*0.506*
	M/C	**0.5**	*0.010*	-	-	**1.3**	*0.184*
	AM/G	**0.3**	*0.006*	**4.6**	*0.028*	**0.7**	*0.098*
	G/H	**0.2**	*0.005*	-	-	**1.1**	*0.148*
	T/L	**0.3**	*0.006*	-	-	**0.5**	*0.070*
	solubilized (sum)	**1.9**	*0.045*	**19.8**	*0.123*	**7.2**	*1.007*
Final residue		**1.9**	*0.043*	**1.9**	*0.012*	**5.8**	*0.804*

RRR		**4.0**	*0.092*	**22.1**	*0.137*	**13.6**	*1.901*
CHAR [1] by HPLC	peak at 31.8 min	-	-	-	-	**0.4**	*0.051*
	other	**0.7**	*0.017*	-	-	**1.3**	*0.184*
	total characterized	**0.7**	*0.017*	-	-	**1.7**	*0.235*
ID [1]	BAS 750 F	-	-	-	-	**1.2**	*0.173*
	M750F009	-	-	-	-	**0.4**	*0.061*
	M750F018 (20.3)	-	-	-	-	**2.1**	*0.294*
	M750F019 (20.9, 21.5, 21.9)	-	-	-	-	**1.2**	*0.164*
	M750F012 / M750F021 (25.6)	-	-	-	-	**0.5**	*0.072*
	M750F012 (26.9)	-	-	-	-	**0.1**	*0.008*
	total identified	-	-	-	-	**5.5**	*0.772*
	identified/characterized (sum)	**0.7**	*0.017*	-	-	**7.2**	*1.007*
Final residue		**1.9**	*0.043*	**1.9**	*0.012*	**5.8**	*0.804*

[1] ID=amount identified, CHAR=amount characterized (information on number and quantities of peaks provided in study report), [2] final residue after solvent extraction and solubilization, [3] sum of amounts characterized and identified as well as final residue , [4] Retention times are provided in parenthesis, M750F019 eluted in three distinct peaks, the peak at 22.5 min and the peak at 25.6 min each did contain two compounds. Note M750F021 is considered an artefact. [5] Solubilization included sequential treatments with ammonia (AM), with macerozyme/cellulase (M/C), with amylase/amyloglucosidase (AM/G), with glucosidase/hersperinidase (G/H) and finally with tyrosinase/laccase (T/L).

3.1 forage

Overall, similar results were observed with both labels. Unchanged parent was with > 84% TRR (2.0 and 2.1 mg/kg for C- and T-label) the predominant component of the residue (and only component present in major amounts) thus similar to straw. The only other components identified were present in amounts of 2% TRR (0.05 mg/kg) or less and were structurally related to the parent (sugar conjugates of the parent molecule and a hydroxylated parent molecule).
Noteably, for C-label and T-label, the malonylglucosyl-O-conjugate of BAS 750 F metabolite M750F012, (including M750F021) accounted for <2% (0.05 mg/kg), M750F018/M750F020 (malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for 1.6% (0.037 mg/kg), and M750F019 (glucosyl-O-conjugate of hydroxylated parent) accounted for 0.1% (0.003 mg/kg).
The RRR after solvent extraction (4.8% and 4.0% TRR (0.11 mg/kg and 0.09 mg/kg) was treated sequentially with ammonia and various enzymes allowing a solubilization of 2.4% and 1.9 % TRR corresponding to approximately 0.05 mg/kg (per treatment at maximum 1% TRR were solubilized).
In total, for C-label and T-label, identification amounted to 88% and 92% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 93.5% and 94.7% TRR were identified/characterized, leaving the final residue at <2% TRR (<0.05 mg/kg).

3.2 grain

Unchanged parent was not detected with the C-label nor the T-label. The radioactive residue detected with the C-label and with the T-label was significantly different both in quantity as well as in composition.
For the C-label the TRR was only low (0.06 mg/kg), 43.9 % (0.027 mg/kg) could be extracted, most of which was characterized by HPLC (42.4% TRR, 0.026 mg/kg) but not identified at the molecular level. The RRR of 56.1%TRR was further investigated allowing characterization of 41.6% TRR with 17% TRR (0.011 mg/kg) as releasable by ammonia, 24.5 % TRR (0.015 mg/kg) as releasable by amylase/amyloglucosidase treatment. Thus, the final residue was reduced to 0.010 mg/kg (15% TRR).
In total, most of the C-labeled residue in grain (84 % of 0.05 mg/kg) was characterized by solvent extraction or enzyme treatment.
In contrast, with the T-label the TRR was significantly higher (0.62 mg/kg) 78% of which could be extracted by solvents (see above). Two metabolites were present in major amounts, triazole alanine (TA, M750F029) at 45.6% TRR (0.282 mg/kg) and triazole acetic acid (TAA, M750F030) at 21.4% TRR (0.133 mg/kg). In addition, 1,2,4-triazole (M750F001) was identified albeit only at very low amounts (1.0% TRR, 0.006 mg/kg). Most of the RRR with 22.1% TRR (0.14 mg/kg) was further characterized (19.8% TRR, 0.123 mg/kg) with ammonia releasing 15.3% TRR (0.10 mg/kg) and amylase/amyloglucosidase releasing 4.6% (0.03 mg/kg). Thus, the final residue was reduced to 0.012 mg/kg (1.9% TRR).
In total, most of the T-labeled residue in grain was identified (67.9% TRR), considering also amounts characterized by solubilization and/or HLPC an amount of 93.1% TRR were identified/characterized.
The results from both labels taken together, indicate that most of the BAS 750 F residue in grain is carrying only the T-label (TA and TAA approximately 0.4 mg/kg) while the components carrying only the C-label (approximately 0.05 mg/kg) account for only a minor part of the total treatment related residue of BAS 750 F.

3.3 straw

Overall, similar results were observed with both labels. Only, a minor difference is seen resulting from metabolite M750F009/M750F010 (see below). Unchanged parent was with 58.6% and 68.5% TRR (14.3 and 9.6 mg/kg for C- and T-label) the predominant component of the residue (and only component present in major amounts), thus similar to forage. The only other components identified were present in amounts of 7% TRR (1.7 mg/kg) or less.
Notably, for C-label and T-label,

- M750F018/F020 (a fraction including malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for 6.9% TRR (1.68 mg/kg) and 4.3% TRR (0.60 mg/kg)
- M750F019 (glucosyl-O-conjugate of hydroxylated parent) accounted for 5.8% (1.41 mg/kg) and 4.8% (0.67 mg/kg)
- M750F018 (malonylglucosyl-O-conjugate of hydroxylated parent) accounted for 2.9% (0.72 mg/kg) and 5.5% (0.77 mg/kg).
- M750F012 (malonylglucosyl-BAS 750 F) accounted for 5.5% TRR (1.34 mg/kg) and 3.5% TRR (0.48 mg/kg). (Note, that amounts of M750F021, a putative artefact are included).

Label-specific components were detected only in low amounts, namely the cleavage product M750F003 (non-quantifiable amounts), as well its conjugated forms M750F009 (glucosyl-O-conjugate) and M750F010 (malonylglucosyl-O-conjugate), both also at very low amounts (1.3% TRR, 0.18 mg/kg). These metabolites result from cleavage at the ether bridge thus containing the T-label while the C-label is absent.
The RRR after solvent extraction (17.0% and 13.6% TRR (4.2 mg/kg and 1.9 mg/kg) was treated sequentially with ammonia and various enzymes allowing a solubilization of 8.2% and 7.2% TRR corresponding to approximately 2.0 and 1.0 mg/kg (with ammonia 4% TRR were released, 1.3-1.7% TRR with macerozyme/cellulase, less with other enzymes).
In total, for C-label and T-label, identification amounted to 88% and 92% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 85.4% and 91.8% TRR were identified/characterized, leaving the final residue at 7.9% TRR (1.9 mg/kg) and 5.8% TRR (0.8 mg/kg).

4. Enantiomer ratio of BAS 750 F

Chiral analysis of BAS 750 F residue in representative samples of the wheat metabolism study revealed absence of any significant change of BAS 750 F enantiomers. Chiral analysis of forage and straw samples (C-label and T-label) confirm that the racemic mixture (1:1 ratio of S-enantiomer and R-enantiomer) of the application formulation is essentially maintained. Chiral analysis was not conducted for grain since BAS 750 F was not present in quantifiable amounts.

Table 6.2.1-10: Determination of isomer ratio of BAS 750 F in wheat matrices

matrix	S-enantiomer [%]	R-enantiomer [%]
C-label		
application formulation	53.6	46.4
forage	47.5	52.5
straw	48.2	51.8
T-label		
application formulation	48.5	51.5
forage	49.0	51.0
straw	47.8	52.2

Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358)

5. Translocation in plants

After foliar application of BAS 750 F to wheat, the unchanged parent BAS 750 F represents the predominant part of radioactive residues in the directly exposed plant parts forage and straw (> 58 % TRR). In contrast, in wheat grain which had not been present yet during the time of application BAS 750 F was not detected. In conclusion, BAS 750 F is not translocated from treated leaves into the cereal grain.

6. Proposed metabolic pathway

Metabolism of BAS 750 F was investigated in foliar treated wheat using two labels, C-label and T-label. Taken results from both labels together the data show a consistent picture of metabolic pathways in the wheat matrices forage, straw and grain.
The metabolic pathway is shown in Figure 6.2-8. Overall, results show that in wheat the parent BAS 750 F, besides its

- O-conjugation of the unchanged parent (at propyl-triazole moiety) with sugars,

is metabolised by two key transformation reactions:

- C-ring hydroxylation (followed by conjugation)
- T-bridge cleavage of the BAS 750 F backbone (between the TFMP-ring and the T-ring)

BAS 750 F hydroxlation can occur at several positions in the C-ring. Conjugation of the hydroxyl group of the propyl-triazole moiety with sugars leads to metabolites M750F011 (glucosyl-O-conjugate), M750F012 (malonyl-O-conjugate), M750F013 (di-glucosyl-O-conjugate), and M750F014 (malonyl-di-glucosyl-O-conjugate).
Alternatively, conjugation of the C-ring hydroxyl-group leads to metabolites M750F019 (glucosyl-O-conjugate), M750F020 (malonyl-glucosyl-O-cojugate). The C-ring can carry a second hydroxyl-group resulting in metabolite M750F018 (malonyl-glucosyl-O-conjugate).

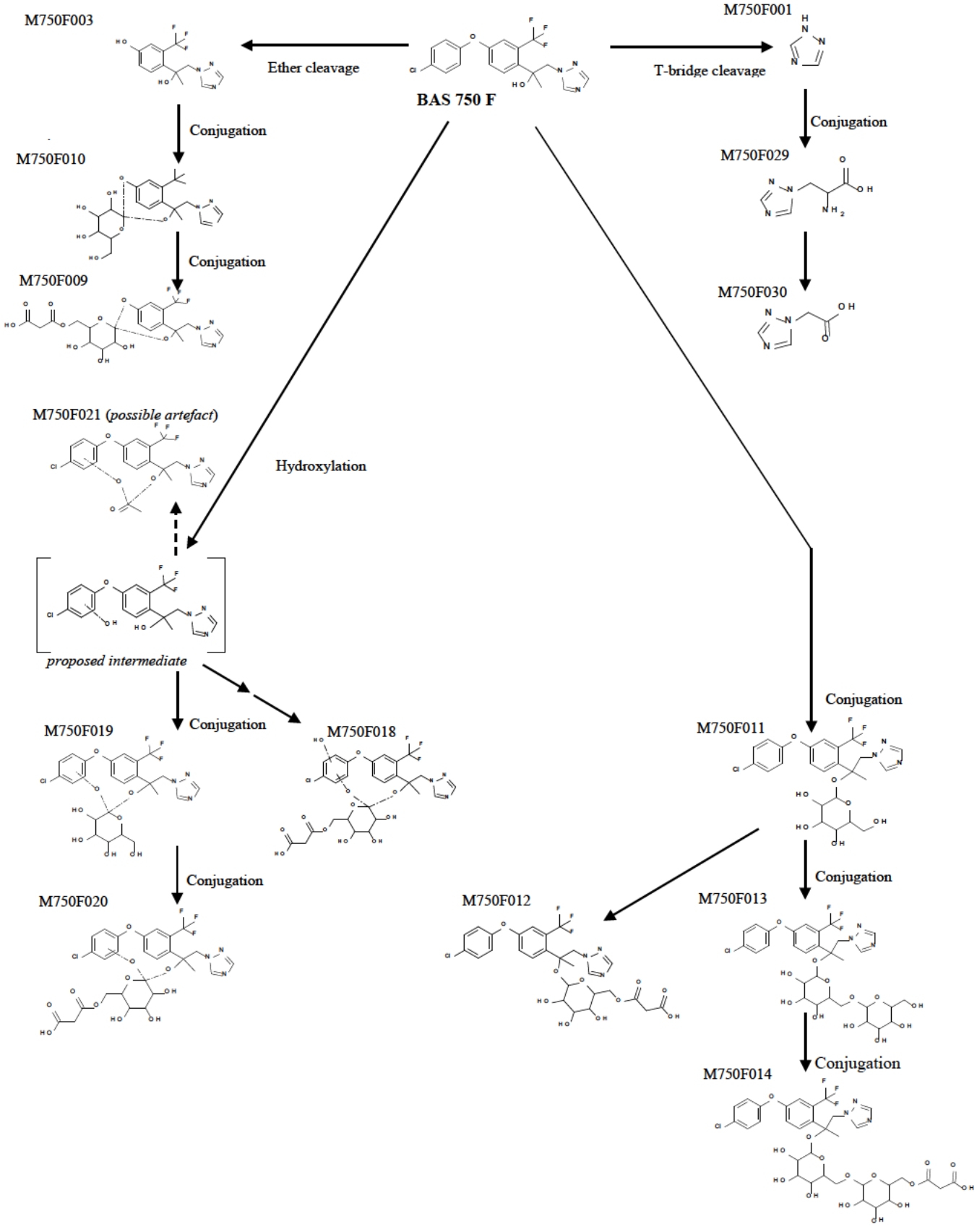
Cleavage of the BAS 750 F backbone generates 1,2,4-triazole (M750F001) which is further conjugated to triazole alanine (TA, M750F029) and triazole acetic acid (TAA, M750F030).

Only to a minor extent cleavage of the backbone occurs at the ether bridge resulting in metabolite M750F003 which is further conjugated to M750F009 (glucosyl-O-conjugate) and M750F010 (malonyl-glucosyl-O-conjugate)

In figure 6.2-8, for some of metabolites generic structures are provided in cases when exact position of hydroxyl group or sugar moiety is not known (indicated by a “dotted” line). The metabolite M750F021 is generated by conjugation of the hydroxylated parent compound with acetate and likely represents an artefact.

In conclusion, the metabolism pathways in foliar treated wheat, representative of the *cereal* crop group, can be considered well elucidated.

Figure 6.2-9: Proposed pathway of BAS 750 F in spring wheat
(note that metabolites M750F003, M750F011, M750F013, and M750F014 were identified but only in non-quantifiable amounts).



7. Extractability of residues according to analytical methods

The extractability of parent BAS 750 F by residue analytical methods is investigated by a separate extractability study, DocID 2014/1261057 (see CA 6.2.1/4). Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F in commodities of plant origin was investigated using samples representative of relevant plant matrices with radiolabeled residue (radiovalidation). Specifically, samples were obtained from three crop metabolism studies, namely wheat samples (see CA 6.2.1/1), soybean samples (see CA 6.2.1/2) and grape samples (see CA 6.2.1/3). Comparison of residue amounts extracted in the metabolism study with the amounts extracted by BASF method 535/1 confirms efficient extraction of BAS 750 F for all matrices investigated, namely forage (wheat), straw (wheat), green pod (soybean) and fruits (grape), amounting to efficiencies of 93% or higher.
The multi-methods also showed good extraction efficiencies in grape and green pod (88% or higher), in forage (80% for QuEChERS) whereas extraction efficiencies were moderate for straw (52-65% for QuEChERS, DFG S 9, and SweEt) and for forage (56-63% for DFG S 9 and SweEt).

8. Storage stability

Storage stability investigations were performed (using both the quantifying and the confirming HPLC method). Taken together the data obtained confirms stability of radioactive residue over the period of the study, both in matrix (prior to extraction) stored deep frozen as well as in extracts stored in refrigerator.

Stability during storage of matrix at ≤-18°C was investigated in detail in green tissue, namely C- and T-labeled forage, by comparing the extractability as well as the resulting metabolic HPLC profiles after extended storage of a plant sample (extraction at two different time points during the study: first extraction was 84/85 days after sampling, second extraction was 623/624 days after sampling, see table below). Samples analyzed were methanol and aqueous extracts of wheat forage.

Stability during storage of extract at ≤-18°C was investigated by comparing the metabolic HPLC profiles after extended storage of the extract (first analysis was after 195 and 36 days (C-label), 12 and 36 days (T-label), second analysis after >540 days of storage (see Table 6.2.1-11). Samples analyzed were methanol and aqueous extracts of wheat forage (both C-labeled and T-labeled samples).

Both for storage of matrix samples and of extract samples, comparison of metabolic HPLC profiles did confirm absence of significant changes. In conclusion, for a period of at least 6 months stability of BAS 750 F residues has been confirmed for both matrix storage and extract storage.

Table 6.2.1-11: Storage intervals of plant samples and extract samples (spring wheat)

	storage of matrix		storage of extract	
matrix	*storage interval (analysis 1)* [1)]	*storage interval (analysis 2)* [1)]	*storage interval (analysis 1)* [2)]	*storage interval (analysis 2)* [2)]
	[days]	*[days]*	*[days]*	*[days]*
C-label				
forage (methanol)	85	624	195	540
forage (water extract)	85	624	36	595
grain (water extract)	183	- [3)]	13	- [3)]
straw (methanol)	43	- [3)]	197	- [3)]
straw (water extract)	43	- [3)]	195	- [3)]
T-label				
forage (methanol)	84	623	12	540
forage (water extract)	84	623	36	595
grain (water extract)	182	- [3)]	62	- [3)]
straw (methanol)	42	- [3)]	197	- [3)]
straw (water extract)	42	- [3)]	195	- [3)]

1) sampling to extraction, 2) extraction to analysis, 3) not analysed

III. CONCLUSION METABOLISM IN <u>WHEAT</u>

The metabolism of BAS 750 F was investigated in wheat by separately applying two labels, C-label and T-label. Data obtained with both labels taken together, shows a consistent picture of the metabolism in foliar applied wheat. Absence of significant cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in wheat. A separate investigation with the TFMP-label would not provide additional information.

For both C- and T-label, similar total radioactivity (TRR) was seen in forage harvested 15 days after the first of two applications (DALA-6) with 2.4 and 2.3 mg/kg, as well as in straw (DALA35) with 24 and 14 mg/kg. In grain, the TRR differed by a factor of 10 between C-label (0.06 mg/kg) and T-label (0.62 mg/kg) correlating with a higher concentration of T-labeled metabolites, namely the triazole derived metabolites (TDM). For both labels, solvent extractability (ERR) was high for forage and straw (at least 83% TRR), as well as T-labeled grain (78% TRR). For C-labeled grain, solvent extraction and enzyme-mediated solubilization retrieved 85% of TRR reducing the final residue to 0.01 mg/kg (15% TRR). Overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring) which introduces a second hydroxyl group which, alike the hydroxy group of the parent, is the site for conjugation leading to an array of sugar conjugates. Cleavage of BAS 750 F at the T-bridge leads to 1,2,4-triazole (TA), triazole alanine (TA) and triazole acetic acid (TAA). These metabolites are part of the group of triazole derivative metabolites (TDM) which are common to a range of azole fungicides. Cleavage at the ether bridge does occur, albeit only at minor amounts leading to the metabolites M750F009 and M750F010 (incidental occurrence only in straw, at 1% TRR). The parent BAS 750 F is present in wheat matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation or translocation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in forage and straw representing at least 80% TRR in forage and at least 59% TRR in straw. A higher proportion of sugar conjugates is observed in straw (BBCH89) with <18% TRR compared with forage (BBCH61) with 3-4% TRR. In contrast, unchanged parent is not detected in grain, where TDM account almost exclusively for the radioactive residue (accounting for 77% TRR). Note that due to the cultivation of the wheat plants in plastic boxes with limited drainage, the accessibility of small polar substances might be enhanced (notably radioactive TDMs generated in the soil). Translocation of TDM into grain is reflected by the fact that TRR level in T-labeled grain are significantly exceeding the TRR in C-labeled grain.

In conclusion, the major components of the residue in wheat were identified as unchanged parent BAS 750 F and the common metabolites TDM which together are representing a large proportion of the residue (>80% in forage, >77% in grain, >50% in straw).

Other components of the residue were sugar conjugates of parent (unchanged or hydroxylated) individually present <6% TRR as well as minor amounts of two metabolites resulting from ether cleavage (individually present at 1.3% TRR). Overall, metabolism of BAS 750 F in wheat, and by extrapolation, in the *cereal* crop group is considered well-elucidated.

Report:	CA 6.2.1/2 Thiaener J.,Bogen C., 2015 a Metabolism of 14C-BAS 750 F in soybean 2014/1224012
Guidelines:	EPA 860.1000, EPA 860.1300, EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, Test No. 501: Metabolism in crops
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in soybean after foliar application of BAS 750 F labeled either in the chlorophenyl-ring (C-ring) or in the triazole-ring (T-ring). Both labels were applied separately. Foliar spray applications of soybean plants cultivated in indoors (plastic containers located in vegetation hall/greenhouse/pythotron) were carried out three times at a target rate of 125 g a.s./ha. Samples of forage (growth stage BBCH 71-72) were taken 19 days after the first application (corresponding to 17 days prior to the last/third application). Samples of seed, hulls and the remaining plant (rest-of-plant) at BBCH89 were collected 47-48 days after the third (last) application. Samples were stored in a freezer at -18°C or below.

Data obtained with C-label and T-label taken together, show a consistent picture of the metabolism in foliar applied soybean. Absence of cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in soybean.

For both C- and T-label, similar total radioactivity (TRR) was seen in forage (6.6, 4.6 mg/kg), in rest-of-plant (16.4, 19.2 mg/kg), and in hulls (3.8, 4.1 mg/kg). In seed, the TRR differed by a factor < 20X between C-label (0.13 mg/kg) and T-label (3.06 mg/kg) correlating with a higher concentration of T-labeled metabolites, namely the triazole derivative metabolites (TDM).

For both labels, solvent extractability (ERR) was high for forage, rest-of-plant (>87% TRR) and hull (at least 68% TRR), as well as T-labeled seed (76% TRR). For C-labeled seed, solvent extraction followed by enzyme-mediated solubilization retrieved 95% of TRR reducing the final residue to 0.007 mg/kg (5% TRR). Thus, overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring), which introduces a second hydroxyl group. This is, alike the hydroxyl group of the parent, the site for conjugation leading to an array of sugar conjugates. Cleavage of BAS 750 F at the T-bridge leads to 1,2,4-triazole (TA), triazole alanine (TA) and triazole lactic acid (TLA). These metabolites are part of the group of triazole derivative metabolites (TDM) which are common to a range of azole fungicides. The parent BAS 750 F is present in soybean matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation or translocation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in forage, rest-of-plant, and hull representing at least 60% TRR.

In contrast, only low amounts (0.4 and 4.0% TRR) unchanged parent are detected in seed where TDM account for a large proportion of the residue (detectable only in T-labeled seed with TA at 48% TRR, TLA at 1.3% TRR, 1,2,4-triazole at 0.3 % TRR). Note that due to the cultivation of the soybean plants in plastic boxes with limited drainage, the accessibility of small polar substances might be enhanced (notably radioactive TDMs generated in the soil). Translocation of TDM into seed is reflected by the fact that TRR level in T-labeled seed are significantly (> 20X) exceeding the TRR in C-labeled grain.
In conclusion, the major components of the residue in soybean were identified as unchanged parent BAS 750 F and the common metabolites TDM which together are representing a large proportion of the residue (>79% in forage and hull, >59% in rest-of-plant, and 50% in T-labeled seed). Other components of the residue were sugar conjugates of parent (unchanged or hydroxylated) individually present < 5% TRR.

Overall, metabolism of BAS 750 F in soybean, and by extrapolation, in the *pulses and oilseeds* crop group is considered well-elucidated.

MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

Description:	BAS 750 F **C-label:** Chlorophenyl-U-C14-BAS 750 F, Chlorophenyl-1-C13-BAS 750 F, **T-label:** Triazole-3(5)-C14-BAS 750 F, Triazole-3(5)-C13-BAS 750 F
Lot/Batch No:	CFQ41561 (Chlorophenyl-U-C14-BAS 750 F, 7.878 MBq/mg) RS4-2012-173A2 (Chlorophenyl-1-C13-BAS 750 F), 1062-2001 (Triazole-3(5)-C14-BAS 750 F, 5.46 MBq/mg) 1077-1001 (Triazole-3(5)-C13-BAS 750 F)
Purity:	Chlorophenyl-U-C14-BAS 750 F: 99.1% (radiochemical 98.9%) Chlorophenyl-1-C13-BAS 750 F: 97.7% Triazole-3(5)-C14-BAS 750 F: 98.9% (radiochemical 98.8%) Triazole-3(5)-C13-BAS 750 F: 97.1%
CAS#:	1417782-03-6 (chlorophenyl labeled-BAS 750 F, triazole-labeled BAS 750 F)
Development code:	BAS 750 F
Spiking levels:	*not applicable*

2. Test Commodity:

Crop:	soybean	
Type:	oilseeds	
Variety:	Sultana	
Botanical name:	G*lycine max*	
Crop parts(s) or processed Commodity:	soya bean (forage, seed, hull, rest-of-plant, green pod)	
Sample size:	forage	(10 plants, 0.14-0.28 kg),
	seed	(0.066-0.155 kg),
	hull	(0.032-0.070 kg),
	rest-of-plant	(3.5 kg),
	green pod	(0.052-0.086 kg)

3. Soil: sandy loam (for soil physicochemical properties see Table 6.2.1-12).

Table 6.2.1-12: Soil physicochemical properties

soil series	soil type [1)]	pH	OM [2)]	sand (%)	silt (%)	clay (%)	moisture at 1/3 bar (%)	CEC cmol/kg
Bruch West	sandy loam	8.0	2.53	70.4	18.6	11.0	N/A [3)]	9.5

1) USDA scheme, 2) organic matter, corresponds to the total organic carbon (TOC), 3) not applicable

B. STUDY DESIGN AND METHODS

1. Test procedure

The study was conducted at the Agricultural Research Center of BASF in Limburgerhof, Germany (2013-2015). Soybean plants were cultivated in containers (0.4 m x 0.6 m, 26 seeds/container) with loamy sand soil. Containers were initially located in a vegetation hall with glass roof, after label application transferred to climatic chambers (phytotrons). Ten containers each were used per label. The maintenance of the plants was performed in accordance with normal agricultural practice. Prior to the first treatment (33 and 34 days before), the number of plants per container was reduced to 13 plants.

For each test item, BAS 750 F labelled in the chlorophenyl-moiety (C-label) or BAS 750 F labeled in the triazole-moiety (T-label), a separate application formulation was prepared for application by automatic spray track. Each C14-labeled test item was mixed with C13-labeled test item (1:1 ratio of chlorophenyl-U-C14 label and chlorophenyl-1-C13-label, 2:1 ratio of triazole-3(5)-C14- and triazole-3(5)-C13-label). For the first application, tank mixes of the test items (taken up in blank formulation and water) were prepared the preceding day. Aliquots of the application formulations were taken for confirmation of identity, for purity check (by radio-HPLC) as well as for determination of the C14:C13 isotope ratio (by mass spectrometry). For the second and third application formulations were prepared as for the first application, purity was confirmed by radio-HPLC.

Both labels were applied separately (see Table 6.2.1-13). Foliar spray applications were carried out three times either with the chlorophenyl-labeled BAS 750 F (C-label) or the triazole-labeled BAS 750 F (T-label) at a target rate of 125 g a.s./ha (BBCH60, BBCH72 and BBCH77). Samples of forage (having received only one application) were taken 19 days after the first application, directly prior to the second application at growth stage BBCH71-72. Samples of seed, hull, green pod and rest-of-plant were collected at harvest growth stage (BBCH89), which was at 47 - 48 days after the last application. Samples were stored in a freezer at -18°C or below.

Table 6.2.1-13: Study design: plant uptake part (soybean)

label	C-label		T-label	
intended use rate [g a.s./ha]	125		125	
application number	3		3	
application interval [days]	18±1		18±1	
application growth stages	BBCH60/BBCH72/BBCH77		BBCH60/BBCH72/BBCH77	
sampled matrices	forage, seed, hull, rest-of-plant, green pod		forage, seed, hull, rest-of-plant, green pod	
sampling timepoints [DALA] [1)]	forage	-17 (=19 DAT) [2)]	forage	-17 (=19 DAT) [2)]
	seed	47	seed	48
	hull	47	hull	48
	rest-of-plant	47	rest-of-plant	48
	green pod	47	green pod	48

[1)] days after last application, [2)] 19 days after the first application (19 DAT) corresponding to directly prior to the second application, and 17 days prior to last application (-17 DALA).

2. Description of analytical procedures

Radioanalysis: For the determination of the TRR and the measurement of solid residues after solvent extraction or solubilization procedures, homogenized subsamples were combusted using a sample oxidizer. The resultant ^{14}C-CO_2 was absorbed, mixed with a scintillation fluid and the radioactivity was determined by liquid scintillation counting (LSC). For liquid samples, aliquots were mixed with a sufficient volume of a scintillation fluid and the radioactivity was determined by LSC.

Homogenization/solvent extraction: Plant samples (forage, rest-of-plant, hull, green pod and seed) were homogenized. Prior to extraction, soya bean forage and rest-of-plant were soaked in water for one hour. Forage, hull and rest-of-plant were extracted three times with methanol and two times with water using a Polytron blender. After each extraction step, the solid material was separated from the extract by centrifugation and filtration. The filtered supernatants of the methanol and water extracts were each combined and adjusted to a defined volume. The methanol extracts were purified by SPE fractionation.

In contrast, seed and green pod were extracted three times with a mixture of acetonitrile and isohexane (1:1). After a centrifugation step, the acetonitrile and isohexane phase were separated using a separatory funnel. Acetonitrile/isohexane extraction of these samples (seed, green pod) was followed by extraction with water (two times). The solid material was separated from the extract by centrifugation and filtration. The residue after solvent extraction was dried in a fume hood, homogenized and radioassayed. The filtered supernatants were combined and adjusted to defined volumes.

Solubilization of the RRR: The post-extraction residue (forage, rest-of-plant, hull and seed of both labels) was re-suspended, incubated in the presence of macerozyme and cellulase (approximately 72 h, except hull 36 h). After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume (for rest-of-plant, forage and seed, an additional purification by SPE fractionation was included).
The resulting residue was re-suspended and incubated in the presence of β-glucosidase and hesperidinase (1:1 ratio, approximately 24 h), then centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue was re-suspended and incubated in the presence of α-amylase, β-amylase and amyloglucosidase (1:1:1 ratio, approximately 24 h or longer), then centrifuged and filtered and adjusted to a defined volume.
The resulting residue was re-suspended. Suitable amounts of laccase and tyrosinase were added and the mixture was incubated for approximately 24 hours, thereafter centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue was re-suspended and incubated in the presence of protease. After overnight incubation, acetonitrile was added. The sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume. The final residue was dried.

3. Identification and characterization of the residue

Components of the residue were identified or characterized by HPLC-MS, co-chromatography and comparison of retention times. In addition, for the parent BAS 750 F enantiomer-specific HPLC analyses were performed in samples of the application solution, as well as extracts of forage, rest-of-plant and hull (samples purified by flash chromatography).

II. RESULTS AND DISCUSSION

1. TOTAL RADIOACTIVE RESIDUE (TRR)

Following foliar application of BAS 750 F to soybean at a rate of three times 125 g as/ha, the total 14C residue (TRR) was measured in soybean forage (BBCH71-72), seed, hull, rest-of-plant (all BBCH89) as well as green pod and ripe seed (see Table 6.2.1-14).

The calculated total radioactive residues (TRR) with the C-label were highest in rest-of-plant (DALA 47) with 16.46 mg/kg, lower in forage (sampled between first and second application, 6.58 mg/kg), green pod (8.72 mg/kg) and hull (3.84 mg/kg), and much lower in seed (0.129 mg/kg).

A similar distribution was seen with the T-label (TRR highest in rest-of-plant, 19.3 mg/kg, lower in forage at 4.61 mg/kg, and hull at 4.12 mg/kg). Notably the TRR in seed (3.06 mg/kg) as well as their immature stage, green pod (16.00 mg/kg), was significantly higher with the T-label compared with the C-label, indicating a difference in composition of the radio-detected residue (see below, part 3).

Table 6.2.1-14: Total radioactive residue after foliar spray application of BAS 750 F

matrix [BBCH]	**DALA** [1)]	**TRR measured** *[mg/kg]*	**TRR calculated** *[mg/kg]* [2)]
C-label			
forage [71-72]	-17 (=19 DAT) [3)]	6.516	6.575
rest-of-plant [89]	47	16.016	16.459
hull [89]	47	3.735	3.838
green pod	47	8.857	8.721
seed [89]	47	0.109	0.129
T-label			
forage [71-72]	-17 (=19 DAT) [3)]	4.416	4.609
rest-of-plant [89]	48	19.934	19.264
hull [89]	48	3.890	4.122
green pod	48	16.005	16.006
seed [89]	48	2.592	3.063

[1)] days after last application, [2)] TRR calculated as the sum of ERR and RRR, [3)] 19 days after the first application (=19DAT) corresponding to 17 days prior to last application (=-17DALA).

2. EXTRACTABILITY

The extractabilities of ^{14}C residues from soya bean forage, plant, straw, and grain are summarized in Table Table 6.2.1-15 and Table Table 6.2.1-16. The extractability was moderate to high depending on the plant matrix.

High extractability with both labels (C-label and T-label) was observed for forage (91.1% and 93.3% TRR), for rest-of-plant (87.1% and 87.8% TRR), for green pod (83.4% and 78.0% TRR) and hulls (68.7% and 74.2% TRR). The resulting RRR was for forage 8.9% and 6.7% TRR (0.59 and 0.31 mg/kg), for rest-of-plant 12.9% and 12.2% TRR (2.13 and 2.34 mg/kg) and for hull 31.3% and 25.8% TRR (1.20 and 1.06 mg/kg). Thus, RRR was further investigated (see below).

Moderate extractability and label-specific differences were observed for seeds (56.6% TRR for C-label, 75.6% TRR for T-label).

In forage, rest-of-plant and hull, extracted with methanol and water, the predominant amount of residue was extracted with methanol. Subsequent water extraction did account only <5% TRR (see Table 6.2.1-15). Also, in green pod, extracted with acetonitrile and isohexane, subsequent water extraction did release only an additional amount of <10% TRR (see Table 6.2.1-16).

In contrast, seed extraction with acetonitrile and isohexane did account for only 21.4% TRR (C-label) and 1.4% TRR (T-label), while the predominant amount was released by subsequent water extraction (C-label: 35.2% TRR, T-label: 74.2% TRR).
The RRR in seed amounted to 43.4% and 24.4% TRR (0.056 and 0.747 mg/kg) and thus was subject to further investigation. Similarly, in the immature stage, green pod, solvent extraction left amounts of 16.6% and 22.0% TRR (1.45 and 3.52 mg/kg) in the RRR.

Table 6.2.1-15: Extractability of radioactive residue from forage, rest-of-plant, hull

matrix	DALA[1)]	TRR [2)]	distribution of radioactive residues							
			methanol extracts [3)]		water extracts [3)]		ERR [2)]		RRR [2)]	
		mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
C-label										
forage	-17 (=19 DAT)	*6.575*	**89.7**	*5.898*	**1.4**	*0.090*	**91.1**	*5.988*	**8.9**	*0.587*
plant [4)]	47	*16.46*	**83.4**	*13.73*	**3.7**	*0.601*	**87.1**	*14.33*	**12.9**	*2.126*
hull	47	*3.838*	**66.7**	*2.558*	**2.0**	*0.078*	**68.7**	*2.637*	**31.3**	*1.201*
T-label										
forage	-17 (=19 DAT)	*4.609*	**92.2**	*4.249*	**1.2**	*0.054*	**93.3**	*4.302*	**6.7**	*0.307*
plant [4)]	47	*19.26*	**83.9**	*16.17*	**3.9**	*0.757*	**87.8**	*16.92*	**12.2**	*2.342*
hull	47	*4.122*	**70.4**	*2.903*	**3.8**	*0.156*	**74.2**	*3.059*	**25.8**	*1.063*

[1)] days after last application, for forage sampling was 19 days after the first application (=19DAT) which was 17 days prior to last application (= -17DALA).[2)] TRR was calculated as the sum of ERR and RRR with. ERR= Extractable Radioactive Residue, RRR=Residual Radioactive Residue (after solvent extraction), [3)] pool of combined repetitive extracts, [4)] rest-of-plant (pods removed)

Table 6.2.1-16: Extractability of radioactive residue from seed and green pod

matrix	DALA[1)]	TRR [2)]	distribution of radioactive residues									
			acetonitrile phase		isohexane phase		water extracts [3)]		ERR [2)]		RRR [2)]	
		mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
C-label												
seed	47	*0.129*	**4.0**	*0.005*	**17.4**	*0.022*	**35.2**	*0.045*	**56.6**	*0.073*	**43.4**	*0.056*
green pod	47	*8.721*	**74.6**	*6.503*	**1.2**	*0.108*	**7.6**	*0.660*	**83.4**	*7.271*	**16.6**	*1.451*
T-label												
seed	48	*3.063*	**0.6**	*0.019*	**0.8**	*0.025*	**74.2**	*2.272*	**75.6**	*2.316*	**24.4**	*0.747*
green pod	48	*16.01*	**67.4**	*10.78*	**0.9**	*0.141*	**9.8**	*1.564*	**78.0**	*12.49*	**22.0**	*3.518*

[1)] days after last application, for forage sampling was 19 days after the first application (=19DAT) which was 17 days prior to last application (= -17DALA). [2)] TRR was calculated as the sum of ERR and RRR with. ERR= Extractable Radioactive Residue, RRR=Residual Radioactive Residue (after solvent extraction), [3)] pool of combined repetitive extracts

3. EXTRACTION, CHARACTERISATION AND IDENTIFICATION OF RESIDUES

An overview over the components of the extractable residue as well as of the residue released from RRR by enzyme treatment is given below in Table 6.2.1-17 (C-label) and Table 6.2.1-18 (T-label). Note on compounds identified, that M750F012 eluted in two distinct peaks, the first eluting at 25.6 min and the second eluting at 26.9 min. One peak contains two compounds (M750F018, M750F020). "Region 1" includes three peaks and several metabolites, namely M750F011/M750F018 (20.3), M750F019 (20.9), M750F012 (21.7), M750F019 (21.5), M750F019 (21.9). "Region 2" includes two peaks with several metabolites: M750F020 (23.0), and M750F013/M750F014 (23.9,24.1). "Peak 29.8" has a retention time characteristic for metabolite M750F021 (a presumed conjugation artefact). The metabolite present in the "6.6 min peak" was characterized as a triazole-like compound, either an (amino) acid conjugated triazole or a polypeptide with triazole alanine-like elution properties. Identification of the molecular structure was not achieved despite intense efforts.

Table 6.2.1-17: Summary of identified/characterized components in soybean (C-label)

C-labeled radioactive component (min) [4] ERR & RRR	**forage**		**rest-of-plant**		**hull**		**green pod**		**seed**	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
TRR	**100.0**	*6.58*	**100.0**	*16.46*	**100.0**	*3.84*	**100.0**	*8.72*	**100.0**	*0.129*
BAS 750 F	**79.9**	*5.257*	**59.8**	*9.848*	**82.8**	*3.179*	**68.5**	*5.978*	**4.0**	*0.005*
M750F018/M750F020	**1.9**	*0.123*	**4.5**	*0.748*	< **0.1**	*0.001*	**3.9**	*0.338*	-	-
M750F012 (25.6)	**3.9**	*0.256*	**4.0**	*0.651*	**0.2**	*0.008*	**2.2**	*0.188*	-	-
M750F012 (26.9)	< **0.1**	*0.003*	**2.0**	*0.321*	**0.2**	*0.006*	-	-	-	-
"region 1"	**0.3**	*0.023*	**4.1**	*0.678*	**0.5**	*0.020*	-	-	-	-
"region 2"	-	-	**5.0**	*0.830*	**0.3**	*0.012*	-	-	-	-
"peak 29.8"	**0.9**	*0.060*	**2.5**	*0.415*	**2.2**	*0.085*	-	-	-	-
"peak 33.9"	**0.5**	*0.032*	**2.3**	*0.379*	-	-	-	-	-	-
ERR	**91.1**	*5.988*	**87.1**	*14.333*	**68.7**	*2.637*	**83.4**	*7.271*	**56.6**	*0.073*
ID [1]	**82.9**	*5.454*	**64.0**	*10.531*	**67.4**	*2.587*	**74.6**	*6.503*	**4.0**	*0.005*
CHAR [1]	**1.8**	*0.116*	**14.7**	*2.425*	**1.3**	*0.051*	**8.8**	*0.767*	**48.5**	*0.063*
sum ID/CHAR	**84.7**	*5.570*	**78.7**	*12.955*	**68.7**	*2.638*	**83.4**	*7.271*	**52.5**	*0.068*
RRR	**8.9**	*0.587*	**12.9**	*2.126*	**31.3**	*1.201*	**16.6**	*1.451*	**43.4**	*0.056*
ID [1]	**2.8**	*0.185*	**6.3**	*1.037*	**15.8**	*0.607*	-	-	-	-
CHAR [1]	**0.5**	*0.034*	**1.6**	*0.259*	**3.0**	*0.115*	-	-	**32.1**	*0.041*
sum ID/CHAR	**3.3**	*0.218*	**7.9**	*1.296*	**18.8**	*0.722*	-	-	**32.1**	*0.041*
SUM ID/CHAR in ERR/RRR [1]	**88.0**	*5.789*	**86.6**	*14.252*	**87.5**	*3.359*	**83.4**	*7.271*	**85.5**	*0.110*
Final residue [2]	**4.8**	*0.316*	**5.8**	*0.954*	**11.0**	*0.420*	**16.6**	*1.451*	**5.2**	*0.007*
Grand Total [3]	**92.8**	***6.104***	**92.4**	***15.206***	**98.5**	***3.780***	***100.0***	***8.721***	**90.7**	***0.117***

[1] ID=amount identified, CHAR=amount characterized, sum ID/CHAR= sum of amounts identified and/or characterized, sum ID/CHAR in ERR/RRR= sum of amounts identified and/or characterized in ERR or in RRR
[2] final residue after solvent extraction and solubilization, [3] Grand Total=sum of amounts characterized and identified as well as final residue, [4] Retention times are provided in parenthesis.

Table 6.2.1-18: Summary of identified/characterized components in soybean (T-label)

T-labeled radioactive component (min) [4] ERR & RRR	forage		rest-of-plant		hull		green pod		seed	
	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg
TRR	**100.0**	*4.61*	**100.0**	*19.26*	**100.0**	*4.12*	*100.0*	*16.01*	**100.0**	*3.06*
BAS 750 F	**79.1**	*3.647*	**71.1**	*13.697*	**79.0**	*3.257*	-	-	**0.4**	*0.013*
M750F001 (1,2,4-T)	-	-	-	-	-	-	-	-	**0.3**	*0.008*
M750F029 (TA)	-	-	-	-	-	-	-	-	**47.7**	*1.461*
M750F031 (TLA)	-	-	-	-	-	-	-	-	**1.3**	*0.040*
"peak 6.6 min"	-	-	-	-	-	-	-	-	**33.6**	*1.029*
"peak 9.1 min"	-	-	-	-	-	-	-	-	**0.3**	*0.008*
M750F018/M750F020	**2.1**	*0.096*	**3.8**	*0.735*	-	-	-	-	-	-
M750F012 (25.6)	**3.1**	*0.141*	**2.9**	*0.554*	**0.7**	*0.030*	-	-	-	-
M750F012 (26.9)	**0.5**	*0.022*	**0.2**	*0.043*	**0.5**	*0.022*	-	-	-	-
"region 1"	-	-	**2.7**	*0.513*	-	-	-	-	-	-
"region 2"	-	-	**2.4**	*0.455*	-	-	-	-	-	-
"peak 29.8 min"	**0.7**	*0.034*	**2.6**	*0.500*	**1.3**	*0.055*	-	-	-	-
"peak 33.9 min"	**0.7**	*0.032*	**1.1**	*0.220*	-	-	-	-	-	-
ERR	**93.3**	*4.302*	**87.8**	*16.922*	**74.2**	*3.059*	-	-	**75.6**	*2.316*
ID [1]	**82.2**	*3.788*	**72.7**	*14.003*	**70.2**	*2.893*	-	-	**33.0**	*1.012*
CHAR [1]	**2.3**	*0.106*	**9.2**	*1.781*	**3.5**	*0.145*	-	-	**35.7**	*1.095*
sum ID/CHAR[1]	**84.5**	*3.894*	**81.9**	*15.783*	**73.7**	*3.038*	-	-	**68.8**	*2.107*
RRR	**6.7**	*0.307*	**12.2**	*2.342*	**25.8**	*1.063*	-	-	**24.4**	*0.747*
ID [1]	**2.6**	*0.118*	**5.3**	*1.026*	**10.1**	*0.417*	-	-	**16.7**	*0.510*
CHAR [1]	**0.2**	*0.007*	**1.3**	*0.251*	**7.9**	*0.326*	-	-	**8.3**	*0.254*
sum ID/CHAR[1]	**2.8**	*0.126*	**6.6**	*1.277*	**18.0**	*0.743*	-	-	**25.0**	*0.764*
SUM ID/CHAR in ERR/RRR[1]	**87.2**	*4.019*	**88.6**	*17.06*	**91.7**	*3.871*	-	-	**93.7**	*2.871*
Final residue [2]	**3.5**	*0.160*	**5.7**	*1.096*	**7.5**	*0.309*	-	-	**0.2**	*0.005*
Grand Total [3]	**90.7**	*4.179*	**94.3**	*18.157*	**99.2**	*4.090*	-	-	**93.9**	*2.877*

[1] ID=amount identified, CHAR=amount characterized, sum ID/CHAR= sum of amounts identified and/or characterized, sum ID/CHAR in ERR/RRR= sum of amounts identified and/or characterized in ERR or in RRR
[2] final residue after solvent extraction and solubilization, [3] Grand Total=sum of amounts characterized and identified as well as final residue, [4] Retention times are provided in parenthesis.

Table 6.2.1-19: Characterization of RRR by enzyme treatment (C-label)

C-labeled radioactive component [4]		forage		rest-of-plant		hull		seed	
		% TRR	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
RRR		**8.9**	*0.587*	**12.9**	*2.126*	**31.3**	*1.201*	**43.4**	*0.056*
CHAR enzyme [5] treatment	M/C	**1.1**	*0.075*	**2.1**	*0.352*	**9.0**	*0.346*	**15.5**	*0.020*
	G/H	**0.5**	*0.032*	**2.5**	*0.407*	**3.6**	*0.137*	**5.0**	*0.006*
	AM/G	**0.4**	*0.026*	**1.0**	*0.170*	**2.8**	*0.106*	**4.0**	*0.005*
	T/L	**0.3**	*0.023*	**0.7**	*0.113*	**1.5**	*0.059*	-	-
	protease	**1.2**	*0.076*	**2.0**	*0.332*	**1.9**	*0.074*	**8.3**	*0.011*
	solubilized (sum)	**3.5**	*0.232*	**8.3**	*1.374*	**18.8**	*0.722*	**32.8**	*0.049*
Final residue		**4.8**	*0.316*	**5.8**	*0.954*	**11.0**	*0.420*	**5.2**	*0.007*
RRR		**8.9**	*0.587*	**12.9**	*2.126*	**31.3**	*1.201*	**43.4**	*0.056*
CHAR[1] (HPLC)	"peak 29.8"	**0.1**	*0.008*	**0.5**	*0.088*	**2.0**	*0.078*	-	-
	"peak 33.9"	**< 0.1**	*0.003*	**0.2**	*0.039*	-	-	-	-
	other	**0.3**	*0.022*	**0.8**	*0.132*	**0.9**	*0.036*	**32.1**	*0.041*
	characterized (sum)[3]	**0.5**	*0.034*	**1.6**	*0.259*	**3.0**	*0.115*	**32.1**	*0.041*
ID [1]	BAS 750 F	**2.7**	*0.179*	**5.8**	*0.962*	**15.8**	*0.607*	-	-
	M750F018/-F020	**< 0.1**	*<0.001*	-	-	-	-	-	-
	M750F012	**1.1**	*0.006*	**0.5**	*0.075*	-	-	-	-
	identified (sum)[3]	**2.8**	*0.185*	**6.3**	*1.037*	**15.8**	*0.607*	-	-
	ID/CHAR (sum)[3]	**3.3**	*0.218*	**7.9**	*1.296*	**18.8**	*0.722*	**32.1**	*0.041*
Final residue [2]		**4.8**	*0.316*	**5.8**	*0.954*	**11.0**	*0.420*	**5.2**	*0.007*

[1] ID=amount identified, CHAR(HPLC) =amount characterized by HPLC (information on number and quantities of peaks provided in study report), [2] final residue after solvent extraction and solubilization, [3] sum of RRR amounts characterized and identified or both, [4] Retention times are provided in parenthesis, one peak did contain two componds (M750F018 and M750F020), [5] Solubilization included sequential treatments with macerozyme/cellulase (M/C), with glucosidase/hersperinidase (G/H), with amylase /amyloglucosidase (AM/G), with tyrosinase/laccase (T/L), and finally with protease. The protease solubilizate was further characterized by SPE fractionation (individual fractions were below or equal to 0.004 mg/kg or 3.4 % TRR).

Table 6.2.1-20: Characterization of RRR by enzyme treatment (T-label)

T-labeled radioactive component [4]		forage		rest-of-plant		hull		seed	
		% TRR	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*	*% TRR*	*mg/kg*
RRR		**6.7**	*0.307*	**12.2**	*2.342*	**25.8**	*1.063*	**24.4**	*0.747*
CHAR enzyme [5] treatment	M/C	**0.7**	*0.033*	**1.8**	*0.340*	**11.0**	*0.452*	**23.1**	*0.706*
	G/H	**0.5**	*0.023*	**1.4**	*0.264*	**2.7**	*0.109*	**3.0**	*0.093*
	AM/G	**0.3**	*0.016*	**0.8**	*0.148*	**2.6**	*0.108*	**0.5**	*0.016*
	T/L	**0.4**	*0.016*	**0.7**	*0.141*	**1.0**	*0.042*	-	-
	protease	**1.0**	*0.044*	**2.3**	*0.434*	**0.8**	*0.032*	**0.3**	*0.009*
	solubilized (sum)[3]	**2.9**	*0.133*	**6.9**	*1.328*	**18.0**	*0.743*	**26.9**	*0.824*
Final residue [2]		**3.5**	*0.16*	**5.7**	*1.096*	**7.5**	*0.309*	**0.2**	*0.005*
RRR		**6.7**	*0.307*	**12.2**	*2.342*	**25.8**	*1.063*	**24.4**	*0.747*
CHAR[1] (HPLC)	"peak 29.8"	**0.1**	*0.003*	**0.6**	*0.119*	**1.3**	*0.055*	-	-
	"peak 33.9"	-	-	**0.2**	*0.03*	-	-	-	-
	other	**0.1**	*0.005*	**0.6**	*0.101*	**6.6**	*0.271*	**8.3**	*0.254*
	total characterized	**0.2**	*0.008*	**1.3**	*0.251*	**7.9**	*0.326*	**8.3**	*0.254*
ID[1]	BAS 750 F	**2.5**	*0.117*	**5.1**	*0.991*	**8.8**	*0.364*	-	-
	M750F018/-F020	-	-	-	-	-	-	-	-
	M750F012	**< 0.1**	*0.001*	**0.2**	*0.035*	**1.2**	*0.052*	-	-
	M750F029	-	-	-	-	-	-	**16.7**	*0.510*
	identified (sum)[3]	**2.6**	*0.118*	**5.3**	*1.026*	**10.1**	*0.417*	-	-
	ID/CHAR (sum)[3]	**2.7**	*0.126*	**6.6**	*1.277*	**18.8**	**0.743**	**25.0**	*0.764*
Final residue [2]		**3.5**	*0.16*	**5.7**	*1.096*	**7.5**	*0.309*	**0.2**	*0.005*

[1] ID=amount identified, CHAR(HPLC) =amount characterized by HPLC (information on number and quantities of peaks provided in study report), [2] final residue after solvent extraction and solubilization, [3] sum of RRR amounts characterized and identified or both, [4] Retention times are provided in parenthesis, one peak did contain two componds (M750F018 and M750F020), [5] Solubilization included sequential treatments with macerozyme/cellulase (M/C), with glucosidase/hersperinidase (G/H), with amylase /amyloglucosidase (AM/G), with tyrosinase/laccase (T/L), and finally with protease. The protease solubilizate was further characterized by SPE fractionation (individual fractions were below or equal to 0.004 mg/kg or 3.4 % TRR).

3.1 forage

Overall, similar results were observed with both labels. Unchanged parent was with >79% TRR (5.3 and 3.6 mg/kg for C- and T-label) the predominant component of the residue (and only component present in major amounts), thus similar to rest-of-plant and hull. The only other components identified were present in amounts of 4% TRR (0.26 mg/kg) or less and were structurally related to the parent (sugar conjugates of the parent molecule and of a hydroxylated parent molecule).
Noteably, for C-label and T-label, M750F012 (malonyl-glucosyl-O-conjugate of BAS 750 F) accounted for 3.9% and 3.6% TRR (0.26 mg/kg and 0.16 mg/kg), M750F018/M750F020 (malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for 1.9% and 2.1% TRR (0.12 mg/kg and 0.10 mg/kg). In addition, several fractions of <1% TRR (<0.06 mg/kg) represented by peaks in HPLC chromatograms were observed.
The RRR after solvent extraction (8.9% and 6.7% TRR (0.59 mg/kg and 0.31 mg/kg) was treated sequentially with various enzymes allowing a solubilization of 3.5% and 2.9% TRR corresponding to 0.23 mg/kg and 0.13 mg/kg (protease treatment solubilized 1.2 and 1.0% TRR, macerozyme/cellulase solubilized 1.1 and 0.7% TRR corresponding to amounts of 0.03 to 0.08 mg/kg). The solubilized residue consisted mainly of the components identified in the ERR, thus BAS 750 F and minor amounts of metabolite M750F018, M750F020 and M750F012.
In total, for C-label and T-label, identification amounted to 86% and 85% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 88% and 87% TRR were identified/characterized, leaving the final residue at 4.8% and 3.5%TRR (<0.32 mg/kg).

3.2 rest-of-plant

Overall, similar results were observed with both labels. Unchanged parent was with 59.8% and 71.1% TRR (9.8 and 13.7 mg/kg for C- and T-label) the predominant component of the residue (and only component present in major amounts) thus similar to forage and hull. The only other components identified were present in amounts of 4.5% TRR (0.75 mg/kg) or less and were structurally related to the parent (sugar conjugates of the parent molecule and a hydroxylated parent molecule).
Notably, for C-label and T-label, M750F012 (malonylglucosyl-O-conjugate of BAS 750 F) accounted for 6.0% and 3.1% TRR (0.97 mg/kg and 0.59 mg/kg), M750F018/M750F020 (malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for 4.5% and 3.8% TRR (0.75 mg/kg and 0.74 mg/kg). In addition, three fractions of 5% TRR (0.83 mg/kg) or less represented by peaks in HPLC chromatograms were observed.
The RRR after solvent extraction was 12.9% and 12.2% TRR (1.23 mg/kg and 2.34 mg/kg) and thus treated sequentially with various enzymes allowing a solubilization of 8.3% and 6.9% TRR corresponding to 1.37 mg/kg and 1.33 mg/kg (solubilization of 2% TRR or higher, was achieved with macerozyme/cellulase, with glucosidase/hesperinidase and with protease). The solubilized residue consisted mainly of the components identified in the ERR, thus predominantly BAS 750 F and minor amounts of metabolite M750F012.
In total, for C-label and T-label, identification amounted to 70% and 78% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 87% and 89% TRR were identified/characterized, leaving the final residue at 5.8% and 5.7%TRR (0.95 mg/kg and 1.10 mg/kg).

3.3 hull

Overall, similar results were observed with both labels. Unchanged parent was with >79% TRR (3.2 mg/kg for C- and T-label) the predominant component of the residue (and only component present in major amounts) thus similar to forage and rest-of-plant. The only other components identified were present in amounts of 1.2% TRR (0.05 mg/kg) or less and were structurally related to the parent (sugar conjugates of the parent molecule and a hydroxylated parent molecule). Notably, for C-label and T-label, M750F012 (malonylglucosyl-O-conjugate of BAS 750 F) accounted for 1.2% TRR (0.05 mg/kg) or less, while M750F018/F020 (malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for <0.1% TRR (<0.001 mg/kg). In addition, three fractions of 2.2% TRR (0.09 mg/kg) or less represented by peaks in HPLC chromatograms were observed.
The RRR after solvent extraction was 31.3% and 25.8% TRR (1.2 mg/kg and 1.1 mg/kg) and thus treated sequentially with various enzymes allowing a solubilization of >18.8% TRR (>0.74 mg/kg). Solubilization of up to 11% TRR (up to 0.45 mg/kg), was achieved with macerozyme/cellulase, of up to 3.6% TRR (up to 0.14 mg/kg) was achieved with glucosidase/hesperinidase. The solubilized residue consisted mainly of the components identified in the ERR, thus predominantly BAS 750 F and minor amounts of metabolite M750F012.
In total, for C-label and T-label, identification amounted to >80% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 87% TRR or higher was identified/characterized, leaving the final residue at 11.0% and 7.5%TRR (0.42 mg/kg and 0.31 mg/kg).

3.4 green pod

In addition to the soybean matrices, forage, rest-of-plant and seeds, also the immature growth stage green pod (C-label, DALA 47) were sampled and analysed to provide further information.
Overall, similar results were observed as with rest-of-plant (DALA 47). Unchanged parent was with 69% TRR (5.98 mg/kg) the predominant component of the residue (and only component present in major amounts). The only other components identified were present in amounts of 4% TRR (0.34 mg/kg) or less and were structurally related to the parent (sugar conjugates of the parent molecule and a hydroxylated parent molecule).
Noteably, M750F012 (malonylglucosyl-O-conjugate of BAS 750 F) accounted for 2.2% TRR (0.19 mg/kg), M750F018/M750F020 (malonylglucosyl-O-conjugate of BAS 750 F and hydroxylated parent) accounted for 3.9% TRR (0.34 mg/kg).
In total, identification amounted to 75% of TRR, considering also amounts characterized by HLPC an amount of 83% TRR were identified/characterized.

3.5 seed

Unchanged parent was detected only in low amounts of 0.01 mg/kg (C-label, 4% TRR and T-label, 0.4% TRR). Rather, the radioactive residue detected with the C-label and with the T-label was significantly different both in quantity as well as in composition.
For the C-label the TRR was only low (0.13 mg/kg), 56.6 % (0.073 mg/kg) could be extracted, most of which was characterized by HPLC (48.5% TRR, 0.063 mg/kg), identification was 4% TRR (unchanged parent BAS 750 F). The RRR of 43.4%TRR (0.056 mg/kg) was further investigated allowing characterization of 32.8% TRR (with 15.5% TRR, corresponding to 0.02 mg/kg, as releasable by macerozyme/cellulase, 8.3 % TRR corresponding to 0.01 mg/kg as releasable by protease treatment). Thus, the final residue was reduced to 0.007 mg/kg (5.2% TRR).
In total, most of the C-label residue in seed (80% of 0.13 mg/kg) was characterized by solvent extraction or enzyme treatment. A smaller portion of the residue was identified (4% TRR, 0.005 mg/kg, BAS 750 F).

In contrast, with the T-label the TRR was significantly higher (3.06 mg/kg) 76% of which could be extracted by solvents (see above). Two fractions were observed to be present in major amounts, one was identified as triazole alanine (TA, M750F029) at 47.7% TRR (1.46 mg/kg), the other one remains characterized as “triazole-like compound” since exact molecular structure identification was not possible despite intense efforts (33.6% TRR, 1.03 mg/kg). In addition, 1,2,4-triazole (M750F001) was identified albeit only at very low amounts (0.3% TRR, 0.008 mg/kg) as well as triazole lactic acid (M750F031) also at low amounts (1.3% TRR, 0.040 mg/kg). The RRR with 24.4% TRR (0.75 mg/kg) was in its entirety characterized (nominal 26.9% TRR) with macerozyme/cellulase releasing 23% TRR (0.71 mg/kg). Thus, the final residue was reduced to (calculated) 0.005 mg/kg (0.2% TRR).
In total, a large proportion of the T-label residue in seed was identified (49.7% TRR, including the “triazole-like compound” 83.3%TRR), when considering also amounts characterized by solubilization and/or HLPC the T-label residue was completely characterized and/or identified (calculated to >100% TRR).
The results from both labels taken together, indicate that most of the BAS 750 F residue in seed is carrying only the T-label and thereby resulting in a higher TRR with the T-label than in a comparable application experiment with the C-label (3.1 mg/kg versus 0.1 mg/kg). This difference in the detectable portion of the BAS 750 F residue in soybean seed is attributed largely to triazole alanine (TA 1.5 mg/kg) and the “triazole-like compound” (“6.6 min peak” with 1.0 mg/kg). Notably, unchanged parent BAS 750 F was detected only in very small amounts (0.01 mg/kg) with both C-label or T-label.

4. Enantiomer ratio of BAS 750 F

Chiral analysis of BAS 750 F residue in representative samples of the soybean metabolism study revealed absence of any significant change of BAS 750 F enantiomers. Chiral analysis of forage, hull and rest-of-plant samples (C-label and T-label) confirms that the racemic mixture (approximately 1:1 ratio of S-enantiomer and R-enantiomer) of the application formulation is essentially maintained in the plant samples. Chiral analysis was not conducted for seeds since BAS 750 F was not present in amounts sufficient to allow chiral analysis.

Table 6.2.1-21: Determination of isomer ratio of BAS 750 F in soybean matrices

matrix	S-enantiomer [%]	R-enantiomer [%]
C-label		
application formulation	50.5	49.5
forage	45.5	54.5
hull	45.6	54.4
rest-of-plant	46.4	53.4
T-label		
forage	51.3	48.7
hull	48.1	51.9
rest-of-plant	42.7	57.3

Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358)

5. Translocation in plants

After foliar application of BAS 750 F to soybean, the unchanged parent BAS 750 F represents the predominant part of radioactive residues in the directly exposed plant parts forage, green pod and rest-of-plant (> 59 % TRR). In contrast, in soybean seed which had not been exposed directly to the application with BAS 750 F, only very low amounts (maximum 0.01 mg/kg) were detected. In conclusion, BAS 750 F is translocated only in trace amounts from treated green plant parts into the seed of soybean.

6. Proposed metabolic pathway

Metabolism of BAS 750 F in foliar treated soybean was investigated using two labels, C-label and T-label. Taken results from both labels together, the data shows a consistent picture of metabolic pathways in the soybean matrices, seed, hull, rest-of-plant, green pod, and forage.
The metabolic pathway is shown in Figure 6.2-9. Overall, results show that in soybean the parent BAS 750 F, besides its

- O-conjugation of the unchanged parent (at propyl-triazole moiety) with sugars

is metabolised by two key transformation reactions:

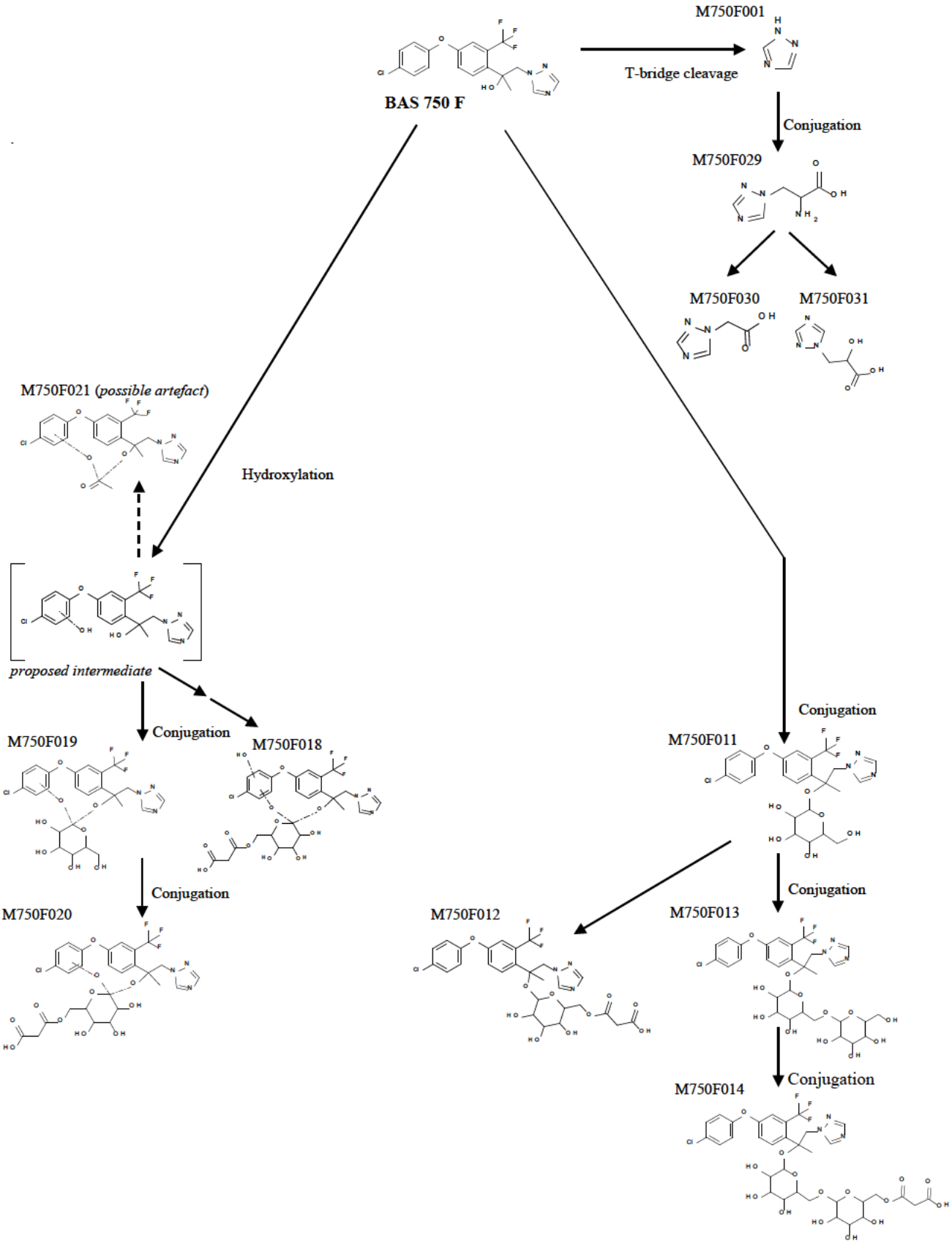
- C-ring hydroxylation (followed by conjugation)
- T-bridge cleavage of the BAS 750 F backbone (between the TFMP-ring and the T-ring)

BAS 750 F hydroxylation can occur at several positions in the C-ring. Conjugation of the hydroxyl group of the propyl-triazole moiety with sugar leads to metabolites M750F011 (glucosyl-O-conjugate), M750F012 (malonyl-glucosyl-O-conjugate), M750F013 (di-glucosyl-O-conjugate), M750F014 (malonyl-di-glucosyl-O-conjugate), while conjugation of the C-ring hydroxyl-group leads to metabolites M750F019 (glucosyl-O-conjugate), M750F020 (malonyl-glucosyl-O-conjugate). The C-ring can carry a second hydroxyl-group resulting in metabolite M750F018 (malonyl-glucosyl-O-conjugate).

Cleavage of BAS 750 F at the T-bridge leads to 1,2,4-triazole (1,2,4-T, M750F001), triazole alanine (TA, M750F029), and triazole lactic acid (TLA, M750F031). These metabolites are part of the group of triazole derivative metabolites (TDM) which are common to a range of azole fungicides. In figure 6.2-9, for some metabolites generic structures are provided, in cases when the exact position of the hydroxyl group or sugar moiety is not known (indicated by “dotted” lines). Importantly, no cleavage of ether bridge of the parent backbone was observed in soybean matrices.

In conclusion, metabolism of BAS 750 F in soybean, and by extrapolation, in the *pulses and oilseeds* crop group is considered well-elucidated.

Figure 6.2-9: Proposed pathway of BAS 750 F in soybean
(Note, that generally at least one isomer per metabolite was identified, except for M750F013, M750F014, M750F019, M750F021 and M750F030 which were only tentatively assigned.)



7. Extractability of residues according to analytical methods

The extractability of parent BAS 750 F by residue analytical methods is investigated by a separate extractability study, DocID 2014/1261057 (see CA 6.2.1/4). Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F in commodities of plant origin was investigated using samples representative of relevant plant matrices with radiolabeled residue (radiovalidation). Specifically, samples were obtained from three crop metabolism studies, namely wheat samples (see CA 6.2.1/1), soybean samples (see CA 6.2.1/2) and grape samples (see CA 6.2.1/3). Comparison of residue amounts extracted in the metabolism study with the amounts extracted by BASF method 535/1 confirms efficient extraction of BAS 750 F for all matrices investigated, namely forage (wheat), straw (wheat), green pod (soybean) and fruit (grape), amounting to efficiencies of 93% or higher.
The multi-methods also showed good extraction efficiencies in grapes and green pods (88% or higher), in forage (80% for QuEChERS) whereas extraction efficiencies were moderate for straw (52-65% for QuEChERS, DFG S 9, and SweEt) and for forage (56-63% for DFG S 9 and SweEt).

8. Storage stability

Storage stability investigations were performed (using both the quantifying and the confirming HPLC method). Taken together the data obtained confirms stability of radioactive residue over the period of the study, both in matrix (prior to extraction) stored deep frozen as well as in extracts stored in refrigerator.

Stability during storage of matrix at ≤-18°C was investigated in detail in forage and rest-of-plant (both, C- and T-labeled), by comparing the extractability as well as the resulting metabolic HPLC profiles after extended storage of a plant sample. Extraction of forage was at two different time points during the study: first extraction was 86 days after sampling, second extraction was 373-374 days after sampling, see table below). Similarly, extraction of rest-of-plant was at two different time points during the study: first extraction was 21-22 days after sampling, second extraction was 350-352 days after sampling).

Stability during storage of extract at ≤-18°C was investigated in detail in forage, rest-of-plant, and seed by comparing the metabolic HPLC profiles after extended storage of the extract.
For seed extract first analysis was after 28 days of storage, second analysis after 294-295 days of storage. Similarly, for rest-of-plant, first and second analysis were after 28 and 362-363 days of storage, for seed, first and second analysis were after 85 and 330 days of storage (C-label) as well as 17 and 330 days (T-label).

Both for storage of matrix samples and of extract samples, comparison of metabolic HPLC profiles did confirm absence of significant changes. In conclusion, for a period of at least 8 months stability of BAS 750 F residues has been confirmed for both matrix storage and extract storage.

Table 6.2.1-22: Storage intervals of plant samples and extract samples (soybean)

matrix	storage of matrix		storage of extract	
	storage interval (analysis 1) [1)]	*storage interval (analysis 2)* [1)]	*storage time (analysis 1)* [2)]	*storage time (analysis 2)* [2)]
	[days]	*[days]*	*[days]*	*[days]*
C-label				
forage	86	374	28	295
rest-of-plant	22	352	28	363
seed	34	- [3)]	85	330
T-label				
forage	86	373	28	294
rest-of-plant	21	350	28	362
seed	32	- [3)]	17	330

[1)] sampling to extraction, [2)] extraction to analysis, [3)] not analysed

III. CONCLUSION METABOLISM IN SOYBEAN

The metabolism of BAS 750 F was investigated in soybean by separately applying two labels, C-label and T-label. Data obtained with both labels taken together, shows a consistent picture of the metabolism in foliar applied soybean. Absence of cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in soybean. A separate investigation with the TFMP-label would not provide additional information.

For both C- and T-label, similar total radioactivity (TRR) was seen in forage (one application, 6.6 and 4.6 mg/kg), in rest-of-plant (16.5 and 19.2 mg/kg) and in hull (3.8 and 4.1 mg/kg). In contrast, the TRR observed with the C-label in seed (0.13 mg/kg) and green pod (8.7 mg/kg) was significantly exceeded by the TRR seen with the T-label in seed (3.06 mg/kg) and green pod (16.0 mg/kg). For both labels, high solvent extractability (ERR) was obtained for forage, rest-of-plant, green pod and hull (>68% TRR). In grain, solvent extraction followed by solubilization reduced the final residue to <0.01 mg/kg (<5% TRR). Thus, overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring) which introduces a second hydroxyl group. This is, alike the hydroxyl group of the parent, the site for conjugation leading to an array of sugar conjugates. Cleavage of BAS 750 F at the T-bridge leads to 1,2,4-triazole (1,2,4-T, M750F001), triazole alanine (TA, M750F029), and triazole lactic acid (TLA, M750F031). These metabolites are part of the group of triazole derivative metabolites (TDM) which are common to a range of azole fungicides. The parent BAS 750 F is present in soybean matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation or translocation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in forage, rest-of-plant and hull representing at least 60% TRR. In contrast, unchanged parent is detected in seed only in low amounts (0.01 mg/kg) with both C-label (4% TRR) and T-label (0.4% TRR), where TDM account almost exclusively for the radioactive residue (T-label, 82% TRR). Note that due to the cultivation of the soybean plants in plastic boxes with limited drainage, the accessibility of small polar substances might be enhanced (notably radioactive TDMs generated in the soil). Translocation of TDM into seed is reflected by the fact that TRR level in T-labeled grain are significantly exceeding the TRR in C-labeled seed.

In conclusion, the major components of the residue in soybean were identified as unchanged parent BAS 750 F and the common metabolites TDM which together are representing a large proportion of the residue (79% in forage, >59% in rest-of-plant and hull, >83% in grain).

Other components of the residue were sugar conjugates of parent (unchanged or C-ring hydroxylated parent) individually present < 5% TRR.

Overall, metabolism of BAS 750 F in soybean and by extrapolation in the "*pulses and oilseed*" crop group is considered well-elucidated.

Report:	CA 6.2.1/3 Birk B.,Bogen C., 2015 a Metabolism of 14C-BAS 750 F in grape 2015/1073822
Guidelines:	EPA 860.1000, EPA 860.1300: Nature of the Residue in Plants Livestock, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants, JMAFF 59 NohSan No 4200, OECD 501 - Metabolism in crops (adopted January 8 2007)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in grapevine after foliar application of BAS 750 F labeled either in the chlorophenyl-ring (C-ring) or in the triazole-ring (T-ring). Both labels were applied separately. Foliar spray applications of grapevine plants (cultivated on outdoor test plots, test area 0.4 m^2, under natural climatic conditions) were carried out three times at a target rate of 150 g a.s./ha. Samples of grapevine leaf were taken immediately prior to the last application (21 days after the first application). Samples of grape and stalks (BBCH 89) were collected 12 days after the third (last) application. Samples were stored in a freezer at -18°C or below.

Data obtained with C-label and T-label taken together, show a consistent picture of the metabolism in foliar applied grapevine. Absence of detectable cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in grapevine.

For both C- and T-label, similar total radioactivity (TRR) was seen in grape (0.35, 0.43 mg/kg), in stalk (0.6, 1.1 mg/kg) and in leaf (7.3 mg/kg). For both labels, high solvent extractability (ERR) was obtained for leaf, stalk and grape (>87% TRR). Thus, overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring) which introduces a second hydroxyl group which, alike the hydroxy group of the parent, is the site for conjugation leading to an array of sugar conjugates. The parent BAS 750 F is present in grape matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in grape (>64% TRR), stalk (>85% TRR) and leaf (60% TRR). Other residue components identified are sugar conjugates of parent (unchanged or hydroxylated), of which two were present in quantifiable amounts, M750F019 (up to 7% TRR in grape and stalk, up to 21% TRR in leaf) and M750F026 (up to 1.3% TRR in leaf). Other sugar conjugates, namely M750F011, M750F027, M750F028 were detected in non-quantifiable amounts.

In conclusion, the major component of the residue in grapevine was identified as unchanged parent BAS 750 F representing a large proportion of the residue in grape (>64% TRR), in stalk (>85% TRR) and in leaf (60% TRR).

Overall, metabolism of BAS 750 F in foliar applied grapevine and by extrapolation in the "*fruit and fruiting vegetables"* crop group is considered well-elucidated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

Description: BAS 750 F
C-label:
Chlorophenyl-U-C14-BAS 750 F,
Chlorophenyl-1-C13-BAS 750 F,
T-label:
Triazole-3(5)-C14-BAS 750 F,
Triazole-3(5)-C13-BAS 750 F

Lot/Batch No: 1075-1001 (Chlorophenyl-U-C14-BAS 750 F, 8.11 MBq/mg)
RS4-2012-173A2 (Chlorophenyl-1-C13-BAS 750 F),
1062-2001 (Triazole-3(5)-C14-BAS 750 F, 5.46 MBq/mg)
1077-1001 (Triazole-3(5)-C13-BAS 750 F)

Purity: Chlorophenyl-U-C14-BAS 750 F: 88% (radiochemical 99.3%)
Chlorophenyl-1-C13-BAS 750 F: 97.7%
Triazole-3(5)-C14-BAS 750 F: 98.9% (radiochemical 98.8%)
Triazole-3(5)-C13-BAS 750 F: 97.1%

CAS No: 1417782-03-6

Development code: BAS 750 F

Spiking levels: *not applicable*

2. Test Commodity:

Crop: grapevine
Type: fruit
Variety: Mueller-Thurgau
Botanical name: *Vitis vinifera*
Crop parts(s) or processed
Commodity: grapevine (leaf, stalk, grape)

Sample size:	leaf	21 days after first application:	0.02-0.04 kg
		DALA12	0.30-0.44 kg
	stalk	21 days after first application:	0.02-0.03 kg
		DALA12	0.25-0.27 kg
	grape	21 days after first application:	0.44-0.50 kg
		DALA12	3.79-4.61 kg

B. STUDY DESIGN AND METHODS

1. Test procedure

The study was conducted at the Agricultural Research Center of BASF in Limburgerhof, Germany (2014-2015). Grapevine plants were cultivated in outdoor test plots (dimension of test area approximately 0.4 m^2) under natural climatic conditions. Maintenance of the crop was performed in accordance with normal agricultural practice.

For each test item, BAS 750 F labeled in the chlorophenyl-moiety (C-label) or BAS 750 F labeled in the triazole-moiety (T-label), an application formulation was prepared for application by manual spray. Each C14-labeled test item was mixed with C13-labeled test item (1:1 ratio of chlorophenyl-U-C14 label and chlorophenyl-1-C13-label, 2:1 ratio of triazole-3(5)-C14- and triazole-3(5)-C13-label). For the first application, spray flask mixes of the test items (taken up in blank formulation and water) were prepared the preceding day. Aliquots of the application formulations were taken for purity check (by radio-HPLC) as well as for determination of the C14:C13 isotope ratio (by mass spectrometry). For the second and third application formulations were prepared as for the first application.

Both labels were applied separately (see Table 6.2.1-23). Foliar spray applications were carried out three times either with the chlorophenyl-labeled BAS 750 F or the triazole-labeled BAS 750 F at a targeted rate of 150 g a.s./ha, respectively. Samples of leaf, stalk and grape were taken 21 days after the first application, directly prior to the last application. Samples of leaf, stalk and grape were collected at harvest growth stage (BBCH89), which was 12 days after the last application. Samples were stored in a freezer at -18°C or below.

Table 6.2.1-23: Study design: plant uptake part (grapevine)

label	C-label		T-label	
intended use rate [g a.s./ha]	150		150	
application number	3		3	
application interval [days]	10±1		10±1	
sampled matrices	leaf, stalk, grape		leaf, stalk, grape	
sampling [DALA] [1)]	leaf	"-0" (21 DAT) [2)]	leaf	"-0" (21 DAT) [2)]
		12		12
	stalk	"-0" (21 DAT) [2)]	stalk	"-0" (21 DAT) [2)]
		12		12
	grape	"-0" (21 DAT) [2)]	grape	"-0" (21 DAT) [2)]
		12		12

1) days after last application, 2) immediately prior to the last (=third) application (DALA"-0") corresponding to 21 after the first application

2. Description of analytical procedures

Radioanalysis: For the determination of the TRR and the measurement of solid residues after solvent extraction or solubilization procedures, homogenized subsamples were combusted using a sample oxidizer. The resultant ^{14}C-CO_2 was absorbed, mixed with a scintillation fluid and the radioactivity was determined by liquid scintillation counting (LSC). For liquid samples, aliquots were mixed with a sufficient volume of a scintillation fluid and the radioactivity was determined by LSC.

Homogenization/solvent extraction: Plant samples (leaf, stalk and grape) were homogenized and extracted three times with methanol and two times with water using a Polytron blender. After each extraction step, the solid material was separated from the extract by centrifugation and filtration. The filtered supernatants of the methanol and water extracts were each combined and adjusted to a defined volume. The methanol extracts of all matrices and the water extracts of leaves were purified by SPE fractionation prior to quantitative HPLC analyses.
The residue after solvent extraction was dried in a fume hood, homogenized and subjected to the sequential solubilization steps by enzyme treatment.

Solubilization of the RRR: The post-extraction residue (leaf, stalk and grape of both labels) was re-suspended in 1 % ammonia and incubated for approximately 24 h. After the addition of water, the sample was centrifuged, filtered and adjusted to a defined volume.
The resulting dried residue was homogenized, re-suspended and incubated in the presence of macerozyme and cellulase (approximately 48 h). After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting dried residue was homogenized, re-suspended and incubated in the presence of α-amylase, β-amylase and amyloglucosidase (3:2:3 ratio, approximately 48 h). After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting dried residue was homogenized, re-suspended and incubated in the presence of β-glucosidase and hesperidinase (approximately 72 h). After addition of acetonitrile, the sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue was re-suspended. Suitable amounts of laccase and tyrosinase were added and the mixture was incubated for approximately 72 hours, thereafter centrifuged and filtered. The solubilizate was adjusted to a defined volume.
The resulting residue was re-suspended and incubated in the presence of protease. After incubation for approximately 72 h, acetonitrile was added. The sample was centrifuged and filtered. The solubilizate was adjusted to a defined volume. The final residue was dried.

3. Identification and characterization of the residue

Components of the residue were identified by HPLC-MS. Additionally, comparison of retention times was performed. For the parent BAS 750 F enantiomer-specific HPLC analyses were performed in samples of the application solution as well as in fractions of extracts of grape (C-Label) and leaf (T-label).

II. RESULTS AND DISCUSSION

1. TOTAL RADIOACTIVE RESIDUE (TRR)

Following foliar application of BAS 750 F to grape at a rate of three times 150 g as/ha, the total 14C residue (TRR) was measured in grapevine leaf (BBCH71-72), as well as grape and stalk (both BBCH89, see Table 6.2.1-24).
The calculated total radioactive residues (TRR) with the C-label were highest in leaf (DALA 12) with 7.37 mg/kg, lower in stalk (0.65 mg/kg) and grape (0.35 mg/kg).
A similar distribution was seen with the T-label (TRR highest in leaf at 7.31 mg/kg, lower in stalk at 1.14 mg/kg, and grape at 0.43 mg/kg).

Table 6.2.1-24: Total radioactive residue after foliar spray application of BAS 750 F

matrix [BBCH]	DALA [1)]	TRR measured *[mg/kg]*	TRR calculated [2)] *[mg/kg]*
C-label			
leaf [71-72]	12	8.860	7.371
stalk [89]	12	0.674	0.648
grape [89]	12	0.435	0.349
T-label			
leaf [71-72]	12	7.245	7.312
stalk [89]	12	1.214	1.136
grape [89]	12	0.400	0.428

[1)] days after last application [2)] TRR calculated as the sum of ERR and RRR

2. EXTRACTABILITY

The extractabilities of 14C residues from grapevine leaf, stalk and grape are summarized in Table 6.2.1-25.

High extractability of 14C residue was seen with both labels in all three matrices, leaf (>89% TRR), stalk (>92% TRR), and grape (>87% TRR). Most of the radioactivity was extracted with methanol (87% TRR or higher) while subsequent water extraction resulted only in additional extraction of 1.6% TRR or less. The RRR from leaf was 11% and 9% TRR (C- and T-label), in stalk 5.9% and 7.4%, and in grape 12.6% TRR (0.048 mg/kg, C-label) and 12.5% TRR (0.045 mg/kg, T-label) and therefore was further investigated (see below). Notably, for leaf, stalk and grape (thus for plant parts directly exposed to the foliar applied BAS 750 F application) no significant label-specific differences were seen.

Table 6.2.1-25: Extraction efficiency in grapevine matrices

matrix	DALA[1)]	TRR [2)]	distribution of radioactive residues							
			methanol extracts [3)]		water extracts [3)]		ERR [2)]		RRR [2)]	
		mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
C-label										
leaf	12	*7.371*	**87.6**	*6.456*	**1.4**	*0.102*	**89.0**	*6.558*	**11.0**	*0.813*
stalk	12	*0.648*	**93.5**	*0.606*	**0.6**	*0.004*	**94.1**	*0.610*	**5.9**	*0.038*
grape [4)]	12	*0.349*	**88.3**	*0.308*	**0.4**	*0.001*	**88.7**	*0.310*	-	-
	12	*0.349*	**87.1**	*0.331*	**0.2**	*0.001*	**87.4**	*0.332*	**12.6**	*0.048*
T-label										
leaf	12	*7.312*	**89.4**	*6.539*	**1.6**	*0.119*	**91.0**	*6.657*	**9.0**	*0.654*
stalk	12	*1.136*	**91.8**	*1.042*	**0.8**	*0.009*	**92.6**	*1.051*	**7.4**	*0.084*
grape [4)]	12	*0.428*	**89.6**	*0.384*	**0.4**	*0.002*	**90.1**	*0.385*	-	-
	12	*0.428*	**87.2**	*0.316*	**0.3**	*0.001*	**87.5**	*0.318*	**12.5**	*0.045*

[1)] days after last application [2)] TRR was calculated as the sum of ERR and RRR with ERR=Extractable Radioactive Residue, RRR=Residual Radioactive Residue (after solvent extraction), [3)] pool of combined repetitive extracts, [4)] For grape only limited sample material was available. Therefore, a first extraction was done without combustion analysis of the resulting RRR. Later, a second extraction was done with the purpose to determine the RRR for grape.

3. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

An overview over the components of the extractable residue is given below in Table 6.2.1-26, Table 6.2.1-27. An overview of the residue released from RRR by enzyme treatment is given in Table 6.2.1-28 and Table 6.2.1-29.

Table 6.2.1-26: Summary of identified/characterized components in grapevine (C-label)

C-labeled radioactive component (min) [4] ERR & RRR	leaf		stalk		grape	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
TRR	**100.0**	*7.371*	**100.0**	*0.648*	**100.0**	*0.349*
BAS 750 F	**60.1**	*4.432*	**85.8**	*0.556*	**64.1**	*0.224*
M750F019 (22.7, 23.2)	**21.1**	*1.554*	**2.3**	*0.015*	**7.0**	*0.024*
M750F026	**1.3**	*0.097*	**-**	-	**-**	-
ERR	**89.0**	*6.558*	**94.1**	*0.610*	**88.7**	*0.310*
ID [1]	**82.5**	*6.082*	**88.1**	*0.571*	**71.0**	*0.248*
CHAR [1]	**2.8**	*0.204*	**0.6**	*0.004*	**5.7**	*0.020*
sum ID/CHAR[1]	**85.3**	*6.286*	**88.8**	*0.575*	**76.7**	*0.268*
RRR	**11.0**	*0.813*	**5.9**	*0.038*	**-**	-
CHAR	**3.9**	*0.285*	**2.0**	*0.013*	**2.1**	*0.007*
SUM ID/CHAR in ERR/RRR[1]	**89.1**	*6.571*	**90.8**	*0.588*	**78.8**	*0.275*
Final residue [2]	**6.1**	*0.448*	**2.9**	*0.019*	**9.2**	*0.032*
Grand Total [3]	**95.2**	*7.019*	**93.7**	*0.607*	**88.0**	*0.307*

[1] ID=amount identified, CHAR=amount characterized, sum ID/CHAR= sum of amounts identified and/or characterized, SUM ID/CHAR in ERR/RRR= sum of amounts of ERR and of RRR which were identified and/or characterized, [2] final residue after solvent extraction and solubilization, [3] Grand Total=sum of amounts characterized and identified as well as final residue, [4] Retention times (in minutes) are provided in parenthesis, M750F019 eluted in two distinct peaks.

Table 6.2.1-27: Summary of identified/characterized components in grapevine (T-label)

T-labeled radioactive component (min) [4] ERR & RRR	leaf		stalk		grape	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
TRR	**100.0**	*7.312*	**100.0**	*1.136*	**100.0**	*0.428*
BAS 750 F	**69.9**	*5.110*	**91.5**	*1.039*	**70.3**	*0.301*
M750F019 (22.7, 23.2)	**14.5**	*1.058*	**-**	-	**6.1**	*0.026*
M750F026	**-**	-	**-**	-	**-**	-
ERR	**91.0**	*6.657*	**92.6**	*1.051*	**90.1**	*0.385*
ID [1]	**84.4**	*6.169*	**91.5**	*1.039*	**76.4**	*0.327*
CHAR [1]	**4.0**	*0.290*	**0.8**	*0.009*	**8.0**	*0.034*
sum ID/CHAR[1]	**88.3**	*6.458*	**92.3**	*1.048*	**84.4**	*0.361*
RRR	**9.0**	*0.654*	**7.4**	*0.084*	**-**	-
CHAR	**3.0**	*0.221*	**3.1**	*0.035*	**2.4**	*0.010*
SUM ID/CHAR in ERR/RRR[1]	**91.4**	*6.680*	**95.3**	*1.083*	**86.7**	*0.371*
Final residue [2]	**5.1**	*0.375*	**3.4**	*0.038*	**7.6**	*0.032*
Grand Total [3]	**96.5**	*7.055*	**98.7**	*1.121*	**94.3**	*0.403*

[1] ID=amount identified, CHAR=amount characterized, sum ID/CHAR= sum of amounts identified and/or characterized, SUM ID/CHAR in ERR/RRR= sum of amounts of ERR and of RRR which were identified and/or characterized, [2] final residue after solvent extraction and solubilization, [3] Grand Total=sum of amounts characterized and identified as well as final residue, [4] Retention times (in minutes) are provided in parenthesis, M750F019 eluted in two distinct peaks.

Table 6.2.1-28: Characterization of RRR by enzyme treatment (C-label)

C-labeled radioactive component (min) [4]		leaf		stalk		grape	
		%TRR	*mg/kg*	*%TRR*	*%TRR*	*%TRR*	*mg/kg*
RRR		**11.0**	*0.813*	**5.9**	*0.038*	**-**	**-** [4]
CHAR *enzyme treatment* [5]	AM	**1.5**	*0.108*	**0.7**	*0.005*	**0.9**	*0.003*
	M	**0.7**	*0.051*	**0.4**	*0.003*	**0.4**	*0.001*
	AM/G	**0.4**	*0.028*	**0.2**	*0.001*	**0.3**	*0.001*
	G/H	**0.3**	*0.023*	**0.1**	*0.001*	**0.2**	*0.001*
	T/L	**0.5**	*0.038*	**0.2**	*0.002*	**0.2**	*0.001*
	protease	**0.5**	*0.035*	**0.3**	*0.002*	**0.1**	*< 0.001*
	solubilized (sum) [5]	**3.9**	*0.285*	**2.0**	*0.013*	**2.1**	*0.007*
Final residue		**6.1**	*0.448*	**2.9**	*0.019*	**9.2**	*0.032*

[1] ID=amount identified, CHAR(HPLC) =amount characterized by HPLC, [2] final residue after solvent extraction and solubilization, [3] sum of RRR amounts characterized and identified, [4] use sum of solubilized and residue as indicative information (RRR not available), [5] Solubilization included sequential treatments with ammonia (AM), with macerozyme (M), with amylase/amyloglucosidase (AM/G), with glucosidase/hersperinidase (G/H) and finally with tyrosinase/laccase (T/L).

Table 6.2.1-29: Characterization of RRR by enzyme treatment (T-label)

T-labeled radioactive component (min) [4]		leaf		stalk		grape	
		%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg
RRR		**9.0**	*0.654*	**7.4**	*0.084*	**-**	**- [4]**
CHAR *enzyme treatment [5]*	AM	**1.1**	*0.079*	**1.1**	*0.013*	**1.2**	*0.005*
	M	**0.6**	*0.040*	**0.6**	*0.007*	**0.5**	*0.002*
	AM/G	**0.4**	*0.028*	**0.3**	*0.004*	**0.3**	*0.001*
	G/H	**0.2**	*0.018*	**0.3**	*0.004*	**0.1**	*0.001*
	T/L	**0.4**	*0.027*	**0.4**	*0.004*	**0.2**	*0.001*
	protease	**0.4**	*0.028*	**0.3**	*0.003*	**0.1**	*< 0.001*
	solubilized (sum) [5]	**3.0**	*0.221*	**3.1**	*0.035*	**2.4**	*0.010*
Final residue		**5.1**	*0.375*	**3.4**	*0.038*	**7.6**	*0.032*

[1] ID=amount identified, CHAR(HPLC) =amount characterized by HPLC, [2] final residue after solvent extraction and solubilization, [3] sum of RRR amounts characterized and identified, [4] use sum of solubilized and residue as indicative information (RRR not available), [5] Solubilization included sequential treatments with ammonia (AM), with macerozyme (M), with amylase/amyloglucosidase (AM/G), with glucosidase/hersperinidase (G/H) and finally with tyrosinase/laccase (T/L).

3.1 grapevine leaf

Overall, similar results were observed with both labels. Unchanged parent was with > 60% TRR (4.4 and 5.1 mg/kg for C- and T-label) the predominant component of the residue thus similar to stalk and grape. The other identified components were structurally related to the parent: The second most abundant component was metabolite M750F019 (O-glucosyl-conjugate of C-ring hydroxylated BAS 750 F) with 21.1% and 14.5% TRR (1.55 mg/kg and 1.06 mg/kg). In addition, metabolite M750F026 (O-di-glucosyl-conjugate of C-ring hydroxylated BAS 750 F) was found with the C-label, albeit at only small amounts with 1.3% TRR (0.097 mg/kg).
The RRR after solvent extraction (11.0% and 9.0% TRR corresponding to 0.81 mg/kg and 0.65 mg/kg) was treated sequentially with various enzymes allowing a solubilization of 3.9% and 3.0% TRR corresponding to 0.29 mg/kg and 0.22 mg/kg (ammonia treatment solubilized 1.5 and 1.1 % TRR, macerozyme/cellulase solubilized 0.7% and 0.6% TRR corresponding to amounts of 0.04 to 0.11 mg/kg, other treatments were less effective).
In total, for C-label and T-label, identification amounted to 83% and 84% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 89% and 91% TRR were identified/characterized, leaving the final residue at 6.1% and 5.1%TRR (0.45 mg/kg and 0.38 mg/kg).

3.2 grapevine stalk

Overall, similar results were observed with both labels. Unchanged parent was with >85% TRR (0.56 and 1.04 mg/kg for C- and T-label) the predominant component of the residue thus similar to leaf and grape. The only other identified component was structurally related to the parent: M750F019 (O-glucosyl-conjugate of C-ring hydroxylated BAS 750 F) with 2.3% TRR (0.02 mg/kg).
The RRR after solvent extraction (5.9% and 7.4% TRR corresponding to 0.04 mg/kg and 0.08 mg/kg) was treated sequentially with various enzymes allowing a solubilization of 2.0% and 3.1% TRR corresponding to 0.01 mg/kg and 0.04 mg/kg (ammonia treatment alone solubilized 0.7 and 1.1 % TRR, other treatments were less effective).
In total, for C-label and T-label, identification amounted to 88% and 92% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 91% and 95% TRR were identified/characterized, leaving the final residue at 3%TRR (<0.04 mg/kg).

3.1 grape

Overall, similar results were observed with both labels. Unchanged parent was with > 64% TRR (0.22 and 0.30 mg/kg for C- and T-label) the predominant component of the residue thus similar to stalk and leaf. The only other identified component was structurally related to the parent: M750F019 (O-glucosyl-conjugate of C-ring hydroxylated BAS 750 F) with 7.0% and 6.1% TRR (0.024 mg/kg and 0.026 mg/kg).
The RRR after solvent extraction was treated sequentially with various enzymes allowing a solubilization of 2.1% and 2.4% TRR corresponding to 0.007 mg/kg and 0.010 mg/kg (ammonia treatment solubilized 0.9% and 1.2% TRR, other treatments were less effective).
In total, for C-label and T-label, identification amounted to 71% and 76% of TRR, considering also amounts characterized by solubilization and/or HLPC an amount of 79% and 87% TRR were identified/characterized, leaving the final residue at 9.2% and 7.6%TRR (0.032 mg/kg for both labels).

4 Enantiomer ratio of BAS 750 F

Chiral analysis of BAS 750 F residue in representative samples of the grape metabolism study revealed absence of any significant change of BAS 750 F enantiomers. Chiral analysis of a grape sample (C-label) and leaf sample (T-label) revealed that the racemic mixture (approximately 1:1 ratio of S-enantiomer and R-enantiomer) of the application formulation is essentially maintained.

Table 6.2.1-30: Determination of isomer ratio of BAS 750 F in grapevine matrices

matrix	S-enantiomer [%]	R-enantiomer [%]
C-label		
application formulation	48.9	51.1
grape	46.7	53.3
T-label		
application formulation	47.4	52.6
leaf	48.1	52.0

Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358)

5. Translocation in plants

The application of BAS 750 F was performed at growth stages while leaf, stalk and grape were already present (days 33, 22 and 12 prior to harvest). Since the application formulation was applied equally resulting in a homogeneous distribution over the entire plant including leaf, stalk and grape, no precise information regarding translocation of BAS 750 F within the plant can be derived from this study.

6. Proposed metabolic pathway

Metabolism of BAS 750 F was investigated in foliar treated grapevine using two labels, C-label and T-label pathways in the grapevine matrices leaf, stalk and grape.
The metabolic pathway is shown in Figure 6.2-10. Overall, results show that in grapevine the parent BAS 750 F, besides its

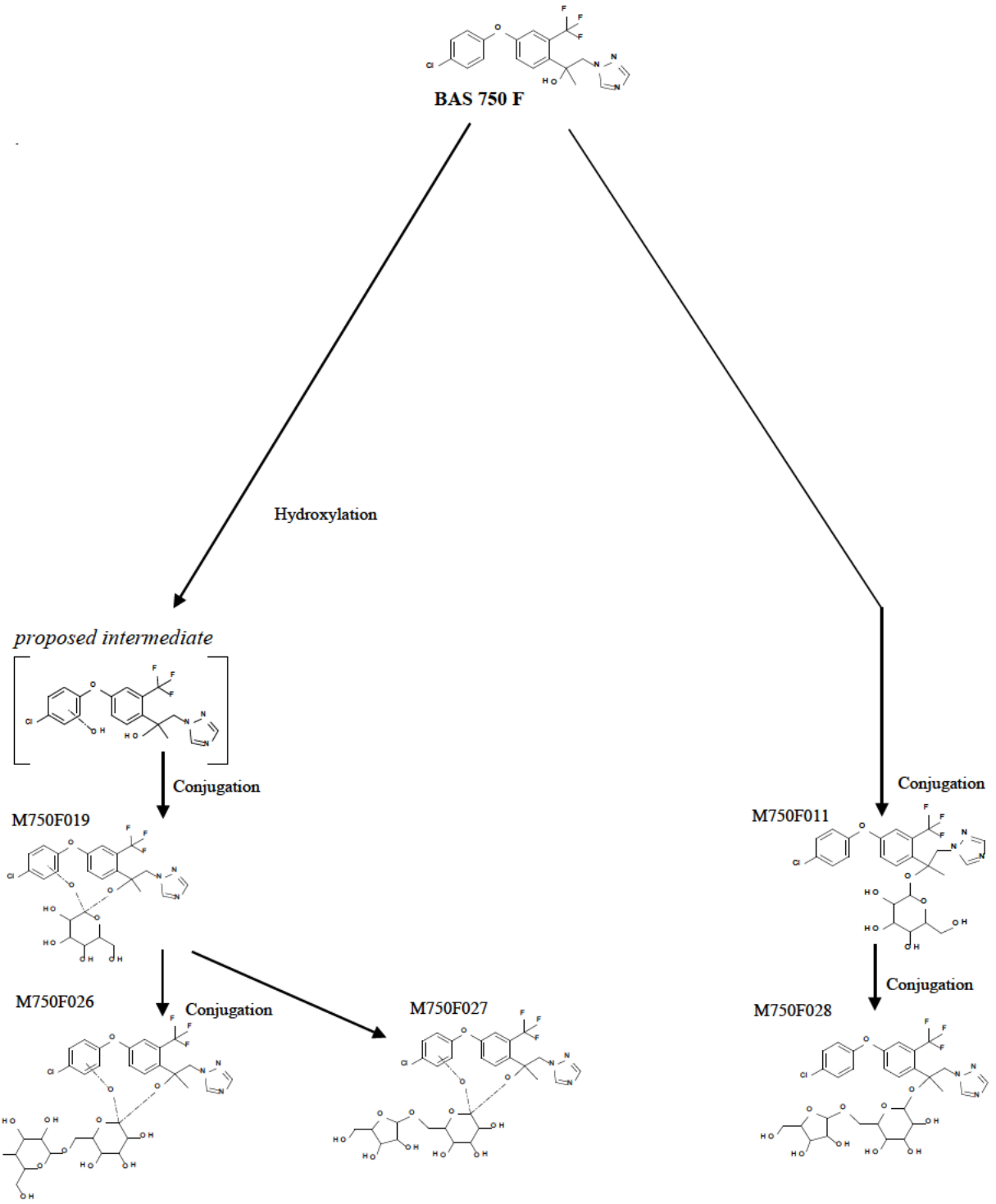
- O-conjugation of the unchanged parent (at propyl-triazole moiety) with sugars

is metabolised by one key transformation reaction:

- C-ring hydroxylation (followed by conjugation)

BAS 750 F hydroxylation can occur at several positions in the C-ring. Conjugation of the hydroxyl group of the propyl-triazole moiety with sugars leads to metabolite M750F028 (pentosyl-glucosyl-O-conjugate).
Alternatively, conjugation of the C-ring hydroxyl-group leads to metabolites M750F019 (glucosyl-O-conjugate), and M750F026 (di-glucosyl-O-conjugate).
In Figure 6.2-10, for some metabolites generic structure are provided in cases when exact position of the hydroxyl group or sugar moiety is not known (indicated by a “dotted” line). Notably, no cleavage of parent backbone was observed in grape matrices. In conclusion, the metabolism pathways in foliar-treated grapevine representative of the crop category of *fruits and fruiting vegetables* can be considered well elucidated.

Figure 6.2-10: Proposed pathway of BAS 750 F in grapevine



7. Extractability of residues according to analytical methods

The extractability of parent BAS 750 F by residue analytical methods is investigated by a separate extractability study, DocID 2014/1261057 (see CA 6.2.1/4). Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F in commodities of plant origin was investigated using samples representative of relevant plant matrices with radiolabeled residue (radiovalidation). Specifically, samples were obtained from three crop metabolism studies, namely wheat samples (see CA 6.2.1/1), soybean samples (see CA 6.2.1/2) and grape samples (see CA 6.2.1/3). Comparison of residue amounts extracted in the metabolism study with the amounts extracted by BASF method 535/1 confirms efficient extraction of BAS 750 F for all matrices investigated, namely forage (wheat), straw (wheat), green pod (soybean) and fruit (grape), amounting to efficiencies of 93% or higher.
The multi-methods also showed good extraction efficiencies in grapes and green pods (88% or higher), in forage (80% for QuEChERS) whereas extraction efficiencies were moderate for straw (52-65% for QuEChERS, DFG S 9, and SweEt) and for forage (56-63% for DFG S 9 and SweEt).

8. Storage stability

Storage stability investigations were performed (using both the quantifying and the confirming HPLC method).

Stability during storage of matrix at ≤-18°C was investigated by comparing the metabolic HPLC profiles after extended storage of a plant sample (extraction at two different time points during the study: first extraction was <2 months, second extraction was >6 months after sampling, see Table 6.2.1-31). Samples analyzed were methanol and aqueous extracts of leaves and methanol extracts of grapes (both C-labeled and T-labeled samples).

Stability during storage of extract at ≤-18°C was investigated by comparing the metabolic HPLC profiles after extended storage of the extract (first analysis 14-27 days, second analysis > 6 months later, Table 6.2.1-31). Samples analyzed were methanol and aqueous extracts of leaves and methanol extracts of grapes (both C-labeled and T-labeled samples).

Both for storage of matrix samples and of extract samples, comparison of metabolic HPLC profiles did confirm absence of significant changes. In conclusion, for a period of at least 6 months stability of BAS 750 F residues has been confirmed for both matrix storage and extract storage.

Table 6.2.1-31: Storage intervals of plant samples and extract samples (grapevine)

matrix	storage of matrix		storage of extract	
	storage interval (analysis 1) [1)]	*storage interval (analysis 2)* [1)]	*storage interval (analysis 1)* [2)]	*storage interval (analysis 2)* [2)]
	[days]	*[days]*	*[days]*	*[days]*
C-label				
leaf (MeOH extract)	57	252	19 (14)	230 (224)
leaf (water extract)	57	252	26 (27)	231 (229)
grape (MeOH extract)	56	203	21 (14)	231 (226)
T-label				
leaf (MeOH extract)	56	251	20 (14)	230 (224)
leaf (water extract)	56	251	26 (27)	230 (229)
grape (MeOH extract)	55	203	20 (16)	231 (226)

[1)] *sampling to extraction,* [2)] *extraction to analysis 3) intervals for a second sample are given in parenthesis*

III. CONCLUSION METABOLISM IN GRAPE

The metabolism of BAS 750 F was investigated in grapevine after foliar application of BAS 750 F labeled either in the chlorophenyl-ring (C-ring) or in the triazole-ring (T-ring). Both labels were applied separately. Data obtained with C-label and T-label taken together, show a consistent picture of the metabolism in foliar applied grapevine. Absence of detectable cleavage at the ether bridge between C-ring and TFMP-ring (trifluoromethylphenyl-ring, linking C-ring and T-ring) confirms that results obtained with C-labeled samples also provide comprehensive information on the metabolic fate of the TFMP-ring. Data obtained with C-label and T-label is therefore sufficiently elucidating the metabolism of BAS 750 F in grapevine.

For both C- and T-label, similar total radioactivity (TRR) was seen in grape (0.35, 0.43 mg/kg), in stalk (0.6, 1.1 mg/kg) and in leaf (7.3 mg/kg). For both labels, high solvent extractability (ERR) was obtained for leaf, stalk and grape (>87% TRR). Thus, overall, the predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F includes hydroxylation of the parent backbone structure (C-ring) which introduces a second hydroxyl group which, alike the hydroxy group of the parent, is the site for conjugation leading to an array of sugar conjugates. The parent BAS 750 F is present in grape matrices as a racemic mixture of the R- and S-enantiomer. Compared to the test substance applied, the ratio remains unchanged and thus indicates absence of preferential metabolisation of one of the two enantiomers.

Similar in both labels, unchanged parent BAS 750 F is the only predominant component of the residue in grape (<64% TRR), stalk (>85% TRR) and leaf (60% TRR). Other components of the residue are sugar conjugates of parent (unchanged or C-ring hydroxylated parent), of which two were present in quantifiable amounts, M750F019 (up to 7% in grape and stalk, up to 21% in leaf) and M750F026 (up to 1.3% in leaf). Other sugar conjugates, namely M750F011, M750F027, M750F028 were detected in non-quantifiable amounts.

In conclusion, the major component of the residue in grapevine was identified as unchanged parent BAS 750 F representing a large proportion of the residue in grape (<64% TRR), in stalk (>85% TRR) and in leaf (60% TRR).

Overall, metabolism of BAS 750 F in grapevine and by extrapolation in the "*fruit and fruiting vegetables"* crop group is considered well-elucidated.

Report:	CA 6.2.1/4 Birk B. et al., 2015 b Investigation of the extractability of BAS 750 F in samples from 14C plant metabolism studies 2014/1261057
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17, OECD 501
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F and in commodities of plant origin as investigated using samples with radiolabeled residue (radiovalidation). Samples from metabolism studies in wheat, soybean and grapevine (see sections CA6.2.1/1, CA6.2.1/2, and CA6.2.1/3), namely forage (wheat), straw (wheat), soybean (green pod), grapevine (grape) were used to investigate extraction efficiency of radiolabeled residues of BAS 750 F.
Extraction procedures used in analytical methods, namely BASF method 535/1 as well as plant multi-methods QuEChERS, DFG S 9, and SweET, were compared to the extracted amounts achieved in the metabolism studies wheat, soybean, and grapevine.

Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction of BAS 750 F achieved with for the analytical methods BAS 535/1.
Extraction efficiencies for BAS 750 F generally were 90% or higher for all matrices investigated, namely wheat forage (98%), wheat straw (111%), soybean green pod (102%) and grapevine grape (93%).

On contrast, with the multi-methods, extraction efficiency was lower for forage (QuEChERS 80%, DFG S 19 63%, SweEt 56%), and for straw (QuEChERS 59%, DFG S 19 52%, SweEt 65%) while similar high extraction efficiency was observed for soybean green pod and grapevine grape (88% or higher).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: *not relevant*

2. Test Commodity: *not relevant*

Samples from metabolism studies in wheat, soybean and grapevine (see sections CA6.2.1/1, CA6.2.1/2, and CA6.2.1/3), namely forage (wheat), straw (wheat), soybean (green pod), grapevine (grape) were used to investigate extraction efficiency of radiolabeled BAS 750 F.

B. STUDY DESIGN AND METHODS

1. Test procedure *not relevant*

2. Description of analytical procedures

Extraction procedures used in analytical methods, namely BASF method 535/1 (see section CA 4.3), as well as plant multi-methods were compared to the extraction procedure used in the wheat, soybean, and grapevine metabolism studies. The plant multi-methods were

QueEChERs	*(Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method. EN 15662:2008)*
DFG S 19	*(Foods of plant origin - Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS - Part 1: General considerations. EN 12393-1:2013)*
SweET	(*Foods of plant origin - Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS - Part 2: Methods for extraction and cleanup. EN 12393-2:2013)*

2.1 Procedure of BAS method 535/1

The extraction procedure is adapted from BASF method number 535/1 (L0076/01), “Technical Procedure: Method for the Determination of BAS 421 F, BAS 480 F, BAS 500 F, 500M07 (BF 500-3), BAS 510 F, BAS 550 F, BAS 555 F and BAS 560 F in Plant Matrices”). In brief, 5 g of homogenised plant sample was extracted once with 100 mL of methanol / water / 2 N HCl (70/25/5, v/v/v) using a homogeniser. 10 mL of the extract were subjected to centrifugation and the resulting supernatant was filtered, diluted with methanol and analysed by LSC and HPLC. For wheat straw, the extraction was repeated to achieve sufficient extractability.

2.2 Procedure of *QuEChERS*

For water-rich matrices (forage, grapes), 10 g of homogenised material was used for extraction. For dry matrices (grain, straw), 5 g of homogenised material was used and 10 g water was added prior to extraction. For fat-rich matrices (soybean green pod), 10 g of homogenised material was used and 3 g water was added prior to extraction. Samples were extracted with 10 mL acetonitrile, centrifuged, filtered, diluted with acetonitrile and analysed by LSC and HPLC. For the extraction of wheat forage and wheat straw, in addition 60 mL acetonitrile/water (1/1, v/v) was added to achieve a suitable solvent volume for extraction. For soybean green pod, 5 g homogenised sample was taken in order to obtain a sufficient ratio of solid material to extraction solvent. After centrifugation, the clear supernatant was subjected to HPLC preparation without further clean-up or dilution steps. Grapevine grape was extracted as described above. After centrifugation, the clear supernatant was subjected to HPLC preparation without further cleanup or dilution steps.

2.3 Procedure of *DFG S 9*

For water-rich matrices (forage, grapes), 25 g material was homogenized after addition of 10 g water and 200 mL acetone, centrifuged, filtered, diluted with acetone and analysed by LSC and HPLC. For dry matrices (grain and straw), 50 g of homogenised material was added to 95 g water (40 °C) and homogenised by mixing. After swelling for 20 min, 200 mL acetone was added for extraction using a homogeniser. After centrifugation, the resulting supernatant was filtered, diluted with acetone and analysed by LSC and HPLC. For fat-rich matrices (soybean), 25 g of homogenised material was mixed with 225 mL acetonitrile and 25 mL acetone and extracted using a homogeniser. After centrifugation, the supernatant was filtered, and analysed by LSC and HPLC. All matrices were extracted as described above, except for wheat straw, where 19.96 g homogenised material was extracted.

2.4 Procedure of *SweEt*

10 g of a homogenised plant sample was mixed with 3 g sodium bicarbonate and at least 10 g sodium sulphate. 20 mL ethyl acetate was added for extraction and further ultra-sonication for 1 min. After centrifugation, the resulting supernatant was filtered, diluted with ethyl acetate and analysed by LSC and HPLC.

For wheat forage, 10 g homogenised material was added to 3 g mixture of sodium bicarbonate and sodium sulphate (3/10, w/w). After adding 50 mL ethyl acetate, due to insufficient solvent volume, the sample was processed as described above. For wheat straw, 5 g homogenised material was added to 3 g mixture of sodium bicarbonate and sodium sulphate (3/10, w/w). After adding 50 mL ethyl acetate, due to insufficient solvent volume, the sample was processed as described above. For soybean green pod, 5 g homogenised material was mixed with 1.5 g sodium bicarbonate and 5 g sodium sulphate to obtain a sufficient ratio of solid material to extraction solvent. The mixture was processed as described above. After centrifugation, the supernatant was subjected to LSC analysis and HPLC sample preparation without further clean-up and dilution steps. Grapevine grape was extracted as described above. After centrifugation, the supernatant was diluted with ethyl acetate without prior filtration.

II. RESULTS AND DISCUSSION

The amount of radioactive residue extracted in the plant metabolism studies (ERR value) was taken as reference value for extraction efficiency (thus, ERR was set to 100%). The amount extracted with an analytical method was compared to this value, and expressed in percentage of the reference value (see Table 6.2.1-32).

Table 6.2.1-32: Summary of extractability: radioactive residues and parent BAS 750 F

extraction procedure	**TRR**	**radioactive residue (in ERR)**			**BAS 750 F**		
	mg/kg	*mg/kg*	*%TRR*	***extraction efficiency (%) 1)***	*mg/kg*	*%TRR*	***extraction efficiency (%) 1)***
forage (wheat, T-label)							
wheat metabolism study	2.31	*2.218*	96.0	100.0	*2.062*	89.3	100.0
BASF Method 535/1 (1a)		*2.161*	93.6	**97.4**	*2.012*	87.1	**97.6**
QuEChERS (2)		*1.769*	76.6	**79.8**	*1.656*	71.7	**80.3**
DFG S 19 (3)		*1.743*	75.5	**78.6**	*1.307*	56.6	**63.4**
SweEt (4)		*1.476*	63.9	**66.6**	*1.149*	49.7	**55.7**
straw (wheat, C-label)							
wheat metabolism study	24.38	*20.241*	83.0	100.0	*13.798*	56.6	100.0
BASF Method 535/1		*18.758*	76.9	**92.7**	*15.351*	63.0	**111.3**
2 QuEChERS		*12.530*	51.4	**61.9**	*8.174*	33.5	**59.2**
3 DFG S 19		*9.310*	38.2	**46.0**	*7.143*	29.3	**51.8**
4 SweEt		*9.123*	37.4	**45.1**	*8.996*	36.9	**65.2**
green pod (soybean, C-label)							
soya metabolism study	8.72	*7.271*	83.4	100.0	*5.978*	68.5	100.0
1a BASF Method 535/1		*6.989*	80.1	**96.1**	*6.101*	70.0	**102.1**
2 QuEChERS		*7.081*	81.2	**97.4**	*5.900*	67.7	**98.7**
3 DFG S 19		*6.006*	68.9	**82.6**	*5.913*	67.8	**98.9**
4 SweEt		*5.890*	67.5	**81.0**	*5.838*	66.9	**97.7**
grape (T-label)							
grape metabolism study	0.42	*0.385*	90.1	100.0	*0.301*	70.3	100.0
1a BASF Method 535/1		*0.366*	85.5	**95.0**	*0.281*	65.8	**93.4**
2 QuEChERS		*0.366*	85.5	**95.0**	*0.296*	69.1	**98.3**
3 DFG S 19		*0.347*	81.2	**81.2**	*0.301*	70.3	**100.0**
4 SweEt		*0.315*	73.7	**73.7**	*0.267*	62.4	**88.7**

1) extraction efficiency = amounts extracted with analytical method compared with amount extracted in metabolism study (set to 100%).

III. CONCLUSION

Efficient extraction for the analytical method, BASF method 535/1 was confirmed by comparison of residue amounts extracted in the metabolism study with the amounts extracted according to extraction procedures of a residue analytical method.

Extraction efficiencies generally were 90% or higher for all matrices investigated, namely wheat forage (98%), wheat straw (111%), soybean green pod (102%) and grapevine grape (93%).

In contrast, with the multi-methods, extraction efficiency was lower for forage (QuEChERS 80%, DFG S 19 63%, SweEt 56%), and for straw (QuEChERS 59%, DFG S 19 52%, SweEt 65%) while similar high extraction efficiency was observed for soybean green pod and grapevine grape (88% or higher).

6.2. OVERALL CONCLUSION METABOLISM IN PLANT

Metabolism was investigated using two radiolabels (BAS 750 F labeled in the C-ring or in the T-ring). Results obtained with both labels show a consistent picture of BAS 750 F metabolism. Investigations were done in three plant species, wheat (cereal crop group), soybean (pulses and oilseed crop group), and grapevine (fruits/fruiting vegetable crop group), foliar applied with BAS 750 F reflecting the cGAP. Altogether, three different EU crop categories were represented. These three crops are all target crops for intended BAS 750 F uses either in Europe (wheat) or in Latin-America / NAFTA (soybean, grape). Comparable results were obtained for all three crop group (see Figure 6.2.11) allowing therefore to extrapolate these results generally to foliar applied crops.

Overall, results show that in plant the parent BAS 750 F, besides its

- O-conjugation of the unchanged parent (at propyl-triazole moiety) with sugars

is metabolised by two key transformation reaction:

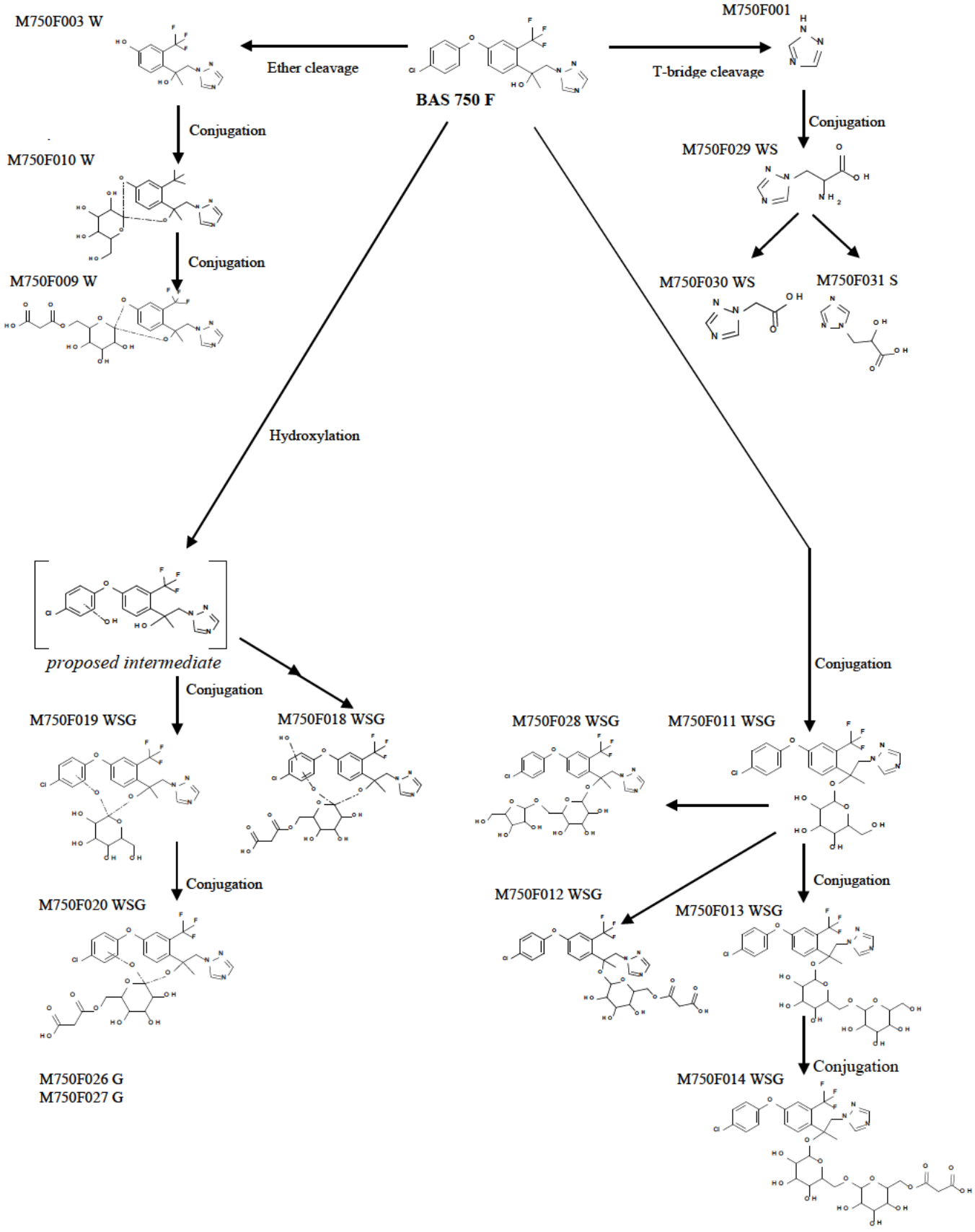
- C-ring hydroxylation (followed by conjugation)
- T-bridge cleavage of the BAS 750 F backbone

The unchanged parent BAS 750 F and the C-ring hydroxy metabolite (proposed necessary intermediate) are further conjugated by sugars resulting in a range of O-glycosylated metabolites. Cleavage at the T-bridge generates 1,2,4-triazole, which together with its conjugation products TA, TAA and TLA forms a group of metabolites (TDM) common to other triazole containing fungicides. In contrast, cleavage at the ether bridge is only a minor transformation step.

In most matrices the unchanged parent is the predominant component of the residue (>60% of the radioactive residue), notably in forage (wheat, soybean), leaf/stalk (grapevine), straw/hull/chaff (wheat, soybean), green pod (soybean) and grape (grapevine). The enantiomers ratio of the two BAS 750 F remains unchanged (racemic mixture). Sugar conjugates of the hydroxy-metabolite are found in major amounts (up to 16% of the radioactive residue in wheat straw, up to 22% in grape leaf), while sugar conjugates of the unchanged parent are found <5% of the radioactive residue.

In contrast, in wheat grain and soybean seed, the predominant component of the residue is the group of TDM with triazole alanine as the most abundant compound. Unchanged parent is absent from wheat grain, and found in lower amounts in soybean seed (up to 4% radioactive residue). The cleavage products resulting from cleavage of the ether bridge followed by conjugation with sugars, are present both only in straw and only in minor amounts (in sum <3% of radioactive residue).

Figure 6.2/11 BAS 750 F: metabolic routes in plant (W wheat, S soybean, G grape)



Metabolites occurring in plant matrices in major amounts (<10% TRR) and in minor amounts (<10% TRR) are listed in the table below. This table groups the metabolites according to their chemical structure together with their corresponding conjugates. The non-conjugated metabolites that were identified in plant matrices are highlighted (underlined).

Table 6.2.1-33: Residue components identified in plant matrices

group definition	**(non-conjugated) group metabolite members in PLANTS**	
	≥ 10% TRR	**< 10% TRR**
a) parent and conjugates		
	BAS 750 F	M750F011 M750F012 M750F013 M750F014 M750F028
b) "C-Ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates		
	M750F018 M750F019 M750F020 M750F026 M750F027	
c) cleavage products & downstream metabolites / conjugates		
metabolites without the C-Ring		M750F009 M750F010
metabolites without 1,2,4-T-ring		
1,2,4-triazole and triazole-derived metabolites (TDM)	**1,2,4-T** **TA** **TAA**	**TLA**

A detailed discussion of any possible relevance for the residue definitions, either for MRL enforcement or for dietary risk assessment, is provided in section CA 6.7.1.

CA 6.2.2 Poultry

Report:	CA 6.2.2/1 [redacted] 2015 a The metabolism of 14C-Reg. No 5834378 (BAS 750 F) in laying hens 2015/1001001
Guidelines:	OECD Test Guideline 503 - Metabolism in livestock, EPA 860.1000: EPA Residue Chemistry Test Guidelines, EPA 860.1300: Nature of the Residue in Plants Livestock, EEC 91/414 (7030(VI/95 Rev. 3), JMAFF 59 NohSan No 4200, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in laying hens following repeated oral administration of 14C-BAS 750 F, labeled either in the Chlorophenyl ring (C-ring), in the Trifluoromethylphenyl ring (TFMP-ring) or in the triazole ring (T-ring). The test item was administered orally by gavage to laying hen (ten animals per label) for 14 consecutive days at a nominal dose of 12 mg/kg feed. The mean actual concentrations were 16.7 mg/kg feed (C-label), 15.9 mg/kg feed (TFMP-label) and 15.0 mg/kg feed (T-label), corresponding to daily means of 1.09, 1.07 and 1.08 mg/kg body weight, respectively.

For the three labels, the overall accountability of the study was good. The radioactive residue was rapidly and extensively excreted. Until sacrifice, the radioactive residues in excreta amounted to 75-89% of the total radioactivity administered. For all labels, only low portions of the administered dose (≤ 0.4 %) were retained in edible tissues or in egg (<1% of dose).

14C residues in egg (sampled on 14 consecutive days) reached a plateau concentration within 5-7 days confirming absence of accumulation of residues in egg. Plateau level in egg yolk were at 0.5 mg/kg, 0.6 mg/kg, and 0.3 mg/kg (C-, TFMP-, T-label). In egg white residues were <0.1 mg/kg (C-and TFMP-label) and 0.3 mg/kg for T-label. Label-specific differences were also seen for TRR from other matrices except kidney (0.43-0.59 mg/kg). C-label and TFMP-label were generally comparable, but distinct from T-label. In muscle and egg white, TRR for C-/TFMP-label (<0.08 mg/kg) was lower than for the T-label (>0.36 mg/kg). In contrast in liver and fat, TRR for C-/TFMP-label (>0.31 mg/kg) was higher than for the T-label (<0.18 mg/kg). Taken together, this data indicates the presence of significant amounts of first, metabolites which carry only the T-label (thus not detectable with C-/TFMP-label) and second, metabolites which do not carry the T-label. For all matrices, TRR of 0.3-0.6 mg/kg (in fat up to 1.2 mg/kg) were observed in at least one label. (Note that each label only detects a fraction of the BAS 750 F residue).

The extractability of radioactive residues from all edible matrices (egg white and yolk, muscle, liver, kidney and fat) was high. Investigation of the RRR obtained after protease treatment reduced the final residue further to mostly <5% TRR, exceeded only for liver 7% (0.02 mg/kg, C-label), and muscle (8%TRR, <0.01 mg/kg, C-label). Overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in laying hen includes two main transformation reactions, cleavage of the parent backbone at the T-bridge generating 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to conjugation by fatty acids (M750F023/M750F024/M750F025). In addition, a transformation reaction seen in liver is the C-ring oxygenation followed by glutathione conjugation (M750F034). The parent BAS 750 F was applied as a racemic mixture of two enantiomers. Chiral analysis of BAS 750 F in egg yolk and in fat showed absence of significant ratio change, while for the other matrices BAS 750 F was present only at very low amounts precluding chiral analysis.

For the metabolite profile, label-dependent differences similar to the observations at TRR level were seen. Generally, results of C-label and TFMP-label were comparable, and distinct from the T-label.

With both C- and TFMP-label, the cleavage metabolite M750F022 together with its fatty acid conjugates the predominant component of the residue, together accounting for >69% TRR in yolk, muscle, and fat, and 20-44% TRR in liver and kidney (egg white with TRR <0.01 mg/kg was not further investigated). M750F022 was the most abundant compound in muscle, liver, kidney and yolk, while in fat the conjugates were present in up to 3X higher amounts. While the sum of M750F022 and its conjugates was similar for both labels, the conjugation ratio however showed some variability between both labels (notably for liver, muscle and fat).

The cleavage metabolite 1,2,4-triazole (M750F001) was found at major level in all matrices, proportions were >65% TRR in egg white, muscle, liver, kidney and fat, and 41% TRR in egg yolk. Parent BAS 750 F was present in all matrices investigated except egg white. Major amounts of BAS 750 F were determined in in egg yolk (7-44% TRR) and fat (5-20% TRR), while proportions were low in muscle, liver, and kidney (<7% TRR). The only other component identified is the liver-specific metabolite M750F034, a glutathione conjugate of parent found at 4-20% TRR (0.01-0.12 mg/kg).

In conclusion, the major components of the residue in hen were identified as 1,2,4-triazole, metabolite M750F022 together with its fatty acid conjugates, parent BAS 750 F as well as a liver-specific metabolite (M750F034). Overall, metabolism of BAS 750 F in laying hen and, by extrapolation in the poultry livestock group, can be considered well-elucidated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

1. C-label

Description: Chlorophenyl-U-C14 (spec. activity 7.88 MBq/mg) added to a 1:2 (w:w) mixture of Chlorophenyl-1-C13-labeled and unlabeled test item

Lot/Batch #:	Chlorophenyl-U-C14:	CFQ41561
	Chlorophenyl-1-C13:	RS4-2012-173A2
	Unlabeled:	COD-001740
Purity:	Chlorophenyl-U-C14:	radiochem. purity 98.9%
	Chlorophenyl-1-C13:	97.7%
	Unlabeled:	98.8%

CAS #: 1417782-03-6

Stability of Test Compound: The stability of BAS 750 F in the vehicle was verified analytically on application days 0 and 14.

Development Code: BAS 750 F

2. TFMP-label

Description: Trifluoromethylphenyl-U-C14 (spec. activity 8.265 MBq/mg) added to a 2:2 (w:w) mixture of Propyl-2-C13-labeled and unlabeled test item

Lot/Batch #:	Trifluoromethylphenyl-U-C14:	CFQ42039
	Propyl-2-C13:	1126-1006
	Unlabeled:	COD-001740
Purity:	Trifluoromethylphenyl-U-C14:	radiochem purity 98.3%
	Propyl-2-C13:	99.5%
	Unlabeled:	98.8%

CAS #: 1417782-03-6

Stability of Test Compound: The stability of BAS 750 F in the vehicle was verified analytically on application days 0 and 14.

Development Code: BAS 750 F

3. T-label

Description: Triazole-3(5)-C14 (spec. activity 5.46 MBq/mg) added to 1:1 (w:w) mix of Triazole-3(5)-C13-labeled and unlabeled test item

Lot/Batch #:	Triazole-3(5)-C14:	1062-2001
	Triazole-3(5)-C13:	1077-1001
	Unlabeled:	COD-001740
Purity:	Triazole-3(5)-C14:	radiochem purity 98.8%
	Triazole-3(5)-C13:	97.1%
	Unlabeled:	98.8%

CAS #: 1417782-03-6

Stability of Test Compound: The stability of BAS 750 F in the vehicle was verified analytically on application days 0 and 14.

Development Code: BAS 750 F

2. Test Animals:

Species:	laying hens ("*Lohmann Brown*")
Gender:	female
Weight at Dosing:	1.5-2.0 kg
Number of Animals:	10 (per label), also 2 hens to obtain control tissues
Acclimatization Period:	14-23 days
Diet:	C- and T-label: Saracen RS Layer Pellets (200 g daily) TFMP-label: Heygates Country Feeds Layers pellets (200 g daily) all labels: OYTA Shells (*ad libitum*)
Water:	Mains drinking water (*ad libitum*)
Housing:	individual metabolism cages
Husbandry:	Husbandry conditions were in accordance with the *United Kingdom Animals (Scientific Procedures) Act 1986*.
Environmental Conditions:	
Temperature:	14-22°C
Humidity:	30-70% relative humidity
Air Changes:	fully air-conditioned rooms; number of air-changes not indicated in the report
Photoperiod:	12 hour light – 12 hour dark cycle

B. STUDY DESIGN AND METHODS

1. Dosing Regime:

Oral:	gelatine dose capsule positioned in gullet using forceps.
Amount of Dose:	nominal dose: 12 mg/kg per dry weight diet per day; mean actual doses (per dry weight diet per day): *C-label: 16.74 mg/kg (1.09 mg/kg bw/d)* *TFMP-label: 15.90 mg/kg (1.07 mg/kg bw/d)* *T-label: 14.98 mg/kg (1.08 mg/kg bw/d)*
Food Consumption:	96.30-141.70 g/day per bird (during application period)
Vehicle:	gelatine dose capsule (under addition of acetone)
Timing:	once daily, in the morning
Duration:	14 consecutive days

2. Sample Collection:

Egg Collection:	24 h prior to first dose administration, twice daily, additionally, whole eggs still in oviduct after termination
Excreta Collection:	24 h prior to first dose administration, once daily
Interval from Last Dose to Sacrifice:	approximately 3-6 h

Tissues/Organs Harvested and Analyzed:
egg white, egg yolk, muscle, fat, liver, kidney and excreta

Matrices Sampled for Balance Analysis:

eggs (white, yolk and partially formed eggs), muscle (breast and thigh), fat (omental, renal and subcutaneous), liver, kidney, excreta, bile, blood, gastrointestinal tract and contents and cage wash

3. Test procedure

The in-life phase of this study was performed Nov 2013 to Nov 2014 (Quotient Bioresearch, Rushden Ltd.), metabolism phase conducted from May 2014 to Nov 2015 (BASF SE). A total of 30 hen was dosed with radiolabeled BAS 750 F at nominal doses of 12 mg/kg for 14 consecutive days (10 birds for each label). Each ^{14}C-labeled test item was mixed with ^{13}C-labeled test item and unlabeled test item (ratios are given in Table 6.2.2-1 below). The test items were prepared in gelatine capsules, orally administered (once daily). The actual dose was based on the average feed consumption (day 1-14). The mean achieved daily dose administered was 15.0-16.8 mg/kg food consumed (dry weight equivalent) corresponding to 1.1 mg/kg bw/d. Details of the study outline are summarized in Table 6.2.2-1.

Table 6.2.2-1: Dosing of laying hen with BAS 750 F

animal no. (10 birds per label)	treat-ment period (days)	isotope ratio ^{14}C : ^{13}C : ^{12}C	nominal daily dose	actual daily dose [1]		time of sacrifice [2] (hours)
			[mg/kg feed]	*[mg/kg feed]*	*[mg/kg bw/d]*	
C-label: 1-10	14	1 : 1 : 2	12	16.8	1.09	3-6
TFMP-label: 21-30	14	1 : 2 : 2	12	15.9	1.07	3-6
T-label: 11-20	14	1 : 1 : 1	12	15.0	1.08	3-6

1) based on mean body weights on study day1 2) hours after last dose

4. Sampling and storage

Excreta, were collected for a 24 h interval prior to first dose and subsequently every 24h until sacrifice. Eggs were collected for a 24h interval prior to first dose and twice daily during the subsequent treatment period of 14 days until sacrifice. The birds were sacrificed after 3-6 h and edible tissues (liver, kidney, muscle (breast, thigh), fat (omental, subcutaneous, and renal) as well as bile, blood and the GI tract collected. All samples were stored deep frozen at - 18 °C.

5. Description of Analytical Procedures

Radiochemical purity of ^{14}C-BAS 750 F was confirmed by HPLC analysis. Stability of ^{14}C-BAS 750 F in dose capsules was confirmed by sequential extractions with acetone and acetonitrile followed by liquid scintillation counting (LSC) measurements. The ratios of ^{14}C: ^{13}C:^{12}C were confirmed by mass spectrometric analyses.

Label-specific pools were prepared for muscle (breast and thigh), for liver, for kidney, for fat (omental, renal and subcutaneous), for egg white (treatment period 168-288h) and egg yolk (treatment period 168-288h). For excreta, C- and T-label specific pools (treatment period 168-288h) were prepared, homogenized and aliquots used for determination of total radioactive residue (TRR measured) by combustion/liquid scintillation counting (LSC).

Egg white of the C- and TFMP-label was not extracted due to low radioactivity. Other matrices were subjected to sequential methanol/water extractions. Muscle (C-label and one extraction in case of the TFMP-label) and liver (TFMP-label) were extracted with methanol only. Fat (T-label and one extraction in case of the TFMP-label) was extracted with a mixture of methanol and isohexane (1/1, v/v). Fat (C-label and one extraction in case of the TFMP-label) was extracted with a mixture of acetonitrile and isohexane (1/1, v/v). Kidney (C-label) was sequentially extracted with methanol, dichloromethane (DCM) and isohexane. The results of the different extractions (LSC of the combined extracts) referred to as extractable radioactive residues (ERR). The residual radioactive residue after solvent extraction (RRR) was determined by combustion analysis. Unless radioactivity was too low, RRR was treated with protease followed by combustion/LSC of the final residue. Extracts were concentrated and analyzed by HPLC (radio-detection). Some extracts were further purified prior to analysis by HPLC-MS/MS.

Generally, identification of metabolites was based analysis by HPLC-MS/MS, on co-chromatography as well as chromatographic comparison of retention times of reference substances. In addition, various HPLC peaks were characterized by their chromatographic properties.
Specifically for TFMP-labeled fat, methanol extract was subjected to lipase treatment or to alkaline hydrolysis (incubation with 2 mol/L NaOH). Both treatments were conducted in order to achieve cleavage of fatty acid conjugates of M750F022 to release the metabolite M750F022.

II. RESULTS AND DISCUSSION

General observations: all birds remained in good health throughout the study. During the acclimatization and application period, no behavioural or physical abnormalities were observed. Bodyweight, feed consumption and egg production remained unchanged during the study period. At sacrifice, macroscopic examinations revealed no abnormalities. The three groups (10 animals each) did receive similar amounts of BAS 750 F dose (C-, TFMP-, T-label: 16.74, 15.90 and 14.98 mg/kg feed corresponding to 1.09, 1.07 and 1.08 mg/kg bw/day).

1. TOTAL RADIOACTIVE RESIDUES (TRR)

Daily egg samples obtained on 14 consecutive days, separated into yolk and egg white, were measured for total radioactive residues (TRR). Plateau levels of radioactive residues in egg were reached within 5-7 days of dosing (see Table 6.2.2-2) indicating absence of accumulation of residues both in yolk and in egg white.

Table 6.2.2-2: TRR in egg white and yolk after administration of ^{14}C-BAS 750 F to hen

	C-label *TRR measured [mg/kg]*		**TFMP-label** *TRR measured [mg/kg]*		**T-label** *TRR measured [mg/kg]*	
application day	**white**	**yolk**	**white**	**yolk**	**white**	**yolk**
1	*0.004*	*0.001*	*0.003*	*0.001*	*0.119*	*0.052*
2	*0.009*	*0.043*	*0.008*	*0.039*	*0.260*	*0.138*
3	***0.013***	*0.121*	*0.009*	*0.138*	*0.300*	*0.178*
4	***0.012***	*0.244*	*0.009*	*0.227*	*0.323*	*0.215*
5	***0.012***	*0.334*	***0.013*** [2)]	*0.384*	*0.314*	*0.234*
6	***0.012***	*0.472*	***0.007***	*0.460*	*0.359*	*0.277*
7 [2)]	***0.011***	***0.571*** [2)]	***0.011***	***0.617*** [2)]	***0.387*** [2)]	***0.301*** [2)]
8	***0.009***	***0.595***	***0.014***	***0.622***	***0.363***	***0.301***
9	***0.006***	***0.556***	***0.009***	***0.666***	***0.415***	***0.322***
10	***0.009***	***0.424***	***0.008***	***0.658***	***0.384***	***0.308***
11	***0.008***	***0.471***	***0.010***	***0.639***	***0.366***	***0.302***
12	***0.007***	***0.454***	***0.010***	***0.665***	***0.390***	***0.311***
13	***0.008***	***0.448***	***0.010***	***0.648***	***0.344***	***0.292***
pool sample [1)]	*0.009*	*0.477*	*0.005*	*0.618*	*0.357*	*0.269*

[1)] refer to Table 6.2.2-3for detailed information on pool samples, [2)] start of plateau phase, indicated by bold typing

Recoveries generally were good and similar for the three labels (80% or higher), with most of the radioactivity being excreta-related (76-91% of dose), only low amounts recovered in GI tract (1-3% dose) or cagewash (2-3% dose). Generally, only low amounts of dose were retained in tissues. Specifically in egg, only very low proportions of the administered dose were found (<1% dose). With C-label and TFMP-label, dose retained was higher in egg yolk compared to egg white (factor of >10X), while for the T-label, dose retained was about 3X higher in egg white compared to egg yolk.

For further analysis, pooled samples (for each matrix and label) of excreta, tissues, yolk and egg white (obtained during the dosing interval days 7 – 12) were generated. “TRR measured” of excreta, egg, muscle, liver, kidney, and fat tissues was obtained by combustion analysis followed by LSC. TRR was also calculated as the sum of the extractable radioactive residues (ERR) and the residual radioactive residues (RRR) after solvent extraction. Since similar values were obtained, generally the available “TRR calculated” was used as 100% TRR for further calculations.

Table 6.2.2-3 provides an overview over the tissue distribution (as “*TRR measured*”), showing that the three labels result in largely similar residue levels (liver 0.15-0.61 mg/kg, kidney 0.43-0.61 mg/kg) while for the other matrices clear differences between the labels are seen. Residues obtained with the C- and TFMP-labeled exceed the residues compared with the T-label for fat (0.68-1.23 mg/kg compared with 0.18 mg/kg) and for egg yolk (0.48-0.62 mg/kg compared with 0.27 mg/kg). Residues obtained with the T-label exceed the residues compared with the C-/TFMP-label for egg white (0.36 mg/kg compared with <0.01 mg/kg) and for muscle (0.38 mg/kg compared with <0.08 mg/kg). The TRR data obtained with all three labels taken together, indicates absence of significant cleavage of the ether bridge (between positions of radiocarbon in C- and TFMP-label) while the T-bridge (between positions of radiocarbon in TFMP- and T-label) appears to be effectively cleaved.

Table 6.2.2-3: Material balance after administration of 14C-BAS 750 F to hen

matrix	C-label		TFMP-label		T-label	
	% of dose	**TRR** *measured [mg/kg]*	**% of dose**	**TRR** *measured [mg/kg]*	**% of dose**	**TRR** *measured [mg/kg]*
excreta	75.30	*2.92*	86.59	-	88.91	*6.34*
egg white	0.01	*0.009* [1)]	0.02	*0.005* [1)]	0.55	*0.357* [1)]
egg yolk	0.22	*0.477* [1)]	0.28	*0.618* [1)]	0.17	0.269 [1)]
partially formed eggs	0.08	-	0.14	-	0.09	-
muscle [2)]	0.03	*0.054*	0.05	*0.078*	0.23	*0.377*
liver	0.06	*0.307*	0.13	*0.611*	0.03	*0.146*
kidney	0.01	*0.431*	0.01	*0.612*	0.01	*0.590*
fat [3)]	0.13	*0.679*	0.10	*1.227*	0.01	*0.183*
GI tract, contents	1.14	-	2.41	-	1.62	-
bile	0.01	-	0.02	-	< 0.00	-
blood	< 0.00	-	< 0.00	-	< 0.00	-
subtotal organs /tissues	1.38	-	2.72	-	1.90	-
cage wash	2.53	-	2.61	-	2.37	-
total recovery	**79.52**	-	**92.36**	-	**93.99**	-

1) refer to table 6.2/34 for detailed information, 2) muscle pool of breast and thigh muscle, 3) fat pool of omental, renal, and subcutaneous fat

2. EXTRACTABILITY

The extractabilities of ^{14}C residue from hen matrices, yolk, egg white, muscle, liver, kidney, and fat are summarized in Table 6.2.2-4 (C-label), Table 6.2.2-5 (TFMP-label), and Table 6.2.2-6 (T-label). Solubilization results of further characterization by protease treatment is provided in Table 6.2.2-10.

High extractability of ^{14}C residue was seen for egg, tissues and excreta (83% TRR or higher).

For all three labels, most of the radioactivity was extracted with methanol (88% TRR or higher) for yolk, egg white (T-label only), muscle, liver and kidney while subsequent water extraction resulted in additional 5% TRR or less. In C-and TFMP-labeled egg white residues were low (TRR <0.01 mg/kg), thus no extraction was done. Methanol extraction also retrieved about 100 % TRR of T-labeled fat, while for C- and TFMP-labeled fat, acetonitrile extraction (83-112% TRR) followed by isohexane extraction (11-18% TRR) was done to achieve complete extraction of the ^{14}C-residue.

Solvent extraction left a RRR of <5% TTR in egg white, fat, T-labeled yolk, TFMP-/T-labeled muscle and thus was not investigated further. Protease treatment allowed to further characterized the RRR of yolk (C-/TFMP-label), of muscle (C-label), of liver and kidney reducing the final residue further to <5% TRR for yolk and kidney, to 8% TRR (0.004 mg/kg) for muscle and to 7.3% TRR (0.02 mg/kg) for liver.

Table 6.2.2-4: TRR and extractability of residues of ^{14C}-BAS 750 F (C-label) in egg, tissues and excreta

matrix [1] **(C-label)**	**TRR** *(measured)*	**methanol extract**		**isohexane extract**		**water extract**		**DCM extracts**		**ERR** [1]		**RRR** [1]		**TRR** [1] *(calculated)*	**recovery** [2] **(extraction)**
	mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*[mg/kg]*	*%*
egg white [3]	*0.009*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
egg yolk (EXTR_1)	*0.477*	**90.2**	*0.430*	-	-	**1.8**	*0.009*	-	-	**92.0**	*0.439*	**6.1**	*0.029*	*0.468*	**98.1**
egg yolk (EXTR_2)	*0.477*	**88.3**	*0.421*	-	-	**1.1**	*0.005*	-	-	**89.4**	*0.426*	**9.1**	*0.043*	*0.469*	**98.4**
muscle (EXTR_1)	*0.050*	**85.0**	*0.043*	-	-	-	-	-	-	**85.0**	*0.043*	**21.5**	*0.011*	*0.053*	**106.4**
liver (WU_1)	*0.320*	**80.6**	*0.258*	-	-	**3.4**	*0.011*	-	-	**84.0**	*0.269*	**14.6**	*0.047*	*0.316*	**98.7**
liver (WU_2)	*0.320*	**79.0**	*0.253*	-	-	**3.7**	*0.012*	-	-	**82.7**	*0.265*	**14.5**	*0.046*	*0.311*	**97.2**
kidney (WU_1)	*0.427*	**82.1**	*0.350*	**0.4**	*0.002*	-	-	**1.2**	*0.005*	**83.7**	*0.357*	**14.5**	*0.062*	*0.419*	**98.1**
kidney (WU_2)	*0.427*	**87.2**	*0.372*	-	-	**5.2**	*0.022*	-	-	**92.4**	*0.394*	**12.9**	*0.055*	*0.449*	**105.3**
excreta	*2.924*	**83.5**	*2.440*	-	-	**5.2**	*0.151*	-	-	**88.6**	*2.592*	**29.0**	*0.847*	*3.439*	**117.6**
	TRR *(measured)*	**acetonitrile extract**		**isohexane extract**		-		-		**ERR** [1]		**RRR** [1]		**TRR** [1] *(calculated)*	**recovery** [2] **(extraction)**
fat (WU_1)	*0.702*	**35.4**	*0.248*	**64.8**	*0.455*	-	-	-	-	**100.2**	*0.703*	**0.7**	*0.005*	*0.708*	**100.9**
fat (WU_2)	*0.702*	**36.0**	*0.253*	**55.8**	0.392	-	-	-	-	**91.9**	*0.645*	**1.1**	*0.007*	*0.652*	**92.9**
fat (WU_3)	*0.702*	**82.6**	*0.580*	**18.2**	*0.128*	-	-	-	-	**100.8**	*0.707*	**1.4**	*0.010*	*0.717*	**102.2**
fat (WU_4)	*0.702*	**83.7**	*0.587*	**14.0**	*0.098*	-	-	-	-	**97.7**	*0.685*	**1.1**	*0.007*	*0.693*	**98.8**

DCM denotes dichloromethan. EXTR_1, EXTR_2 denote extraction 1 and extraction 2, WU_1, WU_2, WU_3, WU_4 denote workup1, workup 2 etc.
[1] The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the respective solvent extracts. [2] recovery is calculated as "TRR calculated" divided by "TRR measured" [3] For egg white residue levels are very low, thus no extraction was performed.

Table 6.2.2-5: TRR and extractability of residues of ^{14C}-BAS 750 F (TFMP-Label) in egg, tissues and excreta

matrix [1] **(TFMP-label)**	**TRR** *(measured)*	**methanol extract**		**isohexane extract**		**water extract**		**DCM extracts**		**ERR** [1]		**RRR** [1]		**TRR** [1] *(calculated)*	**recovery** [2] **(extraction)**
	mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*[mg/kg]*	*%*
egg white [3]	*0.005*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
egg yolk (EXTR_1)	*0.618*	**93.7**	*0.578*	-	-	**0.5**	*0.003*	-	-	**94.2**	*0.582*	**5.1**[4]	*0.032*	*0.613*	**99.3**
muscle (EXTR_1)	*0.066*	**109.2**	*0.072*	-	-	**1.1**	*0.001*	-	-	**110.3**	*0.073*	**2.8**	*0.002*	*0.074*	**113.2**
muscle (EXTR_3)	*0.066*	**114.6**	*0.075*	-	-	-	-	-	-	**114.6**	*0.075*	**3.5**	*0.002*	*0.078*	**118.2**
liver (WU_1)	*0.582*	**92.3**	*0.537*	-	-	**1.8**	*0.010*	-	-	**94.0**	*0.547*	**5.6**[4]	*0.033*	*0.580*	**99.6**
liver (WU_2)	*0.582*	**90.1**	*0.525*	-	-	-	-	-	-	**90.1**	*0.525*	**7.4**	*0.043*	*0.567*	**97.4**
kidney (WU_1)	*0.610*	**97.4**	*0.594*	-	-	**1.6**	*0.009*	-	-	**98.9**	*0.603*	**6.3**[4]	*0.038*	*0.642*	**105.2**
fat (WU_1)	*0.893*	**109.8**	*0.980*	**12.9**	*0.115*	-	-	-	-	**122.7**	*1.095*	**0.1**	*0.001*	*1.096*	**122.8**
		acetonitrile extract		**isohexane extract**		-		-		**ERR** [1]		**RRR** [1]		**TRR** [1] *(calculated)*	**recovery** [2] **(extraction)**
fat (WU_2)	*0.893*	**112.1**	*1.001*	**11.0**	*0.099*	-	-	-	-	**123.1**	*1.100*	**0.3**	*0.002*	*1.102*	**123.4**

DCM denotes dichloromethan. EXTR_1, EXTR_2 denote extraction 1 and extraction 2, WU_1, WU_2, WU_3, WU_4 denote workup1, workup 2 etc.
[1] The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the respective solvent extracts. [2] recovery is calculated as "TRR calculated" divided by "TRR measured", [3] For egg white residue levels are very low, thus no extraction was performed. [4] Samples further investigated by protease treatment.

Table 6.2.2-6: TRR and extractability of residues of ^{14C}-BAS 750 F (T-Label) in egg, tissues and excreta

matrix (T-label)	TRR *(measured)*	methanol extract		isohexane extract		water extract		DCM extracts		ERR [1)]		RRR [1)]		TRR [1)] *(calculated)*	recovery [2)] (extraction)
	mg/kg	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*mg/kg*	*%*
egg white	*0.357*	**97.0**	*0.346*	-	-	**1.2**	*0.004*	-	-	**98.2**	*0.350*	**0.2**	*0.001*	*0.351*	*98.3*
egg yolk	*0.269*	**107.9**	*0.290*	-	-	**1.0**	*0.003*	-	-	**108.9**	*0.293*	**2.6**	*0.007*	*0.300*	*111.4*
muscle	*0.353*	**98.8**	*0.349*	-	-	**2.2**	0.008	-	-	**101.0**	*0.356*	**1.4**	*0.005*	*0.361*	*102.4*
liver	*0.480*	**99.2**	*0.476*	-	-	**1.3**	*0.006*	-	-	**100.5**	*0.482*	**3.3**[4)]	*0.016*	*0.498*	*103.8*
kidney	*0.565*	**98.9**	*0.559*	-	-	**1.1**	*0.006*	-	-	**100.0**	*0.565*	**2.0**	*0.011*	*0.577*	*102.0*
fat	*0.190*	**101.8**	*0.194*	**5.0**	*0.010*	-	-	-	-	**106.8**	*0.203*	**3.2**[4)]	*0.006*	*0.209*	*110.0*
excreta	*6.341*	**99.3**	*6.296*	-	-	**2.3**	*0.148*	-	-	**101.6**	*6.444*	**15.2**	*0.962*	*7.405*	*116.8*

DCM denotes dichloromethan. EXTR_1, EXTR_2 denote extraction 1 and extraction 2, WU_1, WU_2, WU_3, WU_4 denote workup1, workup 2 etc,
[1)] The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the respective solvent extracts. [2)] recovery is calculated as "TRR calculated" divided by "TRR measured". [3)] For egg white residue levels are very low, thus no extraction was performed. [4)]Samples further investigated by protease treatment.

3. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

The following tables provide a summary of the residue components identified and/or characterized in samples obtained with the C-label (Table 6.2.2-7), the TFMP-label (Table 6.2.2-8), and the T-label (Table 6.2.2-9). ERR was subjected to HPLC analysis allowing identification (ID) and characterization (CHAR) of radioactive residue. RRR of selected samples was further treated with protease allowing characterization by solubilization of ^{14}C residue (RRR CHAR).
Overall, largely similar composition of the radioactive residue is found for C-label and TFMP-label, and a significantly different composition with the T-label. Cleavage products account for large proportion of the residue, namely M750F022 and its conjugates in C-/TFMP-labeled samples, and 1,2,4-triazole in T-labeled samples.

3.1. egg yolk

Total extractable residue (ERR) in the pooled sample (day 7-12) accounted for <92% of TRR (C-, TFMP-, T-label) with the residue after extraction (RRR) representing 6.1%, 5.1% and 2.6% TRR (C-TFMP-, T-label). Protease treatment of C- and TFMP-labelled yolk reduced the final residue further to maximum of 4% TRR (<0.02 mg/kg).

The total identified residue in egg yolk accounted for 59-85% TRR. Additional characterization by chromatographic and extraction properties accounted for 6-31% TRR resulting in amounts identified and characterized of >83% TRR.

Metabolite M750F022 was the most abundant component of the residue in the C-label and TFMP-label (39% TRR and 47% TRR, equivalent to 0.19 mg/kg and 0.29 mg/kg). The second most abundant residue components were fatty acid conjugates of M750F022, together accounting for 25.3% TRR (0.12 mg/kg and 0.16 mg/kg). Parent BAS 750 F was detected, albeit at lower amounts (6.5 and 11.5% TRR, equivalent to 0.03 and 0.07 mg/kg). The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples.

In contrast, with the T-label, BAS 750 F was present as the most abundant component (44% TRR, 0.12 mg/kg), while M750F001 (1,2,4-triazole) is found at 41% TRR (0.11 mg/kg). The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples.

3.2. egg white

Total extractable residue (ERR) in the pooled sample (day 7-12) accounted for 98.2% of TRR (only T-label, residues for both C-, TFMP-label were <0.01 mg/kg, and thus not further investigated). The residue after extraction (RRR) represented 0.2% and was thus not further analysed.

The total identified residue in T-labelled egg white accounted for 83% TRR, all of it M750F001 (0.30 mg/kg). No other component was identified, additional characterization by extraction properties accounted for 15% TRR resulting in amounts identified and characterized of 98% TRR. The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples.

Table 6.2.2-7: Summary of BAS 750 F and metabolites and of characterized fractions in hen matrices (C-label)

C-labeled residue component		egg yolk		egg white		muscle		liver		kidney		fat		excreta	
		%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
BAS 750 F		**6.5**	*0.031*	**-**	**-**	**5.6**	*0.003*	**7.2**	*0.023*	**4.0**	*0.017*	**5.4**	*0.038*	**28.6**	*0.835*
M750F034		**-**	-	-	-	**-**	-	**4.3**	*0.014*	-	-	**-**	-	**-**	-
M750F022		**39.0**	*0.186*	**-**	**-**	**49.9**	*0.025*	**36.7**	*0.118*	**20.1**	*0.086*	**25.4**	*0.178*	**3.1**	*0.090*
M750F023		**2.6**	0.012	-	-	**8.0**	*0.004*	**2.0**	*0.006*	**1.7**	*0.007*	**23.7**	*0.166*	-	-
M750F024		**10.6**	0.051	-	-	**-**	-	**1.1**	*0.003*	**0.8**	*0.004*	**13.3**	*0.093*	-	-
M750F024 / M750F025[1)]		**11.4**	0.054	-	-	**11.5**	*0.006*	**-**	-	**-**	-	**27.4**	*0.193*	**-**	-
M750F025		**0.7**	0.003	-	-	**-**	-	**3.8**	*0.012*	**1.4**	*0.006*	**14.2**	*0.099*	**-**	-
ERR	**ID**	**59.4**	*0.283*	**-**	**-**	**75.0**	*0.038*	**55.0**	*0.176*	**28.0**	*0.120*	**81.9**	*0.575*	**31.6**	*0.925*
	CHAR (HPLC)	**30.0**	*0.143*	-	-	**12.9**	*0.006*	**22.7**	*0.072*	**50.5**	*0.215*	**14.7**	*0.103*	**50.8**	*1.485*
	CHAR (other)	**1.1**	*0.005*	**-**	**-**	**-**	-	**3.8**	*0.012*	**1.6**	*0.007*	**2.6**	*0.018*	**5.2**	0.151
	sum ID/CHAR	**90.5**	*0.431*	**-**	**-**	**87.8**	*0.044*	**81.4**	*0.260*	**80.1**	*0.342*	**99.3**	*0.696*	**87.6**	*2.561*
RRR	**CHAR**	-	-	-	-	**7.2**	*0.004*	**7.2**	*0.023*	**-**	-	**-**	-	-	-
Final Residue		**9.1**	*0.043*	**-**	**-**	**8.2**	*0.004*	**7.3**	*0.023*	**14.5**	*0.062*	**1.4**	*0.010*	**29.0**	0.847
Grand Total		**99.6**	*0.475*	**-**	**-**	**103.2**	*0.052*	**95.9**	*0.307*	**94.6**	*0.404*	**100.6**	*0.706*	**116.6**	3.409

ID identification, CHAR characterization, sum ID/CHAR= sum of amounts identified and/or characterized, Final residue = residue after solubilization by protease treatment
[1)] The two metabolites M750F024 and M750F025 were not separated using HPLC.

Table 6.2.2-8: Summary of BAS 750 F and metabolites and of characterized fractions in hen matrices (TFMP-label)

TFMP-labeled residue component		egg yolk		egg white		muscle		liver		kidney		fat		excreta	
		%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
BAS 750 F		**11.5**	*0.071*	-	-	**7.4**	*0.005*	**5.8**	*0.034*	**3.7**	*0.022*	**11.7**	*0.104*	-	-
M750F034		-	-	-	-	-	-	**20.1**	*0.117*	-	-	-	-	-	-
M750F022		**46.7**	*0.288*	-	-	**77.1**	*0.051*	**29.3**	*0.171*	**20.1**	*0.123*	**41.1**	*0.367*	-	-
M750F023		**5.3**	*0.032*	-	-	**5.8**	*0.004*	**3.6**	*0.021*	-	-	**27.5**	*0.245*	-	-
M750F024		**9.0**	*0.056*	-	-	-	-	-	-	-	-	**6.1**	*0.054*		
M750F024 / M750F025[1)]		-	-	-	-	**4.0**	*0.003*	**8.0**	*0.047*	-	-	-	-	-	-
M750F024, F025, other		**10.4** [1)]	*0.064* [1)]	-	-	-	-	-	-	-	-	-	-		
M750F025		**0.6**	*0.003*	-	-	-	-	-	-	-	-	**15.5**	*0.138*		
ERR	**ID**	**73.0**	*0.451*	-	-	**94.3**	*0.062*	**66.9**	*0.389*	**23.8**	*0.145*	**101.8**	*0.909*	-	-
	CHAR (HPLC)	**10.2**	*0.063*	-	-	**4.6**	*0.003*	**17.4**	*0.102*	**62.2**	*0.380*	**12.2**	*0.109*	-	-
	CHAR (other)	**0.6**	*0.004*	-	-	**1.1**	*0.001*	**1.7**	*0.010*	**1.6**	*0.010*	**1.1**	*0.010*	-	-
	sum ID/CHAR	**83.7**	*0.517*	-	-	**100.0**	*0.066*	**86.1**	*0.501*	**87.6**	*0.534*	**115.2**	*1.028*	-	-
RRR	**CHAR**	2.5	0.015	-	-	-	-	**2.8**	*0.016*	**4.2**	*0.026*	-	-	-	-
Final Residue		**2.2**	*0.013*	-	-	**2.8**	*0.002*	**2.4**	*0.014*	**1.7**	*0.011*	**0.3**	*0.002*	-	-
Grand Total		**88.3** [2)]	*0.545* [2)]	-	-	**102.8**	*0.068*	**91.3**	*0.532*	**93.6**	*0.570*	**115.4**	*1.031*	-	-

ID identification, CHAR characterization, sum ID/CHAR= sum of amounts identified and/or characterized, Final residue = residue after solubilization by protease treatment
[1)] The two metabolites M750F024 and M750F025 were not separated using HPLC. [2)] Approximately 10.5 % TRR were lost within a concentration step of the pooled methanol extract (Poo0029) to the HPLC sample (Lab0347).

Table 6.2.2-9: Summary of BAS 750 F and metabolites and of characterized fractions in hen matrices (T-label)

T-labeled residue component		egg yolk		egg white		muscle		liver		kidney		fat		excreta	
		%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
BAS 750 F		**43.7**	*0.117*	-	-	-	-	**3.7**	*0.018*	-	-	**20.1**	*0.038*	-	-
M750F034		-	-	-	-	-	-	**6.7**	*0.032*	-	-	-	-	-	-
M750F001		**41.4**	*0.111*	**83.2**	*0.297*	**91.4**	*0.322*	**85.2**	*0.409*	**65.6**	*0.371*	**73.1**	*0.139*	-	-
ERR	**ID**	**85.1**	*0.229*	**83.2**	*0.297*	**91.4**	*0.322*	**95.7**	*0.459*	**65.6**	*0.371*	**93.2**	*0.177*	-	-
	CHAR (HPLC)	-	-	-	-	-	-	-	-	**26.5**	*0.150*	-	-	-	-
	CHAR (other)	**6.2**	*0.017*	**14.8**	*0.053*	**10.0**	*0.035*	**1.3**	*0.006*	**1.1**	*0.007*	**5.0**	*0.010*	-	-
	sum ID/CHAR	**91.4**	*0.246*	**98.2**	*0.350*	**101.5**	*0.358*	**97.0**	*0.465*	**93.2**	*0.527*	**98.2**	*0.187*	-	-
RRR	**CHAR**	-	-	-	-	-	-	**1.8**	*0.009*	**1.3**	*0.008*	-	-	-	-
Final Residue		**2.6**	*0.007*	**0.2**	*<0.001*	**1.4**	*0.005*	**1.0**	*0.005*	**0.8**	*0.005*	**3.2**	*0.006*	-	-
Grand Total		**93.9**	*0.253*	**98.3**	*0.351*	**102.9**	*0.363*	**99.8**	*0.479*	**95.3**	*0.539*	**101.3**	*0.193*	-	-

ID identification, CHAR characterization, sum ID/CHAR= sum of amounts identified and/or characterized, Final residue = residue after solubilization by protease treatment

Table 6.2.2-10: Characterization of RRR by enzyme treatment (C-/ TFMP-/ T-label)

	RRR		CHAR [1] (protease digestion)		Final residue [2]	
C-label	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
yolk	- [3]	- [3]	**2.1**	*0.023*	**4.0**	*0.019*
muscle	**21.5**	*0.011*	**7.2**	*0.004*	**8.2**	*0.004*
liver	- [3]	- [3]	**7.2**	*0.023*	**7.3**	*0.023*
kidney	**12.9**	*0.055*	**10.4**	*0.044*	**2.2**	*0.010*
TFMP-label	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
yolk	**5.1**	*0.032*	**2.5**	*0.015*	**2.2**	*0.013*
muscle	-	-	-	-	-	-
liver	**5.6**	*0.033*	**2.8**	*0.016*	**2.4**	*0.014*
kidney	**6.3**	*0.038*	**4.2**	*0.026*	**1.7**	*0.011*
T-label	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
yolk	-	-	-	-	-	-
muscle	-	-	-	-	-	-
liver	**3.3**	*0.016*	**1.8**	*0.009*	**1.0**	*0.005*
kidney	**2.0**	*0.011*	**1.3**	*0.008*	**0.8**	*0.005*

[1] CHAR = characterized by protease digestion. [2] final residue after solvent extraction and solubilization
[3] due to limited sample size, the RRR after solvent extraction was not determined. As indicative information refer to data in extractability table (C-label)

3.3 muscle

Total extractable residue (ERR) accounted for >85.0% TRR. The residue after extraction (RRR) represented 21.5% (0.01 mg/kg, C-label), 3.5% (TFMP-label) and 1.4% (T-label). Protease treatment of C-labelled muscle reduced the final residue further to maximum of 8% TRR (0.004 mg/kg).

The total identified residue in muscle accounted for >75 %. Additional characterization by chromatographic and extraction properties accounted for 8.5-20.1% TRR resulting in amounts identified and characterized of >96%.

Metabolite M750F022 was the most abundant component of the residue in the C-label and TFMP-label (50% TRR and 77% TRR, equivalent to 0.03 mg/kg and 0.05 mg/kg). The second most abundant residue components were fatty acid conjugates of M750F022, together accounting for 19.5% and 9.8% TRR (equivalent to 0.01 mg/kg). Parent BAS 750 F was detected, albeit at lower amounts (5.6 and 7.4% TRR, equivalent to <0.01 mg/kg). The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples. In contrast, with the T-label, BAS 750 F was not present at detectable amounts. M750F001 was the only component identified with 91.4% TRR (0.32 mg/kg).

3.4 liver

Total extractable residue (ERR) accounted for >84% of TRR. The residue after extraction (RRR) represented 14.6%, 5.6% and 3.3% (corresponding to 0.046 mg/kg, 0.033 mg/kg and 0.016 mg/kg). A significant amount of the RRR is bound to liver protein as confirmed by release upon up to 7% TRR by protease treatment. For C-labeled liver the final residue was reduced to 7% (0.023 mg/kg), <5% for the TFMP- and the T-label.

The total identified residue in liver accounted for 55%, 67% and 96% TRR. Additional characterization by chromatographic and extraction properties accounted for 3-34% TRR resulting in amounts identified and characterized of >88% TRR.

Metabolite M750F022 was the most abundant component of the residue in the C-label and TFMP-label (36.7% TRR and 29.3% TRR, equivalent to 0.12 mg/kg and 0.18 mg/kg). Fatty acid conjugates of M750F022, taken together were found at 6.9% and 11.6% TRR (equivalent to 0.02 and 0.6 mg/kg). Parent BAS 750 F was detected, albeit at lower amounts (4.0 and 5.8% TRR, equivalent to <0.03 mg/kg mg/kg). The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples. In contrast, with the T-label, M750F001 was the predominant component identified with 85.2% TRR (0.41 mg/kg). BAS 750 F was present at 3.7% TRR (0.02 mg/kg).

In addition, a liver-specific metabolite was identified in all three labels, M750F034 accounting in the three labels for 4.3%, 20.1%, and 6.7% TRR (up to 0.03 mg/kg).

3.5 kidney

Total extractable residue (ERR) accounted for >84% of TRR. The residue after extraction (RRR) represented 13-15%, 6.3% and 2.0% (corresponding to 0.055 mg/kg, 0.038 mg/kg and 0.011 mg/kg). Protease treatment of reduced the final residue further to a maximum of 2% TRR (0.01 mg/kg).

The total identified residue in kidney accounted for 28%, 24%, and 66% TRR. Additional characterization by chromatographic and extraction properties accounted for 28-64% TRR resulting in amounts identified and/or characterized of 80%, 92% and 96% for C-, TFMP-, and T-label.

Metabolite M750F022 was the most abundant component of the residue in the C-label and TFMP-label (20% TRR, equivalent to 0.09 mg/kg and 0.12 mg/kg). In addition, fatty acid conjugates of M750F022 were found at 4% TRR (C-label only, 0.02 mg/kg). Parent BAS 750 F was detected, albeit at lower amounts (4.0 and 3.7% TRR, equivalent to 0.02 mg/kg). The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples.
In contrast, with the T-label BAS 750 F was not present at detectable amounts. M750F001 was the only component identified with 66% TRR (0.37 mg/kg).

3.6 fat

Total extractable residue (ERR) accounted for about 100% of TRR. The residue after extraction (RRR) was <0.01 mg/kg and was thus not further analyzed.
The total identified residue in fat accounted for >82 %. Additional characterization by chromatographic and extraction properties accounted for 2-17% TRR resulting in amounts identified and characterized of >93% TRR.

Metabolite M750F022 was the most abundant component of the residue in the C-label and TFMP-label (25% TRR and 41% TRR, equivalent to 0.18 mg/kg and 0.37 mg/kg). The second most abundant residue components were fatty acid conjugates of M750F022, together accounting for 49.1% and 78.6% TRR (equivalent to > 0.44 mg/kg). Treatment with lipase as well as alkaline treatment resulted in quantitative cleavage of the conjugate, thus releasing M750F022 (performed with TFMP-labelled fat).
The position of the radiocarbon in the T-label explains why M750F022 and its conjugates is not detected in T-label samples. Parent BAS 750 F was detected, albeit at lower amounts (5.4 and 3.7% TRR, equivalent to 0.04 and 0.10 mg/kg). The position of the T-label radiocarbon in the T-ring explains why M750F022 is not detected in T-label samples.
In contrast, with the T-label, M750F001 was the most abundant component identified with 73.1% TRR (0.14 mg/kg). BAS 750 F was present at 20.1% TRR (0.04 mg/kg).

3.7 excreta

Total extractable residue (ERR) accounted for >89% TRR (C- and T-label). The total identified residue accounted for 31.6% TRR (C-label only). Additional characterization by chromatographic and extraction properties accounted for 56% TRR resulting in amounts identified and characterized of 88% TRR. Parent BAS 750 F was the most abundant component of the residue (28.6% TRR) while M750F022 was present at 3.1% TRR.

4. Enantiomer ratio of BAS 750 F

Chiral analysis of BAS 750 F residue in representative samples of the hen metabolism study was restricted to samples where BAS 750 F levels were high enough to allow chiral HPLC analysis. While in whole egg, egg white, muscle, and liver, concentrations were too low, chiral analysis could be done for egg yolk and fat (see Table 6.2.2-7). The ratio of enantiomers in both egg yolk and fat was 43:56, thus comparable to the ratio in the dose administered.

Table 6.2.2-11: Determination of isomer ratio of BAS 750 F in hen matrices

matrix	S-enantiomer [%]	R-enantiomer [%]
T-label		
administered dose	50.4	49.6
egg yolk	43.0	57.0
fat	43.1	56.9

1) Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358)

5. Proposed metabolic pathway

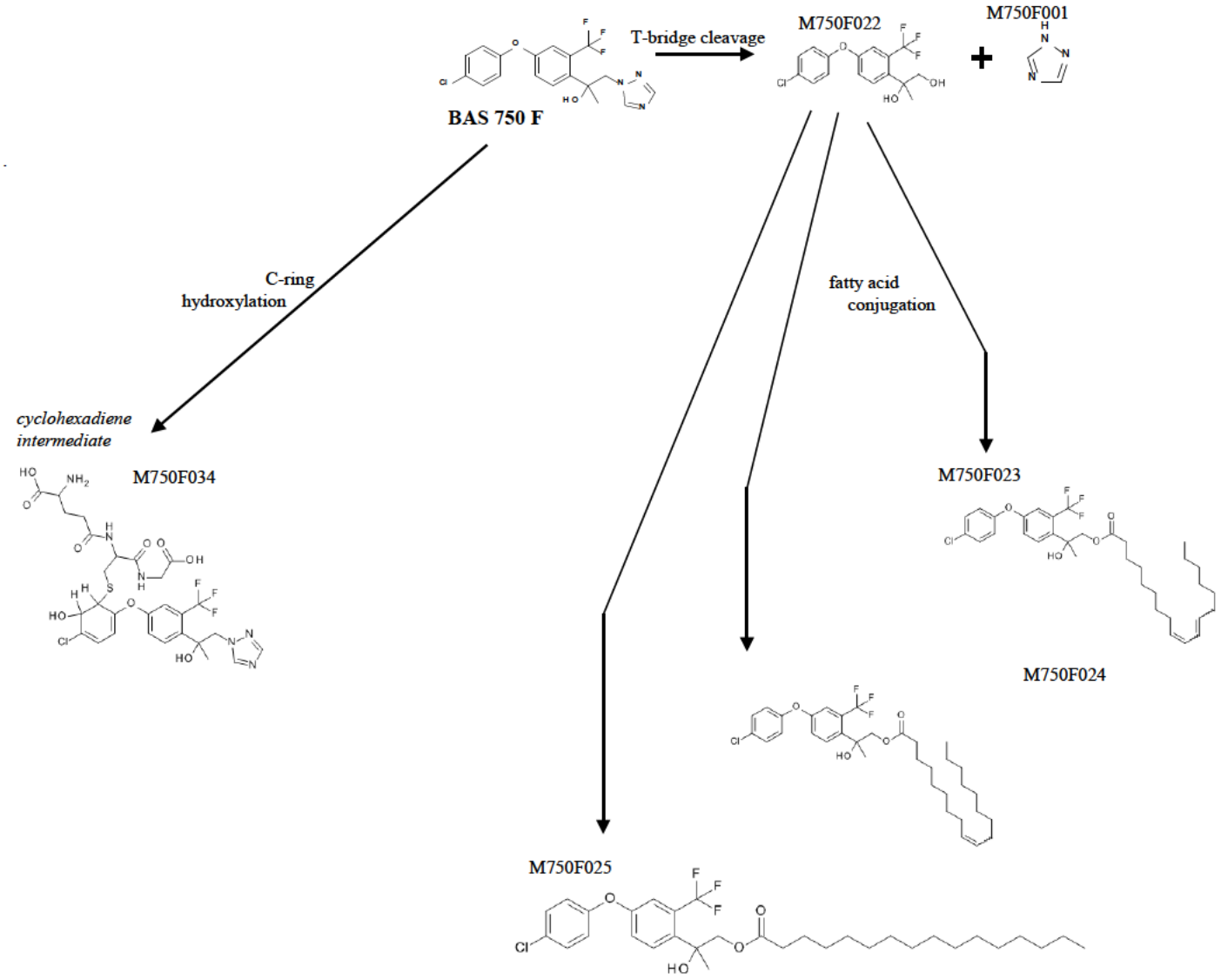
Metabolism of BAS 750 F was investigated in laying hen using three labels, namely the C-label, the TFMP-label and the T-label. Results taken together indicate comparable pathways, the label specific differences observed are consistent with effective cleavage of the parent backbone at the triazole-bridge, cleaving off the triazole ring.

Overall, results show that in laying hen, BAS 750 F is metabolized by two main transformation reactions:

- C-ring hydroxylation (followed by conjugation)
- cleavage of the parent backbone at the triazole bridge (notably followed by fatty acid conjugation of cleavage product M750F022)

Cleavage of BAS 750 F generates the metabolite 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to acylation with various fatty acids resulting in a range of fatty acid conjugates namely as M750F023, M750F024 and M750F025. In addition, exclusively in liver, a metabolite was detected showing that the parent backbone is modified by oxygenation of the C-ring followed by conjugation with glutathione resulting in metabolite M750F034.

Figure 6.2-10: Proposed metabolic pathway of BAS 750 F in laying hens



6. Extractability of residues according to analytical methods

The extractability of parent BAS 750 F and metabolite M750F022 is covered by a separate extractability study, DocID 2015/1161960 (see CA6.2.2/2). Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F and M750F022 in commodities of animal origin was investigated using samples representative of relevant livestock matrices with radiolabeled residue (radiovalidation). Samples were obtained from a study with laying hens, BASF DocID 2015/1001001, and a study with lactating goat, BASF DocID 2015/1078841 (see CA 6.2.2 and CA 6.2.3). Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction for the analytical methods, method L0272/01 for BAS 750 F and L0309/01 for metabolite M750F022.

For BAS 750 F, extraction efficiencies generally were 80% or higher for most matrices (milk, cream, muscle, kidney, fat, egg yolk), and lower for liver (46%).

For M750F022, extraction efficiencies generally were 90% or higher for most matrices (milk, cream, kidney, fat) and lower for egg yolk (66%), for muscle (61%) and for liver (46-50%).

7. Storage stability

Investigation of storage stability of radioactive residue either in matrix (deep frozen storage) or in extract (storage in fridge) was performed for C-labeled and T-labeled samples and extracts representating all edible matrices (see table below). Comparison of chromatographic profiles did show absence of significant changes of composition and amounts indicating both for matrix and extract stability of the radioactive residue over the time investigated. The time interval of confirmed stability for matrix is at least 376 days and for extract at least 354 days. Also stability of TFMP-labeled samples can be assumed for this time interval based on absence of TFMP-specific residue components. Therefore no further stability investigations were conducted with TFMP-labeled samples.

Table 6.2.2-12: Storage stability investigations in hen matrices
(*representative information, further data provided in study report*)

matrix	storage of matrix		storage of extract	
	storage interval (analysis 1) [1)]	storage interval (analysis 2) [1)]	storage interval (analysis 1) [2)]	storage interval (analysis 2) [2)]
	[days]	*[days]*	*[days]*	*[days]*
C-label				
muscle (MeOH extract)	84	399	13	140
egg yolk (MeOH extract)	184	560	77	314
fat (ACN phase)	195	561	3	346
liver (ACN phase)	175	553	-	-
liver (MeOH extract)	274	-	48	274
T-label				
egg white (MeOH extract)	150	-	13	363
egg yolk (MeOH extract)	149	492	18	364
fat (MeOH phase)	161	-	3	345
liver (MeOH extract)	141	-	15	369

ACN denotes acetonitrile phase (of acetonitril extracts or methanol extracts), MeOH denotes methanol,
For egg white and yolk, the sampling date encompasses the treatment interval of 168-188h, the longest storage interval is represented.

III. CONCLUSION METABOLISM IN LAYING HEN

For the three labels, the overall accountability of the study was good. The radioactive residue was rapidly and extensively excreted. Until sacrifice, the radioactive residues in excreta amounted to 75-89% of the total radioactivity administered. For all labels, only low portions of the administered dose (≤ 0.4 %) were retained in edible tissues or in egg (<1% of dose).

14C residues in egg (sampled on 14 consecutive days) reached a plateau concentration within 5-7 days confirming absence of accumulation of residues in egg. Plateau level in egg yolk were at 0.5 mg/kg, 0.6 mg/kg, and 0.3 mg/kg (C-, TFMP-, T-label). In egg white residues were <0.1 mg/kg (C-and TFMP-label) and 0.3 mg/kg for T-label. Label-specific differences were also seen for TRR from other matrices except kidney (0.43-0.59 mg/kg). C-label and TFMP-label were generally comparable, but distinct from T-label. In muscle and egg white, TRR for C-/TFMP-label (<0.08 mg/kg) was lower than for the T-label (>0.36 mg/kg). In contrast in liver and fat, TRR for C-/TFMP-label (>0.31 mg/kg) was higher than for the T-label (<0.18 mg/kg). Taken together, this data indicates the presence of significant amounts of first, metabolites which carry only the T-label (thus not detectable with C-/TFMP-label) and second, metabolites which do not carry the T-label. For all matrices, TRR of 0.3-0.6 mg/kg (in fat up to 1.2 mg/kg) were observed in at least one label. Note that each label only detects a fraction of the BAS 750 F residue.

The extractability of radioactive residues from all edible matrices (egg white and yolk, muscle, liver, kidney and fat was high. Investigation of the RRR obtained after protease treatment reduced the final residue further to mostly <5% TRR, exceeded only for liver 7% (0.02 mg/kg, C-label), and muscle (<6%TRR, <0.01 mg/kg, C-label). Overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in laying hen includes two main transformation reactions, cleavage of the parent backbone at the T-bridge generating 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to conjugation by fatty acids (M750F023/M750F024/M750F025). In addition, a transformation reaction seen in liver is the C-ring oxygenation followed by glutathione conjugation (M750F034). The parent BAS 750 F was applied as a racemic mixture of two enantiomers. Chiral analysis of BAS 750 F in egg yolk and in fat showed absence of significant ratio change, while for the other matrices BAS 750 F was present only at very low amounts precluding chiral analysis.

For the metabolite profile, label-dependent differences similar to the observations at TRR level were seen. Generally, results of C-label and TFMP-label were comparable, and distinct from the T-label.

With both C- and TFMP-label, the cleavage metabolite M750F022 together with its fatty acid conjugates the predominant component of the residue, together accounting for >69% TRR in yolk, muscle, and fat, and 20-44% TRR in liver and kidney (egg white with TRR <0.01 mg/kg was not further investigated). M750F022 was the most abundant compound in muscle, liver, kidney and yolk, while in fat the conjugates were present in up to 3X higher amounts. While the sum of M750F022 and its conjugates was similar for both labels, the conjugation ratio however showed some variability between both labels (notably for liver, muscle and fat).

The cleavage metabolite 1,2,4-triazole (M750F001) was found at major level in all matrices, proportions were >65% TRR in egg white, muscle, liver, kidney and fat, and 41% TRR in egg yolk.

Parent BAS 750 F was present in all matrices investigated except egg white. Major amounts of BAS 750 F were determined in in egg yolk (7-44% TRR) and fat (5-20% TRR), while proportions were low in muscle, liver, and kidney (<7% TRR).

The only other component identified is the liver-specific metabolite M750F034, a glutathione conjugate of parent found at 4-20% TRR (0.01-0.12 mg/kg).

In conclusion, the major components of the residue in hen were identified as 1,2,4-triazole, metabolite M750F022 together with its fatty acid conjugates, parent BAS 750 F as well as a liver-specific metabolite (M750F034). Overall, metabolism of BAS 750 F in laying hen can be considered well-elucidated.

Report:	CA 6.2.2/2 Thiaener J.,Glaessgen W.E., 2015 b Investigation of the extractability of BAS 750 F and M750F022 in samples from 14C animal metabolism studies 2015/1161960
Guidelines:	EPA 860.1300: Nature of the Residue in Plants Livestock, EPA 860.1000: EPA Residue Chemistry Test Guidelines, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 91/414 Annex II (Part A Section 6), EEC 91/414 Annex III (Part A Section 8), EEC 91/414 (7030(VI/95 Rev. 3)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F and M750F022 in commodities of animal origin as investigated using samples with radiolabeled residue (radiovalidation). Samples were obtained in livestock metabolism studies (see CA 6.2 and CA 6.3), namely in laying hens (BASF DocID 2015/1001001) and in lactating goat (BASF DocID 2015/1078841).

Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction for the analytical methods, method L0272/01 for BAS 750 F and L0309/01 for metabolite M750F022.

For BAS 750 F, extraction efficiencies generally were 80% or higher for most matrices (milk, cream, muscle, kidney, fat, egg yolk), and lower for liver (46%). For M750F022, extraction efficiencies generally were 90% or higher for most matrices (milk, cream, kidney, fat) and lower for egg yolk (66%), for muscle (61%) and for liver (46-50%).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: *not relevant*

2. Test Commodity: *not relevant*

Samples from metabolism studies in hen (see section CA 6.2.2, BASF DocID 2015/1001001), namely fat, liver, muscle and egg yolk and in goat (see section CA 6.2.3, BASF DocID 2015/1078841) namely cream, whole milk and kidney were used to investigate extraction efficiency of radiolabeled residues of BAS 750 F.

B. STUDY DESIGN AND METHODS

1. Test procedure *not relevant*

2. Description of analytical procedures

Extraction procedures used in analytical methods, namely BASF method L0272/01 and BASF method L0309/01 (residue analysis in animal matrices) were compared to the extraction procedure used in the hen and goat metabolism studies (see section CA6.2.2 and CA6.2.3).

Principle of the extraction procedures

Analysis of BAS 750 F and M750F022 in the extracts was done by radio-HPLC. Total radioactivity in extract was determined by liquid scintillation counting. The values for “TRR calculated” determined in the goat metabolism study (section CA 6.2.3) were used as reference value for goat matrices (milk, kidney), while “TRR measured” determined in the hen metabolism study was taken as reference value for hen matrices (fat, liver, muscle, egg yolk). For cream (sample of goat metabolism study), the TRR was determined as part of the present study.

2.1 Procedure in goat metabolism study

Milk and cream was extracted with acetonitrile (1x) and isohexane (1x). Kidney was extracted with methanol (3x, extracts combined) and water (2x, extracts combined). Acetonitrile and methanol extracts obtained from cream and kidney were centrifuged and concentrated prior to HPLC analyses. The acetonitrile extract from milk was cleaned-up by SPE, concentrated and analysed by HPLC. Isohexane and water extract were subjected to LSC analysis. The non-extracted residue was dried and subjected to combustion analysis.

2.2 Procedure in hen metabolism study

Fat was extracted twice with acetonitrile/isohexane (1/1, v/v), acetonitrile phases and isohexane phases each were pooled. The isohexane-pool was partitioned against acetonitrile (3x). The resulting acetonitrile phases were pooled with the previous acetonitrile-pool, concentrated, and analysed by HPLC.
Liver and egg yolk were extracted with methanol (3x) and water (2x). Muscle was extracted with methanol (3x). Pooled methanol extracts were concentrated prior to HPLC analysis. Water extract was subjected to LSC analyses.

2.3 Procedure of BAS 750 F-method L0272/01

Fat-rich animal matrices (goat cream, goat milk and hen fat) were extracted with acetonitrile/isohexane (100/40, v/v). The acetonitrile and isohexane phase of the extract were separated.
Protein-rich animal matrices (goat kidney, hen liver, hen muscle, egg yolk) were extracted with methanol/water/2N HCl (75/25/5, v/v/v). The methanol/water/2N HCl extract was cleaned-up by adding 0.2N HCl to the supernatant after centrifugation prior to partition against cyclohexane (2x).
The acetonitrile phase and the combined cyclohexane phase were concentrated to dryness. The residues were re-dissolved in a mixture containing appropriate ratios of acetonitrile, 20 mmol/L ammonium formate and Triton X-100 prior to LSC and HPLC analyses.
The isohexane phase of the extract and the methanol/water/2N HCl phase after partitioning of the extracts were subjected to LSC analysis. The non-extracted residue was dried and subjected to combustion analysis.

2.4 Procedure of M750F022-method L0309/01

Fat-rich animal matrices (goat cream, goat milk and hen fat) were extracted with acetonitrile/isohexane (50/20, v/v). The acetonitrile and isohexane phase of the extract were separated.
Protein-rich matrices (goat kidney, hen liver, hen muscle, egg yolk) were extracted with methanol/water/2N HCl (75/25/5, v/v/v). The methanol/water/2N HCl extract was purified by adding 0.2N NaOH to the centrifuged supernatant prior to partitioning (2x) against dichloromethane (goat kidney) or cyclohexane (hen liver and muscle).
The acetonitrile phase and the combined DCM or cyclohexane phase were concentrated to dryness and the residues were re-dissolved in a mixture containing appropriate ratios either of acetonitrile, 20 mmol/L ammonium formate and Triton X-100 or methanol, acetonitrile, 20 mmol/L ammonium formate and Triton X-100 prior to LSC and HPLC analyses.
The isohexane phase of the extract and the methanol/water/2N HCl phase after partitioning of the extracts were subjected to LSC analysis. The non-extracted residue was dried and subjected to combustion analysis.

II. RESULTS AND DISCUSSION

The amount of radioactive residue extracted in the livestock metabolism study (ERR value) was taken as reference value for extraction efficiency (thus, ERR was set to 100%). The amount extracted with an analytical method was compared to this value, and expressed in percentage of the reference value (see Table 6.2.2-13 and Table 6.2.2-14). Note that, in milk residues of M750F022 are very low, thus a precise calculation of extraction efficiency was not feasible.

Table 6.2.2-13: Summary of extractability: radioactive residues and parent BAS 750 F

extraction procedure	TRR	radioactive residue (in ERR)			BAS 750 F		
	[mg/kg]	*[mg/kg]*	*[% TRR]*	***extraction efficiency (%) 1)***	*[mg/kg]*	*[% TRR]*	***extraction efficiency (%) 1)***
cream (goat)							
goat metabolism study	0.207	0.203	98.4	100.0	0.156	75.6	100.0
L0272/01 2)	0.272	0.288	105.8	**107.4**	0.225	82.5	**109.1**
		0.271	99.3	**100.9**	0.207	75.8	**100.2**
milk (goat)							
goat metabolism study	0.062	0.061	98.1	100.0	0.028	44.5	100.0
L0272/01		0.050	80.8	**82.3**	0.025	40.1	**90.1**
kidney (goat)							
goat metabolism study	0.429	0.422	98.2	100.0	0.198	46.0	100.0
L0272/01		0.386	89.9	**91.5**	0.162	37.7	**82.0**
fat (hen)							
hen metabolism study	0.702	0.707	100.8	100.0	0.038	5.4	100.0
L0272/01		0.690	98.4	**97.5**	0.036	5.1	**95.0**
liver (hen)							
hen metabolism study	0.320	0.265	82.7	100.0	0.023	7.2	100.0
L0272/01 2)		0.221	69.0	**83.5**	0.011	3.3	**46.4**
		0.225	70.2	**84.9**	0.011	3.4	**47.9**
muscle (hen)							
hen metabolism study	0.050	0.043	85.0	100.0	0.003	5.6	100.0
L0272/01		0.033	66.2	**77.9**	0.003	5.2	**94.1**
egg yolk							
hen metabolism study	0.477	0.426	89.4	100.0	0.031	6.5	100.0
L0272/01		0.266	55.7	**62.4**	0.028	6.0	**92.4**

1) *extraction efficiency = amounts extracted with analytical method compared amount extracted in metabolism study (set to 100%). For cream (goat) extraction efficiency was calculated as the %TRR in metabolism study divided by %TRR extracted with analytical method. 2) In the case of cream (goat) and liver (hen), two different subsamples from different containers were extracted for confirmation.*

Table 6.2.2-14: Summary of extractability: radioactive residues and M750F022

extraction procedure	TRR	ERR			M750F022		
	[mg/kg]	*[mg/kg]*	*[% TRR]*	***extraction efficiency (%)*** [1]	*[mg/kg]*	*[% TRR]*	***extraction efficiency (%)*** [1]
cream (goat)							
goat metabolism study	0.207	0.203	98.4	100.0	0.009	4.2	100.0
L0309/01 [2]	0.272	0.289	106.0	**107.6**	0.012	4.5	**106.9**
		0.279	102.5	**104.2**	0.008	3.0	**73.1**
milk (goat)							
goat metabolism study	0.062	0.061	98.1	100.0	0.001	1.2	100.0
L0309/01		0.050	80.4	**81.9**	0.002	2.5	***(213.6)*** [3]
kidney (goat)							
goat metabolism study	0.429	0.422	98.2	100.0	0.046	10.7	100.0
BASF method L0272/01		0.362	84.2	**85.8**	0.042	9.8	**91.7**
fat (hen)							
hen metabolism study	0.702	0.707	100.8	100.0	0.178	25.4	100.0
L0309/01		0.639	91.0	**90.3**	0.167	23.8	**93.7**
liver (hen)							
hen metabolism study	0.320	0.265	82.7	100.0	0.118	36.7	100.0
L0309/01 [2]		0.161	50.3	**60.8**	0.058	18.3	**49.7**
		0.203	63.4	**76.6**	0.057	17.7	**48.2**
		0.196	61.2	**74.0**	0.054	17.0	**46.3**
muscle (hen)							
hen metabolism study	0.050	0.043	85.0	100.0	0.025	49.9	100.0
L0309/01		0.027	53.1	**62.5**	0.015	30.4	**60.9**
egg yolk							
hen metabolism study	0.477	0.426	89.4	100.0	0.186	39.0	100.0
L0309/01 [2]		0.242	50.7	**56.7**	0.123	25.8	**66.2**
		0.211	44.2	**49.5**	0.102 [4]	21.3 [4]	*(54.7)* [4]

1) *extraction efficiency = amounts extracted with analytical method compared to amount extracted in metabolism study (later set to 100%). For cream (goat) extraction efficiency was calculated as the %TRR in metabolism study divided by %TRR extracted with analytical method. 2) As confirmation of the results obtained, for cream (goat) two different subsamples from different containers were extracted for confirmation. For liver (hen) three different subsamples (of the same container) were extracted. For egg yolk (hen), two different subsamples (of the same container) were extracted. 3) The value obtained for milk is indicative only. The value calculated for milk appears to be an overestimation resulting from non-precise data due to low analyte amount. 4) The value calculated for yolk is an underestimation since an additional 0.011 mg/kg (2.4% TRR) was recovers from the contained after extract concentration prior to HPLC analysis.*

Extraction efficiency of method LC0272/01

Concerning “radioactive residue” (ERR), the extraction efficiency achieved with the analytical method L0272/01 was matrix-dependent. For milk, cream, kidney, and fat, the amounts extracted with the analytical method were similar to the amounts extracted in the metabolism study. Thus, extraction efficiency for the analytical method generally was high (82% or higher). Lower extraction efficiencies were determined for liver (84%), for muscle (78%) and for egg yolk (62%).

Analysis specifically of the analyte BAS 750 F, showed that the analytical method L0272/01 achieved high extraction efficiency for all matrices except liver. Comparing amounts of BAS 750 F extracted with the analytical method to the BAS 750 F amounts extracted in the metabolism study, extraction efficiencies of 90% (milk), 100% (cream), 82% kidney, fat (95%), muscle (94%), egg yolk (92%) were obtained while 46-48% was obtained for liver. Taken together, the analytical method for BAS 750 F shows good extraction efficiency in all matrices, and moderate extraction efficiency in liver.

Extraction efficiency of method LC0309/01

Concerning “radioactive residue” (ERR), the extraction efficiency achieved with the analytical method L0309/01 was matrix-dependent. For milk, cream, kidney and fat, the amounts extracted with the analytical method were similar to the amounts extracted in the metabolism study. Thus, extraction efficiency for the analytical method was high (82% or higher). Lower extraction efficiencies were determined for liver (61-77%), for muscle (63%) and for egg yolk (50-57%).

Analysis specifically of the analyte M750F022, showed that the analytical method L0309/01 achieved high extraction efficiency for milk (100%) for cream (73-107%), for kidney (92%), and for fat (94%), good extraction efficiency was determined for muscle (61%) and egg yolk (66%) and only moderate extraction efficiency for liver (46-50%). Note that for milk, the data obtained indicates high extraction efficiency of method L0309/01 for M750F022, a precise determination of extraction efficiency was not feasible due to the very low analyte amounts in milk sample. Taken together, the analytical method for M750F022 shows good extraction efficiency in all matrices, and moderate extraction efficiency in liver.

III. CONCLUSION

Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction for the analytical methods, method L0272/01 for BAS 750 F and L0309/01 for metabolite M750F022.
For BAS 750 F, extraction efficiencies generally were 80% or higher for most matrices (milk, cream, muscle, kidney, fat, egg yolk), and lower for liver (46%). For M750F022, extraction efficiencies generally were 90% or higher for most matrices (milk, cream, kidney, fat) and lower for egg yolk (66%), for muscle (61%) and for liver (46-50%).

CA 6.2.3 Lactating ruminants

Report:	CA 6.2.3/1 [redacted] 2015 a The metabolism of 14C-Reg. No. 5834378 (BAS 750 F) in lactating goats 2015/1078841
Guidelines:	OECD Test Guideline 503 - Metabolism in livestock, EPA 860.1000: EPA Residue Chemistry Test Guidelines, EPA 860.1300: Nature of the Residue in Plants Livestock, EEC 91/414 (7030(VI/95 Rev. 3), PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), JMAFF 59 NohSan No 4200
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in lactating goats following repeated oral administration of ^{14}C-BAS 750 F, labeled either in the Chlorophenyl ring (C-ring), in the Trifluoromethylphenyl ring (TFMP-ring) or in the triazole ring (T-ring). The test item was administered orally by gavage to goat (1-2 animals per label) for 12-14 consecutive days at a nominal dose of 12 mg/kg feed. The mean actual concentrations were 15.5 mg/kg feed (C-label), 23.4 mg/kg feed (TFMP-label) and 17.5 mg/kg feed (T-label), corresponding to daily means of 0.35-0.45 mg/kg body weight, respectively.

For the three labels, the overall accountability of the study was good. The radioactive residue was rapidly and extensively excreted. Until sacrifice, the radioactive residues excreted via urine and feces (including GI tract contents) amounted for all labels to >77% of the total radioactivity administered. The total radioactive residues (TRR) detected in pooled samples of urine ranged from 2.9 - 5.3 mg/kg and the TRR in the investigated feces samples was between 3.2 - 5.5 mg/kg. Bile contained 4.0 - 11.7 mg/kg.

^{14}C residues in milk (sampled on 12-14 consecutive days) reached a plateau concentration within 5 days (<0.1 mg/kg for C- and TFMP-label, around 0.3 mg/kg for T-label) confirming absence of accumulation of residues in milk. Overall, the TRR in whole milk, skim milk and muscle tissues of the C- and TFMP-label were low and ranged from 0.016 mg/kg to a maximum of 0.099 mg/kg. In T-labeled milk, skim milk and cream, higher amounts were detected (0.27 - 0.29 mg/kg). The residues in cream and fat (all labels) were between 0.20 - 0.52 mg/kg. In C-, TFMP- and T-labeled kidney and liver, residues were in the range of 0.35-0.42 mg/kg (kidney) and the range of 0.65-1.33 mg/kg (liver).

The extractability of radioactive residues from all edible matrices (milk samples, muscle, fat, kidney and liver) was high (≥ 89.9 % TRR) for all of the three labels. Therefore, a further investigation of the RRR obtained after solvent extraction was performed only for liver (all labels). Protease treatment of the RRR released 1.8% - 3.4% TRR indicating protein-bound residue in liver. Overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in lactating goat includes, besides O-conjugation of the unchanged parent (M750F068), two main transformation reactions.

The first main transformation reaction is oxygenation of the C-ring of the uncleaved parent BAS 750 F (M750F015, M750F016, M750F017), followed by conjugation (M750F063). The metabolite M750F041 and M750F091 are intermediates with a cyclohexadiene structure of the C-ring prior to re-aromatization. A second transformation reaction is cleavage of the parent backbone at the T-bridge generating 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to oxidation (M750F038), followed by demethylation (M750F040), as well as to C-ring hydroxylation (M750F078), to sulfatation (M750F043), and to glucuronidation (M750F064). A transformation observed only to a minor extend is cleavage of the parent backbone at the ether bridge, generating the two ring metabolite M750F003. Notably, metabolites consisting of only either the C-ring or the TFMP-ring were not observed in any of the samples of the C-label or the TFMP-label. A further minor transformation is the hydroxylation of the methyl group at the quaternary C-atom of BAS 750 F (M750F039) which can be oxidized further (M750F042) or conjugation with sulfate (M750F072).

The parent BAS 750 F was applied as a racemic mixture of two enantiomers. Chiral analysis of BAS 750 F revealed a significant change of the ratio in most matrices, with proportion of the R-enantiomer of 70-80% in cream, muscle, liver, kidney and fat. In contrast, the racemate was maintained in feces, indicating a preferential metabolisation of the S-enantiomer. Note that a comparable change of isomer ratio was also observed in rats dosed with BAS 750 F (rat metabolism study, see section CA 5.1.1).

For the metabolite profile, label-dependent differences similar to the observations at TRR level were seen. Results of C-label and TFMP-label were comparable, and distinct from the T-label.

With both C- and TFMP-label, parent BAS 750 F was seen as the most abundant compound (fat/muscle >85% TRR, milk/liver >45% TRR, kidney 28-46% TRR). Modification of the intact parent backbone was seen at major amounts, in liver (hydroxy metabolite M750F016 up to 15% TRR, 0.20 mg/kg) and in kidney (glucuronide M750F068 up to 18% TRR, 0.06 mg/kg). Other transformed parent metabolites were present at <7% TRR, in kidney (hydroxy metabolites M750F016/M750F015), in liver (glucuronide M750F068), and in milk (sulfate metabolite M750F072 and the intermediate (C-ring)-cyclohexadiene metabolite M750F041).

The cleavage metabolite M750F022 was seen in all matrices, albeit at much lower levels (<8% TRR in milk, muscle, liver, kidney, fat), only in TFMP-labeled kidney at 10.7% TRR (yet corresponding to <0.05 mg/kg). M750F022 appears to be metabolized further effectively in goat, as indicated by its oxidized/glucuronidated products (M750F038/M750F064) present in liver (7-11% TRR) and kidney (14-27% TRR) as well as its sulfation product M750F043 present in milk (14-25% TRR, up to 0.16 mg/kg). In addition, cleavage of the ether bridge did occur to a low extend in kidney only, as indicated by M750F003 (3% TRR, 0.01 mg/kg).

For the T-label, 1,2,4-triazole (non-detectable with the other two labels) was the predominant component in milk, muscle, and kidney (>68% TRR), while this polar component was present only in low amounts in fat (5% TRR, 0.01 mg/kg). In liver, 1,2,4-triazole, together with another triazole derivative metabolite, was found in a fraction of 32% TRR (ratio 2:1). Parent BAS 750 F was present as the most abundant compound only in fat (85% TRR) and liver (26% TRR), in major amounts in muscle and kidney (10-12%TRR), and low amounts in milk (3%). The only other compounds were modified parent (C-ring hydroxy metabolite M750F016 and glucuronide M750F068), found only in liver, where the metabolic profile was consistent with the profile seen with the other labels (M750F022 and M750F038 are non-detectable with the T-label).

In conclusion, the major components of the residue in goat was identified as unchanged parent BAS 750 F and the TDM which together represent a large proportion of the residue. TDM exceed parent in all matrices except fat. Considering the non-TDM residue, parent represents 85% TRR in muscle and fat, >45% of TRR in milk and liver, 28-46% TRR in kidney. The cleavage product M750F022 was present at much lower levels (<7% TRR, except one kidney sample). For both parent and M750F022 presence of several downstream transformation products indicate effective further metabolic transformation. Overall, metabolism of BAS 750 F in lactating goats, and by extrapolation in ruminant livestock, can be considered well-elucidated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

1.1 C-label

Description:	Chlorophenyl-U-C14-BAS 750 F (spec activity 7.88 MBq/mg) with Chlorophenyl-1-C13-labeled and unlabeled test item	
Lot/Batch #:	Chlorophenyl-U-C14:	CFQ41561
	Chlorophenyl-1-C13:	RS4-2012-173A2
	Unlabeled:	COD-001740
Purity:	Chlorophenyl-U-C14:	radiochem. purity:98.9% chemical purity: 99.1%
	Chlorophenyl-1-C13:	97.7%
	Unlabeled:	98.8%
CAS #:	1417782-03-6	
Development Code:	BAS 750 F	

1.2 TFMP-label

Description:	Trifluoromethylphenyl-U-C14-BAS 750 F (spec activity 8.3 MBq/mg) with Propyl-2-C13-labeled and unlabeled test item	
Lot/Batch #:	Trifluoromethylphenyl-U-C14:	CFQ42039
	Propyl-2-C13:	1126-1006
	Unlabeled:	COD-001740
Purity:	Trifluoromethylphenyl-U-C14:	radiochem purity: 98.3% chemical purity: 96.3%
	Propyl-2-C13:	99.5%
	Unlabeled:	98.8%
CAS #:	1417782-03-6	
Development Code:	BAS 750 F	

1.3	**T-label**	
Description:	Triazole-3(5)-C14-BAS 750 F (spec activity 5.46 MBq/mg) with Triazole-3(5)-C13-labeled and unlabeled test item	
Lot/Batch #:	Triazole-3(5)-C14:	1062-2001
	Triazole-3(5)-C13:	1077-1001
	Unlabeled:	COD-001740
Purity:	Triazole-3(5)-C14:	radiochem purity: 98.8% chemical purity: 98.9%
	Triazole-3(5)-C13:	97.1%
	Unlabeled:	98.8%
CAS #:	1417782-03-6	
Development Code:	BAS 750 F	

2. Test Animals

Species:	goat
Variety:	*"British Saanen"*
Gender:	female
Age:	*not reported*
Weight at dosing:	52.5.0-62.0 kg
Number of animals:	5 (C-label and T-label, each 2 goats, TFMP-label 1 goat)
Acclimation period:	23-30 days
Diet:	2 x 0.5 kg non-medicated concentrate + 1 kg hay
Water:	drinking water, *ad libitum*
Housing:	individually in pens (acclimation period) in metabolism cages (dosing period)
Environmental conditions	
Temperature:	14-22°C
Humidity:	30-70% relative humidity
Photoperiod:	12 light, 12 hour dark cycle

B. STUDY DESIGN AND METHODS

1. Dosing regime

Oral:	**amount of dose:**	nominal dose: 12 mg/kg feed/day mean actual doses (per dry weight diet/day) *C-label: 15.5mg/kg (0.36 mg/kg bw/d)* *TFMP-label: 23.4 mg/kg (0.40 mg/kg bw/d)* *T-label: 17.5 mg/kg (0.43 mg/kg bw/d)*
	Food consumption:	0.377-2.780 kg/animal/day (dosing phase)
	Vehicle:	gelatine capsule (applied by dosing gun)
	Timing:	once daily
	Duration:	12-14 days

2. Sample collection

Milk collection: twice daily
Urine and feces collection: daily
Interval from last dose to sacrifice: 23 h

Tissues harvested & analysed:
liver, kidneys, omental fat, renal fat, subcutaneous fat, flank muscle, loin muscle, (blood: samples were taken prior to first dose and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h post first dose; GI tract and contents, bile)

3. Test procedure

The in-life phase of this study was performed Nov 2013 to June 2015 (Quotient Bioresearch (Rushden) Ltd.), the metabolism part from June 2013 to Nov 2015 (BASF SE). A total of 5 goats was dosed with radiolabeled BAS 750 F at nominal doses of 12 mg/kg for 14 consecutive days (dosing with the C-label included 2 goats, dosing with the TFMP-label 1 goat, dosing with the T-label 2 goats). Each ^{14}C-labeled test item was mixed with ^{13}C-labeled test item and unlabeled test item (ratios are given in Table 6.2.3-1 below). The test items were prepared in capsules and administered orally once daily using a dosing gun. The actual dose was based on the feed consumption of the individual goats. The mean achieved daily dose administered was 13.7 - 23.4 mg/kg food consumed (dry weight equivalent). Details of the study outline are summarized in the following table.

Table 6.2.3-1: Dosing of lactating goats with BAS 750 F

animal no.	treatment period (days)	isotope ratio ^{14}C : ^{13}C : ^{12}C	nominal daily dose	actual daily dose [1]		time of sacrifice [2] (hours)
			[mg/kg feed]	*[mg/kg feed]*	*[mg/kg bw/d]*	
C-label: 1, 2	14	1 : 1 : 2	12	13.7, 17.3	0.36	23
TFMP-label: 5	12	1 : 2 : 2	12	23.4	0.40	23
T-label: 3, 4	14	1 : 1 : 1	12	15.7, 19.3	0.43	23

1) based on mean body weights on study day1 2) hours after last dose

4. Sampling and storage

Blood samples were taken prior to first dose and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h after start of dosing. Excreta, urine and feces, were collected for a 24 h interval prior to first dose and subsequently every 24 h until sacrifice. Each goat was milked twice daily with the last sample taken immediately prior to sacrifice. Since the timepoint of maximum residue concentration in plasma (t_{max}) had been determined to be approximately 24 h after the final dose, the goats were sacrificed after 23 h and edible tissues (liver, kidney, loin muscle, flank muscle, omental fat, subcutaneous fat and renal fat), bile, blood and the GI tract collected. All samples were stored at -18 °C.

5. Description of analytical methods

The stability, homogeneity and correctness of the concentration of the test item preparations as well as the radiochemical purity of ^{14}C-BAS 750 F were confirmed by reversed-phase HPLC analysis and LSC analysis. The distribution of ^{12}C : ^{13}C : ^{14}C ratio in the dose formulations was determined by mass spectrometry.

The radioactivity in urine, feces, blood, individual tissue samples, GI tract and in individual milk samples was determined by liquid scintillation counting (LSC). For measurement of radioactivity, solid samples were combusted by means of a sample oxidizer. The resultant $^{14}CO_2$ generated was absorbed and mixed with scintillation fluid. Samples were then subject to LSC analysis. Liquid samples were directly analysed by LSC.

Subsamples of urine, feces and milk of both animals dosed with the C-label and both animals dosed with the T-label were combined to generate label-specific pooled samples. The tissues / organs of both animals (C-label and T-label separately) were combined and homogenized. After determination of radioactivity, the separate flank and loin muscle samples were combined in a ratio of 1 : 2, and the separate omental, subcutaneous and renal fat samples were combined in a ratio of 2 : 1 : 1. Aliquots of each milk sample were separated by centrifugation into fat (cream) and aqueous (skim milk) fractions.

For analyses of the metabolite pattern, pooled samples of urine, feces and milk (time interval: 144-288 h) were investigated. Only for feces of the TFMP-label a time interval of 216-240 h, representing a sample in the plateau area, was analysed. No extraction was necessary for urine and bile prior to HPLC analysis. Subsamples of the pooled feces, liver, kidney and muscle samples were extracted with methanol and water. Whole milk, skimmed milk, cream and fat were extracted with acetonitrile and isohexane. In case of the C-label, whole milk and skim milk were extracted in addition with methanol and skimmed milk was additionally extracted with water. The results of the extractions (LSC of the combined extracts) referred to as extractable radioactive residues (ERR). The residual radioactive residues after solvent extraction (RRR) were determined by combustion analysis. The methanol or acetonitrile extracts with sufficient concentrations of radioactive residues were concentrated and analysed by reversed-phase HPLC with gradient elution and radio-detection using two chromatographic methods.

Specifically for liver (all three labels), the RRR after methanol and water extraction was subjected to a protease treatment that released additional amounts of radioactive residue.

Identification of metabolites was based on HPLC-MS investigations. Some of the samples were purified by liquid / liquid partition and SPE-cleanup. Kidney fractions were obtained by several SPE cleanups and / or HPLC-fractionations. HPLC-MS analyses were performed with urine (all labels), a feces methanol extract (C-label), a cleaned up sample obtained from whole milk (TFMP-label), from bile (C-label), a cleaned up liver methanol extract (C-label) and isolated HPLC fractions obtained from kidney (C-label). The metabolites in the other samples were assigned by co-chromatography experiments and as well by comparison of metabolite patterns and the retention times with those of the samples that were investigated by HPLC-MS analysis and of reference items or reference samples from other studies using two different HPLC methods. Further HPLC peaks were characterized by their chromatographic properties.

For identification of an early eluting peak detected in several extracts of the T-label dose group, the polar region was isolated by SPE fractionation and analysed by two HPLC methods specially developed for analysis of TDM (triazole derivative metabolites). Specifically, these analyses were performed with one aliquot of a whole milk sample, one aliquot each of methanol extracts of liver, kidney and muscle as well as of acetonitrile extract of fat.

Chiral HPLC analysis was done with parent BAS 750 F isolated from extracts of different goat matrices by SPE-fractionation.

Specifically for a C-labeled kidney methanol extract, treatment with ß-glucuronidase / arylsulfatase was done to investigate the cleavability of glucuronic moiety from the glucuronic acid conjugates, namely M750F064 and M750F068.

II. RESULTS AND DISCUSSION

General observations: All goats remained in good general health throughout the study (acclimatization and dosing period). No behavioral or physical abnormalities were observed during duration of the study. Bodyweight did decline initially (adaption to receiving only 2 kg of food per day), then remained constant for the duration of the study. Overall, feed consumption and milk production of the goats was stable throughout the application period.

1. TOTAL RADIOACTIVE RESIDUES (TRR)

Daily samples obtained on 12-14 consecutive days, of milk (two samples per day), urine and feces were measured for total radioactive residues separately for each animal. Plateau levels of radioactive residues in milk were reached within 5-8 days of dosing (see Table 6.2.3-2) indicating absence of accumulation of residues in milk.

Table 6.2.3-2: TRR in milk after administration of ^{14}C-BAS 750 F to goats

	C-label *TRR measured [mg/kg]*		**TFMP-label** *TRR measured [mg/kg]*	**T-label** *TRR measured [mg/kg]*	
application day	goat 1	goat 2	goat 5	goat 3	goat 4
1	- [1)]	- [1)]	- [1)]	- [1)]	- [1)]
2	*0.014*	*0.015*	*0.021*	*0.076*	*0.045*
3	*0.027*	*0.031*	*0.049*	*0.156*	*0.099*
4	*0.029*	*0.030*	*0.058*	*0.220*	*0.262*
5	***0.033*** [2)]	*0.037*	***0.074*** [2)]	***0.261*** [2)]	***0.372*** [2)]
6	***0.031***	***0.041*** [2)]	***0.075***	***0.273***	***0.347***
7	***0.029***	***0.046***	***0.074***	***0.285***	***0.317***
8	***0.028***	***0.042***	***0.080***	***0.311***	***0.310***
9	***0.028***	***0.040***	***0.074***	***0.289***	***0.284***
10	***0.028***	***0.038***	***0.071***	***0.281***	***0.259***
11	***0.028***	***0.035***	***0.062***	***0.285***	***0.275***
12	***0.025***	***0.039***	***0.061***	***0.279***	***0.254***
13	***0.027***	***0.038***	***0.056***	***0.271***	***0.228***
14	***0.028***	***0.053***	-	***0.263***	***0.224***
15	***0.030***	***0.059***	-	***0.253***	***0.224***
pool sample [1)]	*0.020*		*0.065*	*0.284*	

[1)] no determination (i.e. values obtained did not exceed background level) [2)] start of plateau phase, indicated by bold typing

Recoveries generally were good and similar for the three labels. For the C-Label (goats 1, 2) 79% and 82% of the total dose was recovered. The majority of the radioactivity was excreta-related (in feces 47%-49% of dose, in urine 25% and 26% of dose). Only low amounts were recovered in the GI tract contents (2.7-4.0% dose) and the cagewash (ca. 1% dose). For the TFMP-label (goat 5) 82% of the total dose was recovered. The majority of the radioactivity was was excreta-related (in feces 35% of dose, in urine 40% dose). In the GI tract contents 3.8% of dose were found, in the cagewash 0.9% of dose. For the T-label (goats 3, 4) 83-84% of the total dose was recovered. The majority of the radioactivity was excreta-related (in feces 53% and 47% of dose, in urine 24% and 30% of dose). Only low amounts were recovered in the GI tract contents (up to 2.7% dose) and in the cagewash (0.5% dose).

In milk, only very low proportions of the administered dose were found with up to 0.25 % of dose (C-label), 0.35 % of dose (TFMP-label) and up to 2.2 % of dose (T-label).

Table 6.2.3-3: Material balance after administration of ^{14}C-BAS 750 F to goats

matrix	C-label *% of dose*			TFMP-label *% of dose*	T-label *% of dose*		
	goat 1	**goat 2**	***calculated mean***	**goat 5**	**goat 3**	***goat 4***	***calculated mean***
urine	*25.28*	*26.44*	**25.86**	**40.21**	*23.66*	*30.13*	**26.90**
feces	*48.62*	*47.15*	**47.89**	**34.49**	*52.68*	*46.50*	**49.59**
milk	*0.24*	*0.25*	**0.25**	**0.35**	*2.10*	*2.21*	**2.16**
liver	*0.35*	*0.45*	**0.40**	**0.52**	*0.27*	*0.23*	**0.25**
kidney	*0.01*	*0.01*	**0.01**	**0.02**	*0.01*	*0.01*	**0.01**
muscle (flank)	*0.01*	*0.02*	**0.02**	**0.07**	*0.15*	*0.09*	**0.12**
muscle (loin)	*0.01*	*0.01*	**0.01**	**0.03**	*0.05*	*0.06*	**0.06**
fat (subcutan)	*0.03*	*0.04*	**0.04**	**0.22**	*0.03*	*0.02*	**0.03**
fat (omenal)	*0.16*	*0.25*	**0.21**	**0.60**	*0.13*	*0.10*	**0.12**
fat (renal)	*0.05*	*0.12*	**0.09**	**0.16**	*0.03*	*0.02*	**0.03**
G.I. tract contents	*2.66*	*4.04*	**3.35**	**3.76**	*2.66*	*2.60*	**2.63**
G.I. tract	*0.96*	*2.44*	**1.70**	**1.08**	*1.57*	*0.90*	**1.24**
bile	*0.02*	*0.02*	**0.02**	**0.22**	*0.02*	*0.02*	**0.02**
whole blood	*< 0.01*	*< 0.01*	**< 0.01**	**<0.01**	*n.a.*	*n.a.*	**n.a.**
cage wash	*0.73*	*1.15*	**0.94**	**0.87**	*0.58*	*0.47*	**0.53**
sum excreta	-	-	**77.2**	**78.5**	-	-	**79.1**
total recovery	*79.13*	*82.39*	**80.76**	**84.91**	*83.94*	*83.36*	**83.65**

For further analysis, the following pooled samples of urine, feces and milk (per matrix and label) were generated. For the C-label, urine, feces and milk obtained during the dosing interval 144-288h. For the TFMP-label, urine and milk obtained during the dosing interval 144-288h, feces obtained during the interval 216-240 h. For the T-label, urine, feces and milk obtained during the dosing interval 144-288h. Similarly, tissue homogenates of liver, kidney, muscle and fat were used to generate one pooled sample per matrix and label. Aliquots of each whole milk sample were additionally separated by centrifugation into fat (cream) and aqueous (skimmed milk) fractions.

TRR for urine and bile was determined by direct LSC measurement. TRR faeces, milk, liver, kidney, muscle and fat tissues was measured by LSC (direct LSC or combustion analysis followed by LSC). TRR was also calculated as the sum of the Extractable Radioactive Residues (ERR) and the Residual Radioactive Residues (RRR) after solvent extraction. Since similar values were obtained, the available “TRR calculated” was used as 100 % TRR for all further calculations.

Table 6.2.3-4: TRR in tissues, milk, bile and excreta (pool samples)

matrix	C-Label		TFMP-Label		T-Label	
	TRR *measured [mg/kg]*	**TRR** *calculated [mg/kg]*	**TRR** *measured [mg/kg]*	**TRR** *calculated [mg/kg]*	**TRR** *measured [mg/kg]*	**TRR** *calculated [mg/kg]*
muscle [3)]	0.044	0.047	0.099	0.098	0.222	0.223
liver	1.122	1.085	1.468	1.332	0.655	0.650
kidney	0.353	0.352	0.436	0.429	0.386	0.396
fat [4)]	0.307	0.309	0.515	0.532	0.215	0.213
whole milk	0.029	0.029	0.065	0.062	0.284	0.273
skim milk [2)]	0.016	0.016	0.031	0.036	0.286	0.270
cream [2)]	0.204	0.207	0.491	0.521	0.266	0.289
urine	4.154	- [1)]	5.329	- [1)]	2.941	- [1)]
feces	3.823	5.174	4.569	5.543	3.077	3.206
bile	7.393	- [1)]	11.687	- [1)]	3.974	- [1)]

TRR was calculated as the sum of ERR, the radioactivity measured in the residue obtained after protease solubilisation and the radioactivity measured in the protease solubilisate, [1)] not analysed (sample not subjected to extraction), [2)] composite milk sample separated by centrifugation into fat (cream) and aqueous (skim milk) fraction, [3)]muscle types pooled, then pools combined in the ratio 2:1 (w:w) loin: flank muscle, [4)]fat types pooled, then pools combined in the ratio 2:1:1 (w:w:w) omental: subcutaneous: renal fat

Generally, in edible tissues, in organs as well as in milk, similar residue levels were obtained for the C-label (representing only ≤0.45% of total dose) and the TFMP-label (representing only ≤0.52% of the total dose). In contrast, residue levels found in T-labeled samples were significantly different with higher TRR in milk (up to 2.2% of dose) while representing only ≤0.27% of total dose in tissues and organs. The TRR data from the three labels taken together indicates absence of significant cleavage of the ether bridge (between positions of radiocarbon in C- and TFMP-label) while the T-bridge (between positions of radiocarbon in TFMP- and T-label) appears to be effectively cleaved.

Overall, low residue levels were found in milk (except T-labeled milk), muscle, kidney and fat while higher levels were found for liver.

For muscle, the TRR was <0.1 mg/kg (C- /TFMP-label), while 0.22 mg/kg (T-label). Similarly, for milk, the TRR was <0.1 mg/kg (C- /TFMP-label), with predominant partitioning to cream (0.21 and 0.52 mg/kg for C- and TFMP-label) while 0.27 mg/kg (T-label) with similar levels in cream and in skim milk.

With all three labels the TRR in kidney was 0.35-0.42 mg/kg and the TRR in fat 0.21-0.53 mg/kg. In contrast, the TRR in liver was higher with 1.09 mg/kg, 1.33 mg/kg and 0.65 mg/kg (C-label, TFMP-label, T -label).

Regarding excreta, all three labels (C-, TFMP-, T-label) showed similarly high levels in both urine (4.15 mg/kg, 5.33 mg/kg, 2.94 mg/kg) and feces (5.17 mg/kg, 5.54 mg/kg, 3.21 mg/kg). The levels in feces correlate well with high levels found in bile (7.39 mg/kg, 11.69 mg/kg and 3.97 mg/kg, for the C-, TFMP-, T-label, respectively).

2. EXTRACTABILITY

The extractabilities of ^{14}C-residue from milk (including cream and skim milk), from muscle, liver, kidney and feces is summarized in the following tables, namely for C-label, TFMP-label and T-label.

High extractability of ^{14}C residue was seen for milk and tissues (90% TRR or higher) and feces (84% TRR or higher).

For all three labels, most of the radioactivity was extracted with methanol (88% TRR or higher) for muscle, liver, and kidney while subsequent water extraction resulted in additional 2% TRR or less. Solvent extraction left a RRR of <2% TTR in muscle and <3% TRR in kidney which was not further investigated. In liver, RRR amounted to 7.6%, 6.5% and 10.1% TRR and therefore were subjected to protease treatment releasing additional 1.8% - 3.4% TRR.

In fat, the predominant part of the ^{14}C residue was extracted with isohexan (91% TRR or higher), acetonitrile extracted at maximum 5% of TRR. Solvent extraction left a RRR of 4% TRR or less (representing <0.01 mg/kg BAS 750 F equivalents), which was not further investigated.

In milk, the predominant part of the 14C residue was extracted with acetonitrile (86% TRR or higher), isohexane at maximum 5% of TRR, methanol extraction of the C-labeled sample 6% TRR. Similar extraction was seen for skim milk. In contrast, for the C- and TFMP-label, isohexane extracted higher amounts of 14C residue (6-13% TRR) compared with milk correlating with the significant higher TRR in cream compared to milk (an effect not seen with the T-label). RRR in milk fractions were low (for C- and TFMP-label, <5%, corresponding to <0.003 mg/kg, and for the T-label at maximum 7.5 %TRR, corresponding to 0.02 mg/kg) and therefore not further investigated.

Table 6.2.3-5: TRR and extractability of residues of ^{14C}-BAS 750 F (C-label) in milk, tissues and excreta

matrix (C-label)	TRR (measured)	acetonitrile extract		isohexane extract		methanol extract		ERR		RRR		TRR (calculated)	recovery [3] (extraction)
	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	[mg/kg]	%
milk (whole)	**0.029**	**85.6**	*0.025*	**5.0**	*0.001*	**5.8**	*0.002*	**96.5**	*0.028*	**3.5**	*0.001*	**0.029**	100.0
cream	**0.204**	**85.2**	*0.176*	**13.2**	*0.027*	**-**	**-**	**98.4**	*0.203*	**1.6**	*0.003*	**0.207**	101.5
skim milk [1]	**0.016**	**87.8**	*0.014*	**1.0**	*<0.001*	**5.0**	*0.001*	**95.3**	*0.015*	**4.7**	*0.001*	**0.016**	100.0
fat	**0.307**	**1.1**	*0.003*	**99.5**	*0.306*	**-**	**-**	**100.6**	*0.309*	**-** [2]	**-** [2]	**“ 0.307 “** [2]	**-** [2]
		methanol extract		**water extract**		**-**		**ERR**		**RRR**		**TRR (calculated)**	**recovery [3] (extraction)**
muscle	**0.044**	**98.2**	*0.046*	**0.3**	*<0.001*	**-**	**-**	**98.5**	*0.047*	**1.5**	*0.001*	**0.047**	106.8
liver	**1.122**	**91.3**	*0.990*	**1.1**	*0.012*	**-**	**-**	**92.4**	*1.002*	**7.6**	*0.083*	**1.085**	96.7
kidney	**0.353**	**96.4**	*0.340*	**1.0**	*0.003*	**-**	**-**	**97.4**	*0.343*	**2.6**	*0.009*	**0.352**	99.7
faeces	**3.823**	**85.0**	*4.396*	**1.1**	*0.055*	**-**	**-**	**86.0**	*4.452*	**14.0**	*0.723*	**5.174**	135.3

The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the solvent extracts. [1] skim milk (aqueous) of the C-label was also extracted with water. An additional amount of <0.001 mg/kg (1.6 % TRR) was extracted and added to the TRR value. [2] no valid measurement for the RRR was obtained, and thus the “TRR calculated” could not be determined. For further calculations, the TRR measured was used instead. [3] recovery was calculated as “TRR calculated” divided by “TRR measured”

Table 6.2.3-6: TRR and extractability of residues of ^{14C}-BAS 750 F (TFMP-label) in milk, tissues and excreta

matrix (TFMP-label)	TRR (measured)	acetonitrile extract		isohexane extract		ERR		RRR		TRR (calculated)	recovery [3] (extraction)
	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	mg/kg	%
milk (whole)	**0.065**	**95.9**	*0.059*	**2.3**	*0.001*	**98.1**	*0.061*	**1.9**	*0.001*	**0.062**	95.3
cream	**0.491**	**93.8**	*0.489*	**5.9**	*0.031*	**99.7**	*0.520*	**0.3**	*0.001*	**0.521**	106.1
skim milk [1]	**0.031**	**96.0**	*0.035*	**0.3**	*<0.001*	**96.3**	*0.035*	**3.7**	*0.001*	**0.036**	116.1
fat	**0.515**	**1.1**	*0.006*	**98.4**	*0.524*	**99.5**	*0.530*	**0.5**	*0.003*	**0.532**	103.3
		methanol extract		**water extract**		**ERR**		**RRR**		**TRR (calculated)**	**recovery [3] (extraction)**
muscle	**0.099**	**98.8**	*0.097*	**<0.1**	*<0.001*	**98.8**	*0.097*	**1.2**	*0.001*	**0.098**	99.0
liver	**1.468**	**91.5**	*1.219*	**0.3**	*0.003*	**91.7**	*1.222*	**6.5 [1]**	*0.086*	**1.332 [1]**	90.7
kidney	**0.436**	**97.9**	*0.420*	**0.3**	*0.001*	**98.2**	*0.422*	**1.8**	*0.008*	**0.429**	98.3
feces	**4.569**	**81.3**	*4.507*	**3.0**	*0.167*	**84.3**	*4.674*	**15.7**	*0.868*	**5.543**	121.3

The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the solvent extracts. [1] Final Residue after protease solubilisation. Consequently, TRR (calculated) was obtained as the sum of ERR & radioactivity of the post-solubilization solid & solubilisate. [3] recovery was calculated as "TRR calculated" divided by "TRR measured"

Table 6.2.3-7: TRR and extractability of residues of ^{14C}-BAS 750 F (T-label) in milk, tissues and excreta

matrix (T-label)	TRR (measured)	acetonitrile extract		isohexane extract		ERR		RRR		TRR (calculated)	recovery [3] (extraction)
	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR	mg/kg	mg/kg	%
milk (whole)	**0.284**	**92.4**	*0.252*	**0.1**	*<0.001*	**92.5**	*0.253*	**7.5**	*0.020*	**0.273**	96.1
cream	**0.266**	**96.7**	*0.280*	**0.5**	*0.001*	**97.1**	*0.281*	**2.9**	*0.008*	**0.289**	108.6
skim milk	**0.286**	**98.7**	*0.267*	**<0.1**	*<0.001*	**98.7**	*0.267*	**1.3**	*0.003*	**0.270**	94.4
fat	**0.215**	**5.0**	*0.011*	**91.0**	*0.193*	**96.0**	*0.204*	**4.0**	*0.008*	**0.213**	99.1
		methanol extract		**water extract**		**ERR**		**RRR**		**TRR (calculated)**	**recovery [3] (extraction)**
muscle	**0.222**	**98.7**	*0.220*	**0.7**	*0.002*	**99.3**	*0.221*	**0.7**	*0.001*	**0.223**	100.5
liver	**0.655**	**87.9**	*0.571*	**2.0**	*0.013*	**89.9**	*0.584*	**10.1**	*0.066*	**0.650**	99.2
kidney	**0.386**	**98.3**	*0.390*	**0.4**	*0.002*	**98.8**	*0.391*	**1.2**	*0.005*	**0.396**	102.6
feces	**3.077**	**83.6**	*2.680*	**1.2**	*0.040*	**84.9**	*2.720*	**15.1**	*0.486*	**3.206**	104.2

The TRR is calculated as the sum of ERR and RRR. The ERR is calculated as the sum of the solvent extracts. [3] recovery was calculated as "TRR calculated" divided by "TRR measured"

3. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

The following tables provide a summary of the residue components identified and/or characterized in samples obtained with the C-label (Table 6.2/55), the TFMP-label (Table 6.2.3-9), and the T-label (Table 6.2.3-10). ERR was subjected to HPLC analysis allowing identification (ID) and characterization (CHAR) of radioactive residue. RRR of liver samples was further treated with protease allowing characterization by solubilization of 14C residue (RRR CHAR). The data for whole milk represents qualitatively the data obtained for skim milk and cream (see section 3.1. on milk fractions), and therefore is not included in the tables. Overall, largely similar composition of the radioactive residue is found for C-label and TFMP-label, and a significantly different composition with the T-label.

Table 6.2.3-8: Summary of identified/characterized components in goat (C-label)

C-label residue component	whole milk		muscle		liver		kidney		fat		urine		feces		bile	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*
BAS 750 F	**47.5**	*0.014*	**87.9**	*0.042*	**49.9**	*0.541*	**28.3**	*0.100*	**84.6**	*0.260*	**3.0**	*0.124*	**57.2**	*2.962*	**2.8**	*0.206*
M750F068			-	-	**3.0**	*0.033*	**17.8**	*0.063*	-	-			-	-		
M750F039	-	-	-	-	-	-	-	-	-	-	**3.7**	*0.153*	**0.7**	*0.035*	**3.0**	*0.221*
M750F072	**5.9**	*0.002*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M750F041	**6.0**	*0.002*	-	-	-	-	-	-	-	-	**3.7**	*0.154*	-	-	-	-
M750F091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**3.3**	*0.244*
M750F015	-	-	-	-	-	-	-	-	-	-	-	-	**4.7**	*0.244*	**5.5**	*0.403*
M750F015 / -F043	-	-	-	-	-	-	-	-	-	-	**10.1**	*0.421*	-	-	-	-
M750F017	-	-	-	-	-	-	-	-	-	-	**4.2**	*0.173*	**1.5**	*0.079*	-	-
M750F017 /-F078	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2.7**	*0.197*
M750F016	-	-	-	-	**11.8**	*0.128*	-	-	-	-	**7.8**	*0.325*	**3.8**	*0.194*	**6.3**	*0.468*
M750F063	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**26.3**	*1.945*
M750F022	**2.2**	*0.001*	**6.7**	*0.003*	**4.8**	*0.052*	**5.8**	*0.021*	**4.5**	*0.014*	**25.4**	*1.055*	**5.5**	*0.285*	**7.5**	*0.557*
M750F043	**14.2**	*0.004*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M750F038	-	-	-	-	**6.5**	*0.070*	-	-	-	-	-	-	**3.6**	*0.189*	**11.3**	*0.839*
M750F038 /-F042	-	-	-	-	-	-	-	-	-	-	**28.1**	*1.168*	-	-	-	-
M750F038 /-F064			-	-			**26.6**	*0.094*	-	-						
ERR ID-HPLC	**75.7**	*0.022*	**94.6**	*0.045*	**76.0**	*0.824*	**78.5**	*0.277*	**89.1**	*0.274*	**86.0**	*3.573*	**77.1**	*3.988*	**68.7**	*5.080*
ERR CHAR-HPLC	**6.5**	*0.002*	-	-	**8.4**	*0.091*	**19.3**	*0.068*	-	-	**14.0**	*0.581*	**2.5**	*0.127*	**31.3**	*2.313*
ERR CHAR-other	**10.84**	*0.003*	**0.3**	*<0.001*	**1.1**	*0.012*	**1.0**	*0.003*	**1.1**	*0.003*	-	-	**1.1**	*0.055*	-	-
ERR SUM-ID/CHAR	**93.1**	*0.027*	**94.9**	*0.045*	**85.5**	*0.927*	**98.8**	*0.348*	**90.2**	*0.277*	**100.0**	*4.154*	**80.6**	*4.170*	**100.0**	*7.393*
RRR-CHAR	-	-	-	-	**3.4**	*0.037*	-	-	-	-	-	-	-	-	-	-
Final Residue	**3.5**	*0.001*	**1.5**	*0.001*	**4.5**	*0.049*	**2.6**	*0.009*	-	-	-	-	**14.0**	*0.723*	-	-
Grand Total	**96.7**	*0.028*	**96.4**	*0.046*	**93.4**	*1.013*	**101.5**	*0.358*	**90.2**	*0.277*	-	-	**94.6**	*4.892*	-	-

ID-HPLC= amounts identified by HPLC, CHAR-HPLC= amounts characterized by HPLC, CHAR-other=amounts characterized by extraction, SUM-ID/CHAR=sum of amounts identified and/or characterized, RRR-CHAR=amounts of RRR which were characterized. Data on skim milk and cream are provided in study report.

Table 6.2.3-9: Summary of identified/characterized components in goat (TFMP-label)

TFMP-label residue component	whole milk		muscle		liver		kidney		fat		urine		feces		bile	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*
BAS 750 F	**44.5**	*0.028*	**95.7**	*0.094*	**46.7**	*0.622*	**46.0**	*0.198*	**88.1**	0.469	-	-	**26.6**	*1.473*	**1.8**	*0.213*
M750F068	-	-	-	-	**4.2**	*0.056*	-	-	-	-	-	-	-	-	-	-
M750F039	-	-	-	-	-	-	-	-	-	-	-	-	**2.1**	*0.117*	-	-
M750F072	**5.8**	*0.004*	-	-	-	-	**3.0**	*0.013*	-	-	-	-	-	-	-	-
M750F041	**7.2**	*0.004*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M750F091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2.7**	*0.319*
M750F015	-	-	-	-	-	-	**2.6**	*0.011*	-	-	**2.3**	*0.122*	**8.8**	*0.488*	**0.5**	*0.064*
M750F017	-	-	-	-	-	-	-	-	-	-	-	-	**2.9**	*0.162*	-	-
M750F016	-	-	-	-	**15.0**	*0.200*	**3.7**	*0.016*	-	-	**3.5**	*0.186*	**13.8**	*0.766*	**0.7**	*0.087*
M750F063	-	-	-	-	-	-	-	-	-	-	**26.6**	*1.417*	-	-	**58.3**	*6.808*
M750F022	**1.2**	*0.001*	-	-	**7.6**	*0.101*	**10.7**	*0.046*	**5.8**	0.031	-	-	**8.7**	*0.482*	-	-
M750F043	**25.0**	*0.016*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M750F038	-	-	-	-	**11.2**	*0.149*	**14.0**	*0.060*	-	-	-	-	**6.2**	*0.342*	-	-
M750F038 /-F064	-	-	-	-	-	-	-	-	-	-	**47.1**	*2.511*	-	-	-	-
M750F003	-	-	-	-	-	-	**3.2**	*0.014*	-	-	-	-	**3.0**	*0.166*	-	-
ERR ID-HPLC	**83.7**	*0.052*	**95.7**	*0.094*	**84.7**	*1.128*	**83.3**	*0.357*	**93.9**	*0.500*	**79.5**	*4.24*	**72.1**	*3.996*	**64.1**	*7.491*
ERR CHAR-HPLC	**3.0**	*0.002*	-	-	**4.5**	*0.060*	**11.0**	*0.047*	-	-	**20.5**	*1.093*	**9.1**	*0.504*	**33.9**	*3.957*
ERR CHAR-other	**2.3**	*0.001*	**<0.1**	*<0.001*	**0.3**	*0.003*	**0.3**	*0.001*	**4.5**	*0.024*	-	-	**3.0**	*0.167*	-	-
ERR SUM-ID/CHAR	**88.9**	*0.055*	**95.7**	*0.094*	**89.4**	*1.191*	**94.6**	*0.406*	**98.4**	*0.524*	**100.0**	*5.329*	**84.2**	*4.667*	**98.0**	*11.448*
RRR-CHAR	-	-	-	-	**1.8**	*0.023*	-	-	-	-	-	-	-	-	-	-
Final Residue	**1.9**	*0.001*	**1.2**	*0.001*	**6.5**	*0.086*	**1.8**	*0.008*	**0.5**	*0.003*	-	-	**15.7**	*0.868*	-	-
Grand Total	**90.8**	*0.056*	**96.9**	*0.095*	**97.7**	*1.301*	**96.3**	*0.414*	**99.0**	*0.527*	-	-	**99.9**	*5.535*	-	-

ID-HPLC= amounts identified by HPLC, CHAR-HPLC= amounts characterized by HPLC, CHAR-other=amounts characterized by extraction, SUM-ID/CHAR=sum of amounts identified and/or characterized, RRR-CHAR=amounts of RRR which were characterized. Data on skim milk and cream are provided in study report.

Table 6.2.3-10: Summary of identified/characterized components in goat (T-label)

T-label residue component	whole milk		muscle		liver		kidney		fat		urine		feces		bile	
	%TRR	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	%TRR	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*	*mg/kg*	*%TRR*
BAS 750 F	**3.0**	*0.008*	**11.9**	*0.027*	**26.2**	*0.170*	**10.3**	*0.041*	**84.9**	*0.180*	-	-	**49.5**	*1.586*	-	-
M750F068	-	-	-	-	**4.4**	*0.028*	-	-	-	-	-	-	-	-		
M750F039	-	-	-	-	-	-	-	-	-	-	-	-	**2.8**	*0.090*	-	-
M750F091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1.9**	*0.076*
M750F015	-	-	-	-	-	-	-	-	-	-	**7.4**	*0.217*	**8.9**	*0.285*	-	-
M750F017	-	-	-	-	-	-	-	-	-	-	-	-	**2.5**	*0.080*	-	-
M750F016	-	-	-	-	**10.0**	*0.065*	-	-	-	-	**6.9**	*0.202*	**8.3**	*0.266*	-	-
M750F063	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**73.5**	*2.920*
M750F001	**78.4**	*0.214*	**87.3**	*0.194*	-	-	**68.1**	*0.270*	**4.7**	*0.010*	**69.2**	*2.036*	**4.6**	*0.147*	**8.0**	*0.319*
M750F001/derivate[1)]	-	-	-	-	**31.8**	*0.207*	-	-	-	-	-	-	-	-	-	-
M750F003	-	-	-	-	-	-	-	-	-	-	**16.5**	*0.486*	**5.2**	*0.167*	-	-
ERR ID-HPLC	**81.4**	*0.222*	**99.2**	*0.221*	**72.4**	*0.470*	**78.4**	*0.311*	**89.6**	*0.190*	**100.0**	*2.94*	**81.7**	*2.621*	**83.4**	*3.316*
ERR CHAR-HPLC	**1.8**	*0.005*	-	-	**8.0**	*0.052*	**9.9**	*0.039*	-	-	-	-	-	-	**19.7**	*0.782*
ERR CHAR-other	**0.1**	*<0.001*	**0.7**	*0.002*	**2.0**	*0.013*	**0.4**	*0.002*	**2.7**	*0.006*	-	-	**1.2**	*0.040*	-	-
ERR SUM-ID/CHAR	**83.3**	*0.228*	**99.9**	*0.223*	**82.4**	*0.536*	**88.7**	*0.352*	**92.2**	*0.196*	**100.0**	*2.941*	**83.0**	*2.661*	**103.1**	*4.098*
RRR-CHAR	-	-	-	-	**2.2**	*0.014*	-	-	-	-	-	-	-	-	-	-
Final Residue	**7.5**	*0.020*	**0.7**	*0.001*	**6.7**	*0.044*	**1.2**	*0.005*	**4.0**	*0.008*	-	-	**15.1**	*0.486*	-	-
Grand Total	**90.8**	*0.248*	**100.6**	*0.224*	**91.3**	*0.594*	**89.9**	*0.356*	**96.2**	*0.205*	-	-	**98.1**	*3.146*	-	-

ID-HPLC= amounts identified by HPLC, CHAR-HPLC= amounts characterized by HPLC, CHAR-other=amounts characterized by extraction, SUM-ID/CHAR=sum of amounts identified and/or characterized, RRR-CHAR=amounts of RRR which were characterized. Data on skim milk and cream are provided in study report. 1) The metabolites 1,24-triazole and a triazole-derivate metabolite were detected in a ratio of 2:1 in the confirmatory HPLC analysis.

3.1 milk

The nature of the ^{14}C residue was investigated in whole milk (section 3.1.1) as well as in its fat-enriched and a fat-reduced fractions, skim milk (section 3.1.2) and cream (section 3.1.3).

3.1.1 milk (whole)

Total extractable residue (ERR) in the pooled sample (days 6-12) accounted for 96.5, 98.1 and 92.5% of TRR (C-, TFMP-, T-label). The residue after extraction (RRR) represented 3.5%, 1.9% and 7.5% (max 0.02 mg/kg) and was thus not further analysed.
The total identified residue in milk accounted for >75%. Additional characterization by chromatographic and extraction properties accounted for 2-17% TRR resulting in amounts "identified and/or characterized" of 93.1%, 88.9% and 83.3% for C-, TFMP-, and T-label.
Parent BAS 750 F was the most abundant component of the residue in the C-label and TFMP-label (47.5% TRR and 44.5% TRR, equivalent to 0.014 mg/kg and 0.028 mg/kg). In contrast, in the T-label BAS 750 F was present only at 0.008 mg/kg (and 3%TRR), while the metabolite 1,2,4-triazole (M750F001) was the predominant (and only other) component (78.4% TRR, 0.21 mg/kg). The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples. In the C-label and TFMP-label, the second most abundant component was metabolite M750F043 (14.2% and 25.0 % TRR), albeit at only low absolute amounts (0.004 mg/kg and 0.016 mg/kg). In addition, small amounts of M750F072 and M750F041 were identified (maximal 7.2 % TRR, 0.004 mg/kg).

3.1.2 skim milk

The data for skim milk is highly comparable to whole milk. Total extractable residues (ERR) in the pooled sample (days 6-12) accounted for 95.3, 96.3 and 98.7% of TRR (C-, TFMP-, T-label). The residue after extraction (RRR) represented 4.7%, 3.7% and 1.3% (max 0.003 mg/kg) and was thus not further analysed.
The total identified residue in skim milk accounted for >72%. Additional characterization by chromatographic and extraction properties accounted for 0.1-15% TRR resulting in amounts "identified and/or characterized" of 88.3%, 87.4% and 95.2% for C-, TFMP-, and T-label. The metabolite pattern obtained from skim milk samples of the C-label and the TFMP-label were almost identical with those of whole milk samples. Metabolites M750F041, M750F072 and M750F043 were detected in somewhat higher quantities (up to 36.8% TRR). The parent compound BAS 750 F was less prominent (23.3% TRR) compared to whole milk and M750F022 was not detected. The only metabolite detected in T-labeled skim milk was the metabolite M750F001 (95.2% TRR).

3.1.3 cream

Total extractable residues (ERR) in the pooled sample (days 6-12) accounted for 98.4, 99.7 and 97.1% of TRR (C-, TFMP-, T-label). The residue after extraction (RRR) represented 1.6%, 0.3% and 2.9% (max 0.008 mg/kg) and was thus not further analysed.
The total identified residue in cream accounted for >85 %. Additional characterization by chromatographic and extraction properties accounted for 0.5-13% TRR resulting in amounts "identified and/or characterized" of 98.4%, 103.9% and 90.8% for C-, TFMP-, and T-label.
Parent BAS 750 F was the most prominent component (up to 80.3 % TRR) for the C-label and the TFMP-label. In addition M750F043 and M750F022 were detected (up to 12.3 % TRR). For the T-label, M750F001 was the most prominent metabolite (74.5 % TRR) and the parent compound accounted for 15.8 % TRR.

3.2 muscle

Total extractable residue (ERR) accounted for >98% of TRR for all three labels, the residue after extraction (RRR) < 1.5% (max 0.002 mg/kg) and was thus not further analysed.
The total identified residue in muscle amounted to 95 - 99 % or higher, further characterization did not result a significant increase (<0.002 mg/kg).
Parent BAS 750 F was the most abundant component of the residue in the C-label and TFMP-label (87.9% TRR and 95.7% TRR, equivalent to 0.042 mg/kg and 0.094 mg/kg). In contrast, in the T-label sample, the BAS 750 F amount present (0.027 mg/kg) accounts for only 11.9 % of TRR, while the metabolite 1,2,4-triazole (M750F001) was the predominant (and only other) component (87.3% TRR, 0.19 mg/kg). The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples. In the C-label, the only other component identified was metabolite M750F022 (6.7%), albeit at only very low absolute amounts (0.003 mg/kg). In the TFMP-label, no further component was detected.

3.3 liver

Total extractable residue (ERR) accounted for 92.4, 91.7 and 89.9% of TRR (C-, TFMP-, T-label). For all labels, the residue after extraction (RRR) was further treated with protease releasing up to 3.4% TRR or 0.037 mg/kg. Taken together, the RRR represented 7.6% and 10.1% (0.083 mg/kg, 0.066 mg/kg) for the C- and T-label. For the TFMP-label, the RRR was not determined.
The total identified residue accounted for 76.0% TRR, 84.7% TRR, and 72.4% TRR. Additional characterization by protease treatment, chromatographic and extraction properties accounted for 6.6 – 10.2% TRR resulting in amounts "identified and/or characterized" of 85.5%, 89.5% and 82.6% for C-, TFMP-, and T-label.
Parent BAS 750 F was the most abundant component of the residue in the C-label and TFMP-label (49.9% TRR and 46.7% TRR, equivalent to 0.54 mg/kg and 0.62 mg/kg). In contrast, in the T-label sample, BAS 750 F was present at lower amounts (0.17 mg/kg, 26.2% TRR), while the metabolite 1,2,4-triazole (M750F001) was slightly higher (31.8% TRR, 0.21 mg/kg). The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples.

In all three labels, significant amounts of metabolites resulting from glucuronidation (M750F068, 3.0-4.4 %TRR) or C-ring hydroxylation (M750F016, 10.0–11.8 % TRR) of the parent backbone were detected.
In the C-label and TFMP-label, the cleavage metabolite M750F022 was detected (4.8 and 7.6% TRR, 0.05 and 0.10 mg/kg), as well as its derivative M750F038 (6.5 and 11.2% TRR, 0.07 and 0.15 mg/kg).

3.4. kidney

Total extractable residue (ERR) accounted for 97.4, 98.2 and 98.8% of TRR (C-, TFMP-, T-label). The residue after extraction (RRR) represented at maximum 2.6 % TRR (0.009 mg/kg or lower) and thus was not further analyzed.
The total identified residue accounted for >78.5, 83.3 and 78.4%. Additional characterization by chromatographic and extraction properties accounted for 10.4 - 20.3% TRR resulting in amounts "identified and/or characterized" of 98.8%, 94.6% and 88.7% for C-, TFMP-, and T-label.
Parent BAS 750 F was the most abundant component of the residue in the C-label and TFMP-label (28.3% TRR and 46.0% TRR, equivalent to 0.10 mg/kg and 0.20 mg/kg). In contrast, in the T-label sample, BAS 750 F was present only at 0.041 mg/kg (and 10.3%TRR), while the metabolite 1,2,4-triazole (M750F001) was the predominant (and only other) component identified (68.1% TRR, 0.27 mg/kg. The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples.
In the C-labeled kidney, 17.8% of M750F068 (glucuronide of parent compound) were detected, whereas this metabolite accounted only for up to 4.4% in TFMP- and T-labeled kidney.
In the C-label and TFMP-label, the second most abundant component was metabolite M750F038 (26.6% and 14.0 % TRR), at absolute amounts of 0.094 mg/kg and 0.060 mg/kg. For the C-label, metabolite M750F038 co-eluted with metabolite M750F064. HPLC-MS investigations and enzymatic treatment of an aliquot of the methanol extract of kidney resulted in a 1:1 ratio of both metabolites. The enzyme treatment effectively cleaved off the glucuronic acid moiety from both M750F064, thus generating M750F022, as well as off M750F038 generating parent BAS 750 F.
In the TFMP-label, several low level metabolites resulting from modification of the parent backbone were identified: the hydroxy-metabolites M750F015 (2.6% TRR) and M750F016 (3.7% TRR) as well as a sulfate conjugate of parent M750F072 (3.0% TRR) at absolute levels of 0.011 – 0.016 mg/kg.
The cleavage metabolite M750F022 was found at low levels of 5.8% TRR (C-label) and 10.7% (TFMP-label), while M750F003 was found at 3.2% TRR (only TFMP-label). Characterization by chromatographic properties detected 2 further peaks accounting for at maximum 8.4% TRR (0.036 mg/kg).

3.5. fat

Total extractable residue (ERR) accounted for about 100% of TRR for all three labels while the residue after extraction (RRR) was maximal 4.0% TRR representing 0.008 mg/kg, thus was not further analysed.
The total identified residue in fat accounted for >89%, including additionally characterized amounts, the residue "identified and/or characterized" amounts to 90% TRR or higher.
Parent BAS 750 F was the most abundant component with 84.6% TRR, 88.1% TRR, 84.9% TRR (C-, TFMP-, T-label). In the C- and TFMP-label, small amounts of metabolite M750F022 were found (4.5% and 5.8% TRR, corresponding to 0.014 and 0.031 mg/kg). In the T-label, small amounts of the metabolite 1,2,4-triazole (M750F001) were found (4.7% TRR, 0.010 mg/kg). The position of the radiocarbon in the C-label and TFMP-label explains why M750F001 is not detected in C-labeled or TFMP-label samples. The position of the radiocarbon in the T-label explains why M750F022 is not detected T-labeled samples.

3.6. urine

No extraction was necessary. The total identified residue accounted for 86.0, 79.5 and 100.0% TRR (C-, TFMP-, T-label). Additional characterization by chromatographic and extraction properties accounted for 14.0 and 20.5% TRR for C- and TFMP-label.
According to HPLC analyses, M750F038 (co-eluting with either M750F042 or M750F064 for the C- and the TFMP-label) was one of the main components detected in urine of the C- and the TFMP-label (28.1% and 47.1 % TRR) while undetectable in T-labeled samples due to absence of the T-ring.
The metabolites M750F022, M750F040, M750F041 and M750F043 (up to 25.4% TRR) were only detected in C-labeled urine, while the two glucuronic acid conjugates, M750F063 (26.6% TRR) and M750F064 (co-eluting with metabolite M750F038) were found at high amounts only in TFMP-labeled urine. In T-labeled urine, M750F001 (69.2% TRR) and metabolite M750F003 (16.5 % TRR) were the main components. M750F003 is a cleavage product (ether bridge cleavage, absence of C-ring). The C-ring hydroxyl-metabolites M750F015 (up to 10.1% TRR), M750F016 (up to 10.17.8% TRR), M750F017 (up to 4.2% TRR) were detected with all three labels.

3.7. feces

Total extractable residue (ERR) accounted for 86.0, 84.3 and 84.9% of TRR (C-, TFMP-, T-label). The residue after extraction (RRR) represented at maximum 15.7% TRR (0.87 mg/kg or lower) and was not further analysed.
The total identified residue accounted for 77.1, 72.1 and 81.7% TRR. Additional characterization by chromatographic and extraction properties accounted for 1.2 – 12.1% TRR resulting in amounts of 80.6, 84.2 and 83.0% TRR (C-, TFMP-, and T-label).
According to HPLC analyses, the metabolite patterns of the feces methanol extracts of all three labels were very similar. The main component was the parent compound BAS 750 F (26.6 - 57.2% TRR). In addition, the metabolites M750F015 (up to 8.9 % TRR), M750F016 (up to 13.8% TRR), M750F017 (up to 2.9% TRR) and M750F039 (up to 2.8% TRR) were detected in the extracts of all three labels. Metabolites M750F038 (up to 6.2% TRR) and M750F022 (up to 8.7 % TRR) were found in methanol extracts of C- and TFMP-labeled feces. Furthermore the label specific metabolites M750F001 (T-label) and M750F003 (T- and TFMP-label) were found at minor quantities (up to 5.2% TRR).

3.8. bile

Extraction was not done for bile. The total identified residue accounted for 68.7%, 64.1% and 83.4% TRR. Additional characterization by chromatographic and extraction properties accounted for 31.3%, 33.9% and 19.7% TRR for C-, TFMP- and T-label.
According to HPLC analyses, the main component detected in bile of all three labels was the glucuronic acid conjugate M750F063 (up to 26.%3, 58.3% and 73.5% TRR). In bile of the C-label additionally the metabolites M750F038 (11.3% TRR) and M750F022 (7.5% TRR) and the metabolites M750F017 and M750F078 (co-eluting in one peak, 2.7 % TRR) were detected. Metabolites M750F091 (up to 3.3% TRR), M750F015 (up to 5.5% TRR), M750F016 (up to 6.3% TRR) and BAS 750 F (up to 2.8% TRR) were found at minor amounts (C-label, TFMP-label). For the T-label, besides M750F063, only M750F001 and M750F091 were found (up to 8.0 % TRR).

4. Enantiomer ratio of BAS 750 F

Chiral analysis of BAS 750 F residue in representative samples of the goat metabolism study revealed a change of the ratio of the S-enantiomer and R-enantiomer in several matrices. While in the dose administered the ratio was approximately 50:50, in all matrices the proportion of R-enantiomer increased significantly, namely in cream (72%), muscle (76%), liver (70%), kidney (80%), and fat (80%). Note, that in feces the enantiomer ratio was comparable to the administered dose.

Table 6.2.3-11: Determination of isomer ratio of BAS 750 F in goat matrices

Matrix	S-enantiomer **[%]**	R-enantiomer **[%]**
TFMP-label		
administered dose	50.60	49.40
muscle	23.68	76.32
kidney	19.60	80.40
T-label		
administered dose	50.28	49.72
cream	28.34	71.66
liver	30.04	69.96
fat	20.67	79.33
feces	50.54	49.46

Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358).

5. Proposed metabolic pathway

Metabolism of BAS 750 F was investigated in lactating goats using three labels, namely C-label, TFMP-label, and T-label. Results taken together indicate comparable pathways, the label specific differences observed are consistent with effective cleavage of the parent backbone, notably the triazole ring, to minor extend also the ether bridge between the C-ring and the TFMP-ring.

Overall, results show that in lactating goats, BAS 750 F is metabolized by two main transformation reactions:

- C-ring hydroxylation (followed by conjugation)
- cleavage of the parent backbone at the T-bridge (followed by conjugation)

In addition, minor transformation steps were observed in goat:

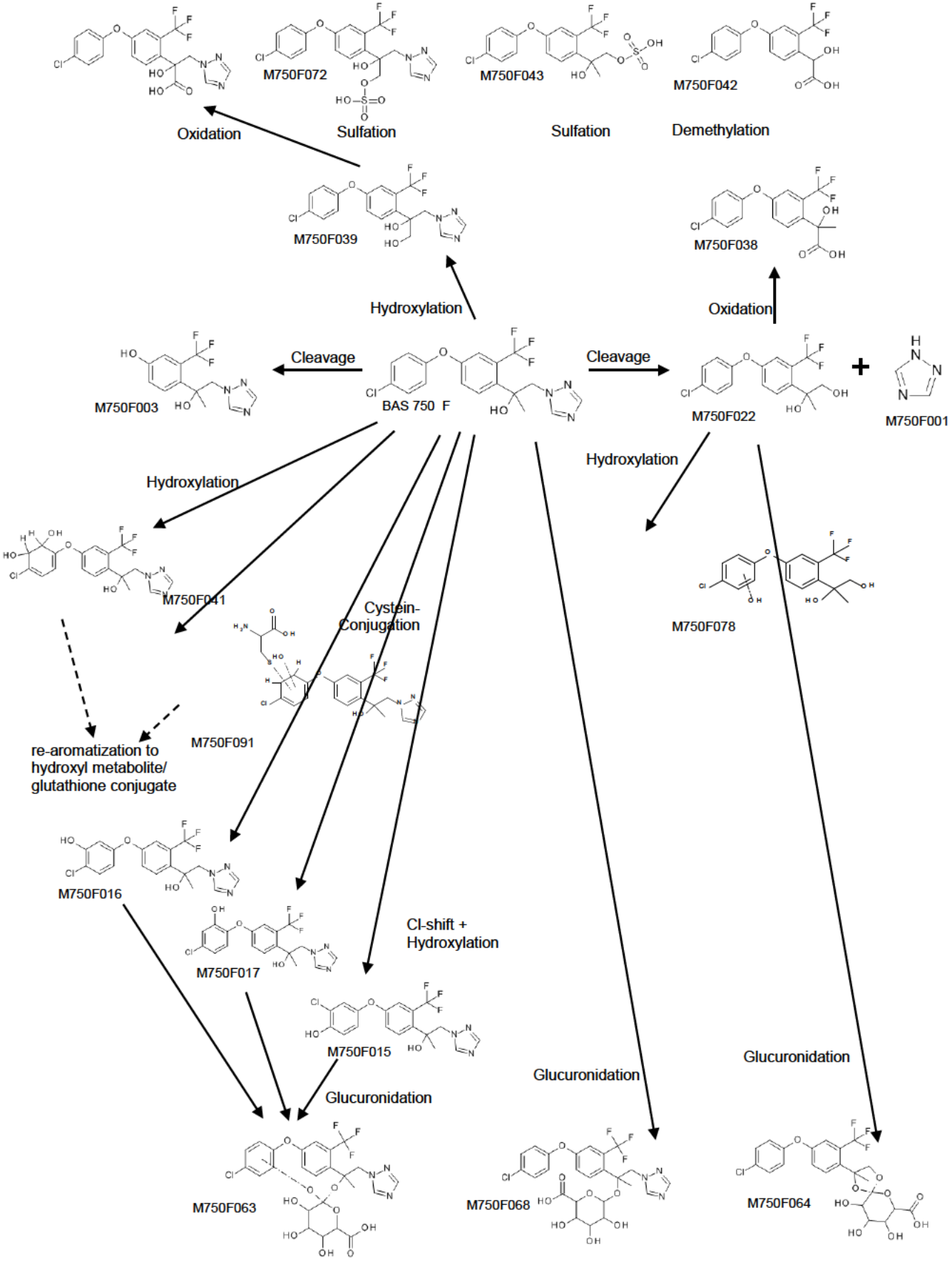
- cleavage at the ether bridge
- hydroxylation of the methyl group (at quaternary C-atom, followed by conjugation)

Hydroxylation at the chlorophenyl-ring leads to M750F015, M750F016, M750F017, further glucuronidation generates M750F063 and M750F068. M750F041 and M750F091 are intermediates of the C-ring oxygenation reaction leading to C-ring hydroxyl metabolites (notably M750F016).

Cleavage of BAS 750 F at the T-bridge generates 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to oxidation (M750F038), followed by demethylation (M750F040), as well as to hydroxylation (M750F078), to sulfatation (M750F043), and to glucuronidation (M750F064).

A transformation reaction observed only to a minor extend is cleavage of the parent backbone at the ether bridge, generating the two-ring metabolite M750F003. Notably, metabolites consisting of only either the C-ring or the TFMP-ring were not observed in any of the samples of the C-label or the TFMP-label. The proposed metabolic pathway of BAS 750 F in lactating goats is shown in figure below. Also seen only to a minor extend is hydroxylation at the methyl of the triazole bridge (M750F039, which is further sulfated to M750F072 or oxidized to M750F042).

Figure 6.2-11: proposed metabolic pathway of BAS 750 F in lactating goats



6. Extractability of residues according to analytical methods

The extractability of parent BAS 750 F and metabolite M750F022 is covered by a separate extractability study, DocID2015/1161960 (see CA6.2.2/2). Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F and M750F022 in commodities of animal origin was investigated using samples representative of relevant livestock matrices with radiolabeled residue (radiovalidation). Samples were obtained from a study with laying hens, BASF DocID 2015/1001001, and a study with lactating goat, BASF DocID 2015/1078841 (see CA 6.2.2 and CA 6.2.3).

Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction for the analytical methods, method L0272/01 for BAS 750 F and L0309/01 for metabolite M750F022.

For BAS 750 F, extraction efficiencies generally were 80% or higher for most matrices (milk, cream, muscle, kidney, fat, egg yolk), and lower for liver (46%).

For M750F022, extraction efficiencies generally were 90% or higher for most matrices (milk, cream, kidney, fat) and lower for egg yolk (66%), for muscle (61%) and for liver (46-50%).

7. Storage stability

Investigations of storage stability were performed for selected goat matrices at the beginning and at the end of the study. For this purpose, extractions of the stored material were usually performed within eight months and initial analyses within 8 – 13 months. Re-analyses of stored extracts were performed for the matrices milk, skim milk (only for T- and TFMP-label), cream, liver, kidney, muscle and fat (all three labels). Re-extraction was performed for kidney (C-label).

For all matrices, the chromatograms obtained from stored extracts and re-analysed samples were in good accordance with the initial analysis. Altogether, stability of extracts was proven for the period of investigation.

Table 6.2.3-12: Storage stability investigations in goat matrics
(*representative information, further data provided in study report*)

matrix	matrix storage interval (analysis 1) *[days]*	extract storage interval (analysis 1) *[days]*	extract storage interval (analysis 2) *[days]*
C-label			
milk (ACN phase)	394	6	264
cream (ACN phase)	234	10	411
liver (MeOH extract)	211	12	432
kidney (MeOH extract)	214 (607)	8	429
muscle (MeOH extract)	210	7	435
fat (isohexane phase)	243	2	400
TFMP-label			
milk (ACN phase)	217	23	178
skim milk (ACN phase)	219	33	176
cream (ACN phase)	219	21	176
liver (MeOH extract)	212	39	183
kidney (MeOH extract)	212	27	183
muscle (MeOH extract)	224	1	171
fat (isohexane phase)	209	38	186
T-label			
milk (ACN phase)	207	131	334
skim milk (ACN phase)	210	148	331
cream (ACN phase)	212	140	329
liver (MeOH extract)	197	134	407
kidney (MeOH extract)	198	103	406
muscle (MeOH extract)	204	95	335
fat (isohexane phase)	201	156	338

ACN denotes acetonitrile phase (of acetonitril extracts or methanol extracts), MeOH denotes methanol

III. CONCLUSION METABOLISM LACTATING GOAT

BAS 750 F and its metabolites were rapidly excreted by lactating goats, observed similarly with C-label, TFMP-label and T-label. Until sacrifice, the radioactive residues excreted via urine and feces (including GI tract contents) amounted for all labels to >77% of the total radioactivity administered. The total radioactive residues (TRR) detected in pooled samples of urine ranged from 2.9 - 5.3 mg/kg and the TRR in the investigated faeces samples was between 3.2 - 5.5 mg/kg. Bile contained 4.0 - 11.7 mg/kg.

14C residues in milk (sampled on 12-14 consecutive days) reached a plateau concentration within 5 days (<0.1 mg/kg for C- and TFMP-label, around 0.3 mg/kg for T-label) confirming absence of accumulation of residues in milk. Overall, the TRR in whole milk, skim milk and muscle tissues of the C- and TFMP-label were low and ranged from 0.016 mg/kg to a maximum of 0.099 mg/kg. In T-labeled milk, skim milk and cream, higher amounts were detected (0.27 - 0.29 mg/kg). The residues in cream and fat (all labels) were between 0.20 - 0.52 mg/kg. In C-, TFMP- and T-labeled kidney and liver, residues were in the range of 0.35-0.42 mg/kg (kidney) and the range of 0.65-1.33 mg/kg (liver).

The extractability of radioactive residues from all edible matrices (milk samples, muscle, fat, kidney and liver) was high (≥ 89.9 % TRR) for all of the three labels. Therefore, a further investigation of the RRR obtained after solvent extraction was performed only for liver (all labels). Protease treatment of the RRR released 1.8% - 3.4% TRR indicating protein-bound residue in liver. Overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in lactating goat includes, besides O-conjugation of the unchanged parent (M750F068), two main transformation reactions. The first main transformation reaction is oxygenation of the C-ring of the uncleaved parent BAS 750 F (M750F015, M750F016, M750F017), followed by conjugation (M750F063). The metabolite M750F041 and M750F091 are intermediates with a cyclohexadiene structure of the C-ring prior to re-aromatization. A second transformation reaction is cleavage of the parent backbone at the T-bridge generating 1,2,4-triazole (M750F001) as well as the two-ring metabolite M750F022, which itself is subject to oxidation (M750F038), followed by demethylation (M750F040), as well as to C-ring hydroxylation (M750F078), to sulfatation (M750F043), and to glucuronidation (M750F064). A transformation observed only to a minor extend is cleavage of the parent backbone at the ether bridge, generating the two ring metabolite M750F003. Notably, metabolites consisting of only either the C-ring or the TFMP-ring were not observed in any of the samples of the C-label or the TFMP-label. A further minor transformation is the hydroxylation of the methyl group at the quaternary C-atom of BAS 750 F (M750F039) which can be oxidized further (M750F042) or conjugation with sulfate (M750F072).

The parent BAS 750 F was applied as a racemic mixture of two enantiomers. Chiral analysis of BAS 750 F revealed a significant change of the ratio in most matrices, with proportion of the R-enantiomer of 70-80% in cream, muscle, liver, kidney and fat. In contrast, the racemate was maintained in feces, indicating a preferential metabolism of the S-enantiomer. Note that a comparable change of isomer ratio was also observed in rats dosed with BAS 750 F (rat metabolism study, see section CA 5.1.1).

For the metabolite profile, label-dependent differences similar to the observations at TRR level were seen. Results of C-label and TFMP-label were comparable, and distinct from the T-label.

With both C- and TFMP-label, parent BAS 750 F was seen as the most abundant compound (fat/muscle >85% TRR, milk/liver >45% TRR, kidney 28-46% TRR). Modification of the intact parent backbone was seen at major amounts, in liver (hydroxy metabolite M750F016 up to 15% TRR, 0.20 mg/kg) and in kidney (glucuronide M750F068 up to 18% TRR, 0.06 mg/kg). Other transformed parent metabolites were present at <7% TRR, in kidney (hydroxy metabolites M750F016/M750F015), in liver (glucuronide M750F068), and in milk (sulfate metabolite M750F072 and the intermediate C-ring-cyclohexadiene metabolite M750F041).

The cleavage metabolite M750F022 was seen in all matrices, albeit at much lower levels (<8% TRR in milk, muscle, liver, kidney, fat), only in TFMP-labeled kidney at 10.7% TRR (yet corresponding to <0.05 mg/kg). M750F022 appears to be metabolized further effectively in goat, as indicated by its oxidized/glucuronidated products (M750F038/M750F064) present in liver (7-11% TRR) and kidney (14-27% TRR) as well as its sulfation product M750F043 present in milk (14-25% TRR, up to 0.16 mg/kg). In addition, cleavage of the ether bridge did occur to a low extend in kidney only, as indicated by M750F003 (3% TRR, 0.01 mg/kg).

For the T-label, 1,2,4-triazole (non-detectable with the other label) was the predominant component in milk, muscle, and kidney (>68% TRR), while this polar component was present only in low amounts in fat (5% TRR, 0.01 mg/kg). In liver, 1,2,4-triazole, together with another triazole derivative metabolite, was found in a fraction of 32% TRR. Parent BAS 750 F was present as the most abundant compound only in fat (85% TRR) and liver (26% TRR), in major amounts in muscle and kidney (10-12%TRR), and low amounts in milk (3%). The only other compounds were modified parent (C-ring hydroxy metabolite M750F016 and glucuronide M750F068), found only in liver, where the metabolic profile was consistent with the profile seen with the other labels (M750F022 and M750F038 are non-detectable with the T-label).

In conclusion, the major components of the residue in goat was identified as unchanged parent BAS 750 F and the TDM which together represent a large proportion of the residue. TDM exceed parent in all matrices except fat. Considering the non-TDM residue, parent represents 85% TRR in muscle and fat, >45% of TRR in milk and liver, 28-46% TRR in kidney. The cleavage product M750F022 was present at much lower levels (<7% TRR, except one kidney sample). For both parent and M750F022 presence of several downstream transformation products indicate effective further metabolic transformation. Overall, metabolism of BAS 750 F in lactating goats, and by extrapolation in ruminant livestock, can be considered well-elucidated.

Report:	CA 6.2.3/2 Thiaener J.,Glaessgen W.E., 2015 b Investigation of the extractability of BAS 750 F and M750F022 in samples from 14C animal metabolism studies 2015/1161960
Guidelines:	EPA 860.1300: Nature of the Residue in Plants Livestock, EPA 860.1000: EPA Residue Chemistry Test Guidelines, PMRA Residue Chemistry Guidelines Section 97.2 Nature of the Residue - Plants - Livestock (Canada), EEC 91/414 Annex II (Part A Section 6), EEC 91/414 Annex III (Part A Section 8), EEC 91/414 (7030(VI/95 Rev. 3)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

This study applies to metabolism investigations in poultry as well as ruminants and is summarize in section CA 6.2.2/2. For information, the *Executive Summary* is copied below.

EXECUTIVE SUMMARY

Extraction efficiency of analytical methods for quantitative analysis of BAS 750 F and M750F022 in commodities of animal origin as investigated using samples with radiolabeled residue (radiovalidation). Samples were obtained in livestock metabolism studies (see CA 6.2 and CA 6.3), namely in laying hens (BASF DocID 2015/1001001) and in lactating goat (BASF DocID 2015/1078841).
Comparison of residue amounts extracted in the metabolism study with the amounts extracted by the extraction procedures of a residue analytical method confirms efficient extraction for the analytical methods, method L0272/01 for BAS 750 F and L0309/01 for metabolite M750F022.
For BAS 750 F, extraction efficiencies generally were 80% or higher for most matrices (milk, cream, muscle, kidney, fat, egg yolk), and lower for liver (46%). For M750F022, extraction efficiencies generally were 90% or higher for most matrices (milk, cream, kidney, fat) and lower for egg yolk (66%), for muscle (61%) and for liver (46-50%).

CA 6.2.4 Pigs

The metabolisation of BAS 750 F in rodents (rats) and ruminants (goats) did not reveal significant qualitative differences. Therefore, investigations of the metabolism in pigs are not required. Consequently, the metabolism of BAS 750 F in pigs was not investigated.

CA 6.2.5 Fish

Report: CA 6.2.5/1
[redacted] 2015 a
The metabolism of 14C-BAS 750 F in rainbow trout (Oncorhynchus mykiss)
2015/1106141

Guidelines: EPA 860.1300: Nature of the Residue in Plants Livestock, SANCO/11187/2013 (31 January 2013), OECD 305

GLP: yes
(certified by Ministerium fuer Arbeit, Integration und Soziales des Landes Nordrhein-Westfalen, Duesseldorf)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F was investigated in rainbow trout following repeated oral administration of ^{14}C-BAS 750 F, labeled either in the Chlorophenyl ring (C-ring), or in the triazole ring (T-ring). The test item was administered orally at a nominal dose of 5 mg/kg feed. Per label, 5 fish were dosed (4 fish for 10 consecutive days, 1 fish for 14 consecutive days with the purpose to confirm steady state). The actual concentrations were 5.82 mg/kg feed (C-label) and 5.40 mg/kg feed (T-label). In a pre-test radioactivity in tissue had reached a plateau after 10 days of dosing (the pre-test dose of 10 mg/kg feed showed insufficient acceptance by fish and therefore was reduced to 5 mg/kg in the study). Feces was collected (siponed daily prior to and after feeding). Radioactivity in tanks only at low levels ensured absence of bioconcentration effects. Sampling was done 6 hours after the last dose and included the matrices filet, filet with skin and liver.

For both labels, the overall accountability of the study was good. Recovery was high with 90-93% of dose the predominant part present in active coal (68-62% of dose). In tissues only low amounts of dose were present (filet up to 1.5% dose, in filet skin up to 0.25% dose and in liver up to 0.5% dose).

The TRR (determined during the plateau phase, see above) in edible tissues, filet and filet skin, was low compared to the TRR observed in liver. In filet the TRR accounted for 0.029 mg/kg (C-label) and 0.053 mg/kg (T-label). Slightly lower levels were observed when dosing was extended by 4 days (0.024 mg/kg for C-label, 0.036 mg/kg for T-label). Similar results were obtained for filet skin with 0.038 mg/kg (14 day, 0.026 mg/kg) for the C-label and 0.054 mg/kg (14 day 0.048 mg/kg) for the T-label. In contrast, TRR in liver was significantly higher for the C-label with 0.34 mg/kg, (14 days, 0.28 mg/kg) and also for the T-label with 0.23 mg/kg (14 days, 30 mg/kg). Overall, TRR in C-labeled filet and filet skin, was lower than in T-labeled filet and filet skin, whereas in liver no significant difference between the labels was seen.

The extractability of radioactive residues from all matrices (filet, filet skin and liver) was similarly high for both labels (≥ 79 % TRR, with two exceptions, first C-labeled fish dosed 14d with 61% TRR, and second, T-labeled fish dosed 14d with 58% TRR). A further investigation of the RRR obtained after solvent extraction was not performed, since overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in rainbow trout includes one main transformation reaction, cleavage of the parent backbone at the T-bridge, generating the cleavage product 1,2,4-triazole (M750F001). A further minor transformation is the hydroxylation of the T-ring of uncleaved parent BAS 750 F (M750F086) which was however observed only in non-edible tissue (fish liver).

Unchanged parent was seen in edible tissues (filet, filet skin) at comparable absolute amounts (filet up to 0.02 mg/kg, filet skin up to 0.03 mg/kg) corresponding to >60% TRR with C-label and >30% of T-label. With the T-label, also the cleavage product 1,2,4-triazole was detected, amounting to 0.03 mg/kg (>48% TRR). In order to investigate biotransformation in fish, also the composition of the radioactive residue in liver was analysed. Besides unchanged parent and 1,2,4-triazole, an additional transformation product was identified, resulting from hydroxylation of the T-ring. This liver-specific compound was found only in minor amounts (7% TRR with the C-label, 6% TRR with the T-label, corresponding to levels <0.03 mg/kg).

In conclusion, the major components of the residue in edible fish matrices, filet and filet skin, was identified as unchanged parent BAS 750 F and the TDM which together represent a large proportion of the residue (80% TRR or higher). Taking into consideration that the highest residue level for BAS 750 F in edible fish matrices of 0.027 mg/kg (filet skin of C-labeled 10d-fish) represents the plateau level resulting from a daily dose of 5 mg/kg, it can be concluded that for fish feed burden of <1.8 mg/kg no significant residues (0.01 mg/kg) are expected in dietary commodities of fish. Overall, metabolism of BAS 750 F in rainbow trout, and by extrapolation in fish raised in aquaculture, can be considered well-elucidated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description:	**C-label**
	Chlorophenyl-U-^{14}C-BAS 750 F, specific activity 8.11 MBq/mg
	Chlorophenyl-^{13}C-BAS 750 F
	T-label
	Triazole-3(5)-^{14}C-BAS 750 F, specific activity 5.57 MBq/mg
	Triazole-^{13}C-BAS 750 F
Lot/Batch #:	[Chlorophenyl-U-^{14}C]-Label: 1075-1001
	[Triazole-3(5)-^{14}C]-Label: 1062-2101
	Chlorophenyl-^{13}C-BAS 750 F: RS4-2012-173A2
	Triazole-^{13}C-BAS 750 F: 1077-1001
Purity:	[Chlorophenyl-U-^{14}C]-Label: 88.0% (CoA)
	[Triazole-^{14}C]-Label: 566-5101: 95.7% (CoA)
	Chlorophenyl-^{13}C-BAS 750 F: 97.7% (CoA)
	Triazole-^{13}C-BAS 750 F: 97.1% (CoA)
CAS#:	1417782-03-6
Development code:	BAS 750 F
Stability of test compound:	tested after 0, 1, 2 and 3 weeks when storing at 4°C: stable over the whole dosing period

2. Test animals: fish
Test species: rainbow trout (*Oncorhynchus mykiss*)
Body weight: 310 – 415 g (between study days -4 and -11; no measurement at the start of experiment was conducted to avoid stress)
Body length: 34 -37 cm at sacrifice

B. STUDY DESIGN AND METHODS

1. Test procedure

Prior to the dosing period, the fish were acclimatized to the laboratory environment for at least 8 weeks in de-chlorinated tap water. During this time period, the animals raised up to the experimental weight of at least 250 g. The fish were transferred to the test basin and acclimatized to the experimental conditions applied in the exposure for at least 14 days. In this acclimatization phase, the experimental animals were fed on the non-enriched diet. The experiment was carried out in two experimental tanks, each filled with approximately 1400 L of pre-conditioned tap-water under flow-through conditions with a flow rate of approximately 130 L/h. Each tank was stocked with 5 experimental animals. Water in the tank was constantly recirculated through two filter columns to avoid the accumulation of dissolved test item and metabolites. The water temperature was kept constant at around 15ºC (±2°C) throughout the experiment. Fish were housed under a 16 hour light/8 hour dark cycle throughout the experiment.
The dose formulation for the C-label was prepared by mixing ^{14}C-labelled, ^{13}C-labelled and non-radiolabelled test items (molecular structures are provided in section 6.2) in a ratio 2:1:1, leading to an actual specific activity of 4.01 MBq/mg. The dose formulation for the T-label was prepared by mixing ^{14}C-labelled, ^{13}C-labelled and non-labelled test item in a ratio 4:1:1 leading to an actual specific activity of 3.71 MBq/mg. A specific activity of 4.01 MBq/mg for the C-label and 3.71 MBq/mg for the T-label was used for the purpose of calculating TRR.

During the study, the test item was dosed to the fish for 10 consecutive days for both labels at a nominal level of 5 mg/kg. The daily dose administered was 5.82 mg [Chlorophenyl-U-14C]-BAS 750 F and 5.40 mg [Triazole-3(5)-14C]-BAS 750 F respectively per kg food consumed. One fish per label was kept under this conditions for 14 consecutive days to obtain data for steady state confirmation. The daily ration was distributed on the surface of the water. The daily ration was equivalent to 2.5% of body weight at the start of exposure period. On day 7 the daily ration was adjusted to take account of the growth increment equivalent to a food conversion ratio of 1.2.

A pre-test showed that radioactivity in tissue reached a maximum plateau 10 days after the first dose. Thus, sacrifice time was set to the day 10 (6h after dose10). Filet, filet skin, liver as well as pyloric caeca removed post mortem. Samples not analyzed immediately were stored frozen (-20°C) until analysis. After analysis, samples were returned to storage at -20°C.

2. Description of analytical procedures

Aliquots of the homogenized filet, the entire homogenized filet skins and unmodified livers were extracted with acetonitrile. The number of acetonitrile extractions depended on the sample weight. After evaporation (nitrogen stream) the residues were taken up in a mixture of acetonitrile and water (1:1, v:v) and sonicated. The suspension was centrifuged and the supernatant was separated. The extracts were combined and filtered. These concentrated samples were then analyzed by LSC, HPLC and LC-MS.

3. Identification and characterization of the residue

Concentrated extracts of filet, skin and liver were analyzed with radio-HPLC, one metabolite was identified using high resolution LC-MS. Reference substances used include chlorophenyl-U-^{14}C-BAS 750 F and triazole-3(5)-^{14}C-BAS 750 F, as the TDM (M750F001, M750F029, M750F030 and M750F031).

II. RESULTS AND DISCUSSION

General observations: All animals remained in good general health throughout the study. No behavioral or physical abnormalities were observed during acclimatization period and dosing period except for reduction of feed consumption towards end of the study (see below). The body weight during acclimatization reached the experimental body weight of 250 g. During the dosing period the weight variation did not exceed +/- 25% of the average weight.
Animals showed a reduced feed consumption starting day 8 of the dosing period. To avoid the risk of a decrease of tissue radioactivity resulting from reduced feed consumption, the dosing period was reduced to 10d for 4 of the 5 animals per label (while one animal per label was held until day 14 in order to confirm the establishment of steady state condition at 10d.) Note, the dose of 5 mg/kg had been chosen based on results of a pre-test where doses of 1 mg/kg and 5 mg/kg had shown no effect on acceptance of the diet, whereas a dose of 10 mg/kg had indicated reduced uptake of the diet over a 14d period.

Generally, edible tissues did contain only very low levels of radioactivity. To allow quantitative and qualitative evaluation of the composition of the radioactive residue pooled samples in their entirety had to be extracted and extracts concentrated. As a consequence, some repetitive analyses was in cases not feasible and also, some detections near the LOQ showed certain variability. Nevertheless, consistent results could be obtained as summarized.

1. TOTAL RADIOACTIVE RESIDUES (TRR)

The total mass balance recovered was 89.6% of the C-label, and 93.3% of the T-label. The majority was present in charcoal (68% and 62%, C-and T-label). Radioactivity in the experimental tanks was very low throughout the study. A significant effect on tissue concentrations caused by bioconcentration processes can be thus excluded.
Overall, similar recoveries were obtained for both labels. In faeces, 4.75% and 7.36% of dose were contained (C- and T-label). In contrast, the recovery from edible tissues was much lower with, for filet 0.88% and 1.52% (C-and T-label), for filet skin 0.11% and 0.25% (C-and T-label), for liver 0.52% and 0.40% (C-and T-label).

Table 6.2.5-1: Recovery of radioactivity following dosing trout with ^{14}C-BAS 750 F

C-label	sample	total sample weight *(fresh weight, [g] or [L])*	dose recovered *[%]*
	filet	959.4	0.88
	filet skin	97.1	0.11
	liver	48.9	0.52
	pyloric caeca	417.5	7.03
	feces	105.8	4.75
	carcass	1 086.2	2.04
	active coal	20 940	67.67
	water	34 744.0 L	6.57
	TOTAL	-	**89.57**
T-label	**sample**	**total sample weight** *(fresh weight, [g] or [L])*	**dose recovered** *[%]*
	filet	925.5	1.52
	filet skin	143.6	0.25
	liver	65.8	0.40
	pyloric caeca	161.0	2.10
	feces	119.0	7.36
	carcass	1 294.4	2.74
	active coal	14 110.0	62.03
	water	36 369.4 L	16.93
	TOTAL	-	**93.33**

Total radioactive residue (Table 6.2.5-1) obtained with both labels show that for both labels similar data is obtained after 10 day exposure (4 fish per label) and 14 day exposure (1 fish per label) indicating that 10 day data in fact represents a steady state level. In tissues, TRR was only low with higher levels for the T-label compared with the C-label. The TRR in filet accounted for 0.03 and 0.05 mg/kg, in filet skin 0.04 and 0.05 mg/kg (C- and T-label). Higher residues were found in liver with 0.35 mg/kg and 0.23 mg/kg (C- and T-label).

2. EXTRACTABILITY

Extractability generally was high, in filet above 90% TRR, in filet skin above 85.9% TRR and in liver above 89.5% TRR with RRR in filet at <3.0% (maximum 0.002 mg/kg), in filet skin <7.5% (maximum 0.004 mg/kg) and in liver <5.5% (maximum 0.018 mg/kg) and thus was not further analysed. For the two control animals (14d dosing, C- and T-label) results were not significantly different, variability appears rather due to the low amounts in radioactivity present in the samples.

Table 6.2.5-2: TRR and extractability of residues of 14C-BAS 750 F (C- and T-label)

matrix		**sample**	**TRR** *measured*	**ERR**		**RRR**		**recovery** [1)]
			mg/kg	*[%]*	*mg/kg*	*[%]*	*mg/kg*	*[%]*
C-label	filet	10 d	*0.029*	**90.6**	*0.026*	**3.2**	*0.001*	93.8
		14 d	*0.024*	**92.2**	*0.022*	**6.4**	*0.002*	98.6
	filet skin	10 d	*0.038*	**85.9**	*0.033*	**7.0**	*0.003*	92.9
		14 d	*0.026*	**60.8**	*0.017*	**17.3**	*0.005*	78.1
	liver	10 d	*0.345*	**89.5**	*0.308*	**5.3**	*0.018*	94.8
		14 d	*0.275*	**-**[2)]	**-**[2)]	**13.3**	*0.036*	**-**[2)]
T-label	filet	10 d	*0.053*	**92.8**	*0.049*	**3.1**	*0.002*	95.9
		14 d	*0.036*	**95.1**	*0.035*	**3.8**	*0.001*	98.9
	filet skin	10 d	*0.054*	**93.2**	*0.050*	**7.4**	*0.004*	100.6
		14 d	*0.048*	**79.4**	*0.038*	**8.6**	*0.004*	88.0
	liver	10 d	*0.234*	**97.7**	*0.229*	**5.4**	*0.007*	103.1
		14 d	*0.303*	**57.5**	*0.174*	**8.8**	*0.012*	66.3

[1)] recovery was calculated as sum of ERR and RRR, [2)] not analyzed

3. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

The following table provides a summary of the residue components identified and/or characterized in samples obtained with the C-label and the T-label, each for fish dosed over 10 consecutive days (4 fishes per label) and for fish dosed 14 consecutive days (1 fish per label). The ERR was subject to HPLC analysis, the RRR was not further analysed. Overall, similar level of BAS 750 F were seen with both labels, the T-label detected in addition one cleavage product resulting from T-bridge cleavage (M750F001). Furthermore, a liver-specific metabolite was identified (M750F086).

3.1 filet

Unchanged parent BAS 750 F was the predominant component of the radioactive residue in pooled samples (C-labeled 10d-fish) with 74.2% TRR corresponding to 0.022 mg/kg (C-labeled 14d-fish, 68.8% TRR). T-labeled 10d-fish contained in a lower proportion of BAS 750 F with 35.5% TRR corresponding to 0.018 mg/kg (T-labeled 14d-fish, 14.6% TRR).
The sum of identified residue from ERR for the C-label was 74.2% TRR (14d, 68.8%TRR), for the T-label was 97.8% TRR (14d, 86.7% TRR). Including the “calculated loss” the Grand Total was 93.8% TRR (14d, 98.6% TRR), for the T-label was 100.3% TRR (14d, 98.9% TRR). The loss during work up was low with 0.006 mg/kg, due to the overall low levels resulted in a proportion of 23.4% TRR (C-label, 14d).

3.2 filet skin

Unchanged parent BAS 750 F was the predominant component of the radioactive residue in samples of C-labeled 10d-fish with 71.9% TRR corresponding to 0.027 mg/kg (C-labeled 14d-fish, 63.9% TRR). T-labeled 10d-fish contained parent BAS 750 F, albeit only as the second most abundant compound. Proportions were lower accounting for 35.3% TRR (14 d fish: 31.1% TRR) corresponding to 0.019 mg/kg. The most abundant compound in the T-label was the metabolite 1,2,4-triazole (M750F001) with 58.1% TRR (14 d fish: 48.4% TRR) corresponding to 0.031 mg/kg. The sum of identified residue from ERR for C-labeled 10d-fish was 71.9% TRR (C-labeled 14d-fish, 63.9% TRR), for T-labeled 10d-fish, 93.4% TRR (T-labeled 14d-fish, 79.5% TRR). Including the “calculated loss” the Grand Total was 92.9% TRR (14d, 81.2% TRR), for the T-label was 100.8% TRR (14d, 88.1% TRR).

3.3 liver

Unchanged parent BAS 750 F was the predominant component of the radioactive residue in samples of the C-labeled 10d-fish with 58.2% TRR (C-labeled 14d-fish, 59.1% TRR) corresponding to 0.204 mg/kg. With the T-labeled 10d-fish proportions were in a similar order of magnitude: 69.3% TRR (14 d fish: 35.5% TRR) corresponding to 0.162 mg/kg. In the T-label, the second most abundant compound was the metabolite 1,2,4-triazole (M750F001) with proportions of 16.8% TRR (14d, 11.4% TRR) corresponding to 0.039 mg/kg (14d, 0.034 mg/kg). In both labels, one additional metabolite was identified, albeit below 10% TRR and at only low absolute amounts (C-labeled 10d-fish and 14d-fish: 7.2 and 7.2% TRR (0.025 and 0.020 mg/kg), T-labeled 10d-fish and 14d-fish: 5.7% TRR (0.013 mg/kg).
The sum of identified residue from ERR for the C-labeled 10d-fish was 65.4% TRR (14d, 66.1% TRR), for the T-labeled 10d-fish was 91.8% TRR (14d, 46.9% TRR). Including the “calculated loss” the Grand Total was 94.8% TRR (14d, 79.4% TRR), for the T-label was 103.1% TRR (14d, 88.1% TRR).

Table 6.2.5-3: Summary of identified/characterized components in fish (C- and T-label)

C-label	filet-10 [1]		filet-14 [2]		filet skin-10 [1]		filet skin-14 [2]		liver-10 [1]		liver-14 [2]	
	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg
BAS 750 F	**74.2**	*0.022*	**68.8**	*0.016*	**71.9**	*0.027*	**63.9**	*0.018*	**58.2**	*0.204*	**59.1**	*0.173*
M750F001	-	-	-	-	-	-	-	-	-	-	-	-
M750F086	-	-	-	-	-	-	-	-	**7.2**	*0.025*	**7.0**	*0.020*
ERR [3]												
ID [3]	**74.2**	*0.022*	**68.8**	*0.016*	**71.9**	*0.027*	**63.9**	*0.018*	**65.4**	*0.229*	**66.1**	*0.193*
CHAR [3]	**16.4**	*0.004*	**23.4**	*0.006*	**14.0**	*0.006*	-	-	**24.1**	*0.079*	-	-
ID/CHAR	**90.6**	*0.026*	**92.2**	*0.022*	**85.9**	*0.033*	**63.9**	*0.018*	**89.5**	*0.308*	**66.1**	*0.193*
RRR [3]	**3.2**	*0.001*	**6.4**	*0.002*	**7.0**	*0.003*	**17.3**	*0.005*	**5.3**	*0.018*	**13.3**	*0.036*
Grand Total	**93.8**	*0.027*	**98.6**	*0.024*	**92.9**	*0.036*	**81.2**	*0.023*	**94.8**	*0.326*	**79.4**	*0.229*

T-label	filet [1]		filet [2]		filet skin [1]		filet skin [2]		liver [1]		liver [2]	
	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg	TRR	mg/kg
BAS 750 F	**35.5**	*0.018*	**14.6**	*0.005*	**35.3**	*0.019*	**31.1**	*0.015*	**69.3**	*0.162*	**35.5**	*0.108*
M750F001	**62.3**	*0.033*	**72.1**	*0.026*	**58.1**	*0.031*	**48.4**	*0.023*	**16.8**	*0.039*	**11.4**	*0.034*
M750F086	-	-	-	-	-	-	-	-	**5.7**	*0.013*	-	-
ERR [3]												
ID [3]	**97.8**	*0.051*	**86.7**	*0.031*	**93.4**	*0.050*	**79.5**	*0.038*	**91.8**	*0.214*	**46.9**	*0.142*
CHAR [3]	-	-	**8.4**	*0.004*	-	-	-	-	**5.9**	*0.015*	**10.6**	*0.032*
ID/CHAR	**97.8**	*0.051*	**95.1**	*0.035*	**93.4**	*0.050*	**79.5**	*0.038*	**97.7**	*0.229*	**57.5**	*0.174*
RRR [3]	**3.1**	*0.002*	**3.8**	*0.001*	**7.4**	*0.004*	**8.6**	*0.004*	**5.4**	*0.007*	**8.8**	*0.012*
Grand Total	**101.0**	*0.053*	**98.9**	*0.036*	**100.8**	*0.054*	**88.1**	*0.042*	**103.1**	*0.236*	**66.3**	*0.186*

[1] data obtained from a pool sample of 4 fishes dosed for 10 consecutive days, [2] data obtained from one fish dosed for 14 consecutive days, [3] ERR = extractable radioactive residue, RRR= residual radioactive residue, equivalent to PES=post extraction solid, ID = amount identified, CHAR= amount characterized, ID/CHAR = sum of amount identified and/or characterized, Grand Total = sum of RRR+amounts identified and/or characterized.

4. Proposed metabolic pathway

Metabolism of BAS 750 F was investigated in fish using two labels, C-label and T-label. Results taken together indicate comparable pathways, the label-specific differences observed are consistent with effective cleavage of the parent backbone at the triazole bridge, cleaving off the triazole ring.

Overall, results show that in fish, BAS 750 F is metabolized by one main transformation reaction:

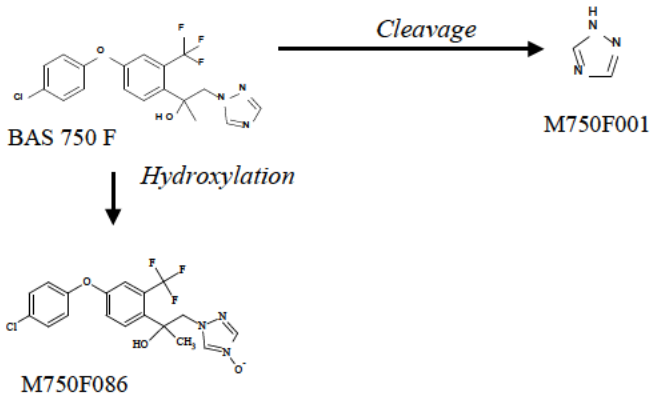
- cleavage of the parent backbone at the triazole bridge (T-bridge)

In addition a minor transformation reaction was observed:

- T-ring hydroxylation of the uncleaved parent molecule

Cleavage of the T-bridge generates 1,2,4-triazole (M750F001), while T-ring hydroxylation generates the fish-liver-specific metabolite M750F086.

Figure 6.2-12: Proposed metabolic pathway of BAS 750 F in rainbow trout



5. Sample storage stability

Analysis of samples including extraction and workup was completed within 6 months after sampling (Table 6.2.5-4), and therefore within the time of assumed stability (deep frozen storage).

Table 6.2.5-4: Storage intervals of C- and T-labeled fish samples

Step	storage interval [months]					
	10d group (C-and T-label)			14d animal (C-and T-label)		
	filet	filet skin	liver	filet	filet skin	liver
sampling to extraction	2	2	2	4	4	4
extraction to analysis	1	1	1	(directly)	(directly)	(directly)
sampling to analysis	3	3	3	4	4	4
assumed	<6	<6	<6	<6	<6	<6

6. Lipid analysis

Filets had an average fat content of 7.5% (C-labeled 10d-fish) and of 9.7% (C-labeled 14d-fish) and of 8.7% (T-labeled 10d-fish) and 11.5% (T-labeled 14d-fish). The higher fat content at day 14 compared with day10 most likely reflects the fact the largest animal in each of the two groups was selected for the extended dosing period. The values for fat content are in the range of natural variance and are not impacting the study results (Table 6.2.5-5).

Table 6.2.5-5: Fat content in C- and T-labeled filet samples

samples	fat content [% total weight]			
	C-label		T-label	
	10d-4 fishes	14d-1 fish	10d-4 fishes	14d-1 fish
1	7.2	9.3	8.4	11.6
2	7.4	10.0	8.9	11.5
3	7.8	9.9	8.9	11.4
mean	**7.5**	**9.7**	**8.7**	**11.5**

III. CONCLUSION METABOLISM IN FISH

The TRR in edible tissues, filet and filet skin, was low compared to the TRR observed in liver. In filet the TRR accounted for 0.029 mg/kg (C-label) and 0.053 mg/kg (T-label). Slightly lower levels were observed when dosing was extended by 4 days (0.024 mg/kg for C-label, 0.036 mg/kg for T-label). Similar results were obtained for filet skin with 0.038 mg/kg (14 day, 0.026 mg/kg) for the C-label and 0.054 mg/kg (14 day 0.048 mg/kg) for the T-label. In contrast, TRR in liver was significantly higher for the C-label with 0.34 mg/kg, (14 days, 0.28 mg/kg) and also for the T-label with 0.23 mg/kg (14 days, 30 mg/kg). Overall, TRR in C-labeled filet and filet skin, was lower than in T-labeled filet and filet skin, whereas in liver no significant difference between the labels was seen.

The extractability of radioactive residues from all matrices (filet, filet skin and liver) was similarly high for both labels (≥ 79 % TRR, with two exceptions, first C-labeled fish dosed 14d with 61% TRR, and second, T-labeled fish dosed 14d with 58% TRR). A further investigation of the RRR obtained after solvent extraction was not performed, since overall, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

Metabolism of BAS 750 F in rainbow trout includes one main transformation reaction, cleavage of the parent backbone at the T-bridge, generating the cleavage product 1,2,4-triazole (M750F001). A further minor transformation is the hydroxylation of the T-ring of uncleaved parent BAS 750 F (M750F086) which was however observed only in non-edible tissue (fish liver).

Unchanged parent was seen in edible tissues (filet, filet skin) at comparable absolute amounts (filet up to 0.02 mg/kg, filet skin up to 0.03 mg/kg) corresponding to >60% TRR with C-label and >30% of T-label. With the T-label, also the cleavage product 1,2,4-triazole was detected, amounting to 0.03 mg/kg (>48% TRR). In order to investigate biotransformation in fish, also the composition of the radioactive residue in liver was analysed. Besides unchanged parent and 1,2,4-triazole, an additional transformation product was identified, resulting from hydroxylation of the T-ring. This liver-specific compound was found only in minor amounts (7% TRR with the C-label, 6% TRR with the T-label, corresponding to levels <0.03 mg/kg).

In conclusion, the major components of the residue in edible fish matrices, filet and filet skin, was identified as unchanged parent BAS 750 F and the TDM which together represent a large proportion of the residue (80% TRR or higher). Taking into consideration that the highest residue level for BAS 750 F in edible fish matrices of 0.027 mg/kg (filet skin of C-labeled 10d-fish) represents the plateau level resulting from a daily dose of 5 mg/kg, it can be concluded that for fish feed burden of <1.8 mg/kg no significant residues (0.01 mg/kg) are expected in dietary commodities of fish. Overall, metabolism of BAS 750 F in rainbow trout, and by extrapolation in fish raised in aquaculture, can be considered well-elucidated.

OVERALL CONCLUSION LIVESTOCK

Metabolism was investigated with in rat, goat and hen with three labels, and in fish with two labels (the labeling strategy capturing all possible cleavage products as detailed in section CA 6.2). The residue was rapidly and extensively eliminated via excreta, reaching a plateau in milk and egg within 7 days. The animal metabolism studies taken together, confirm that livestock animals, namely ruminants, poultry and fish, have common basic metabolic routes of BAS 750 F which are highly comparable to the metabolic routes identified in rat (see section 6.7.1).

The metabolic pathway is largely based on two main transformation steps in livestock animals:

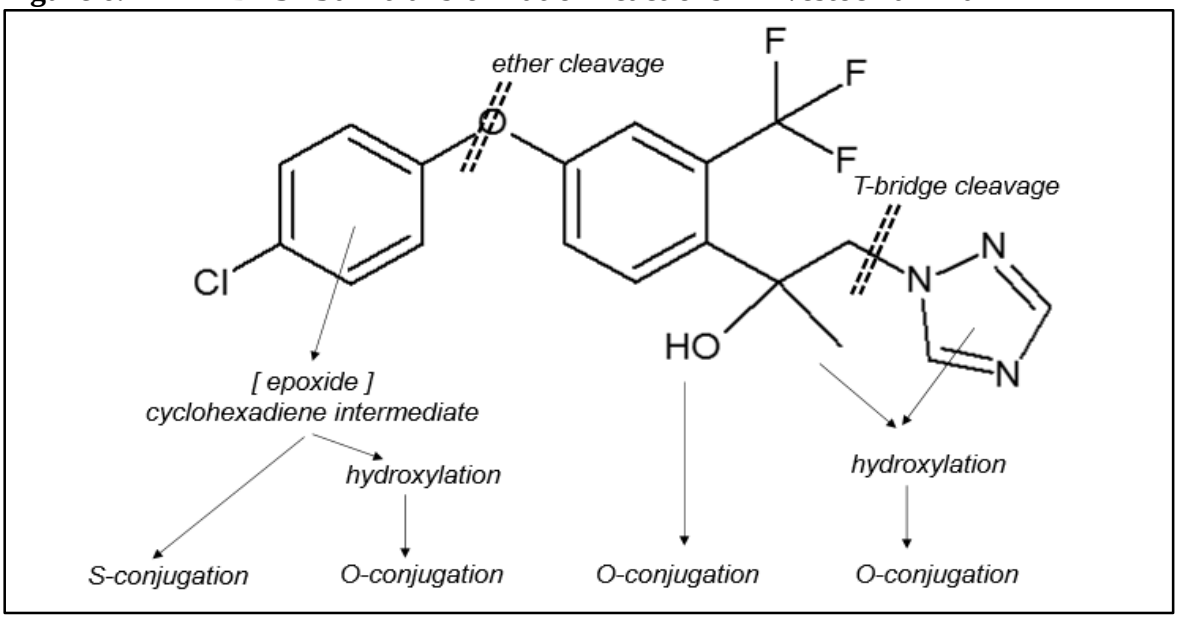
- hydroxylation at the C-ring (followed by conjugation)
- cleavage at the T-bridge (followed by conjugation)

In addition, minor transformation steps were observed in livestock animals:

- cleavage at the ether bridge (followed by conjugation)
- hydroxylation at the T-ring
- hydroxylation of the methyl group (at quaternary C-atom, followed by conjugation)

Differences seen in species and/or matrices are the result of quantitative differences of transformation reactions as well as species-typical conjugation reactions (sulfatation, glucuronidation, methylation, glutathion conjugation).

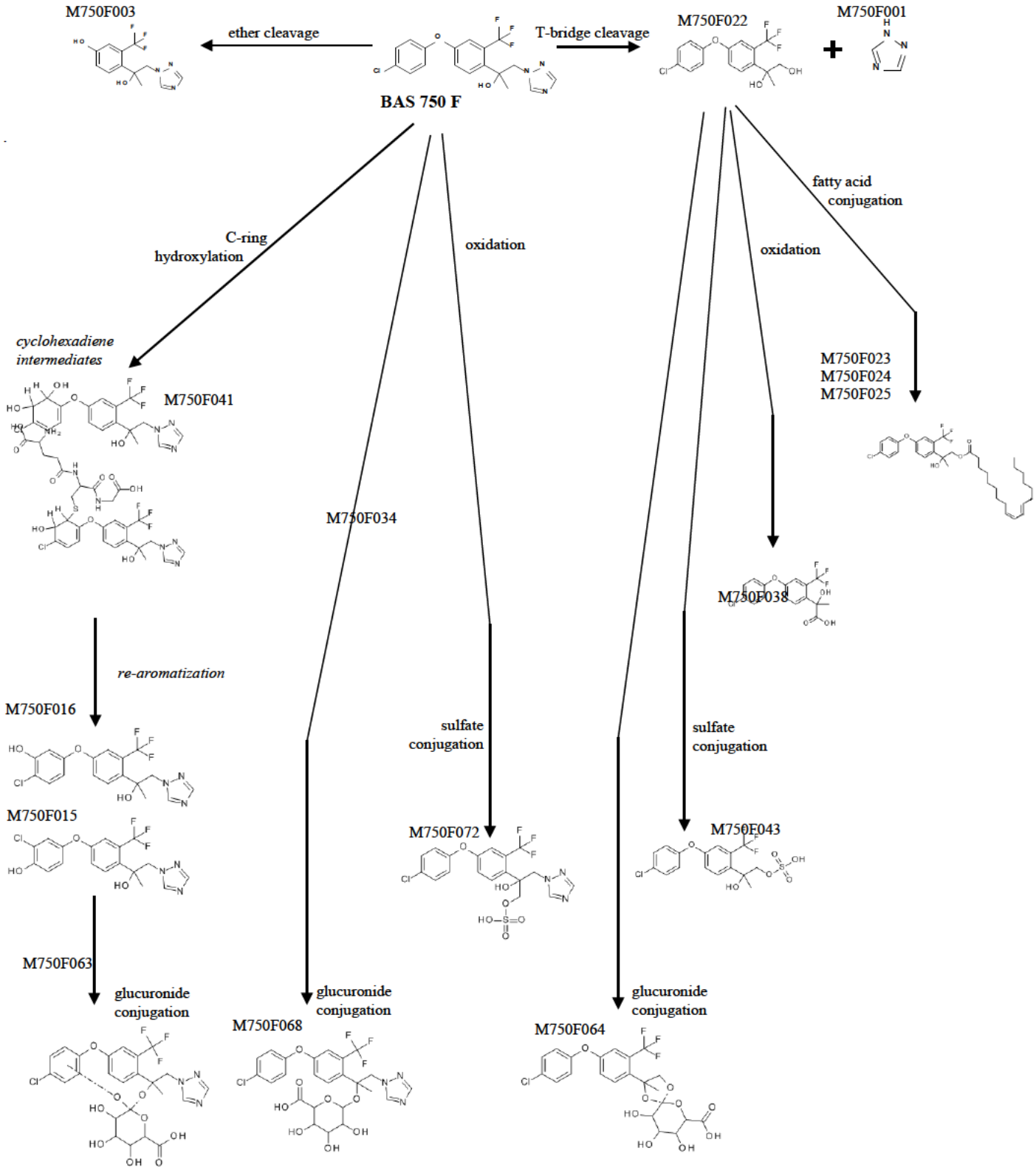
Figure 6.2-12 BAS 750 F transformation reactions in livestock animal



In rat, as a metabolically highly active species, due to intensively thorough investigations a very detailed metabolic profile was obtained, encompassing identification of over 100 metabolites. In contrast, while livestock metabolism studies confirm presence of comparable metabolic routes, the number of pertinent residue components in animal commodities is limited (see Table 6.2.5-6).

Figure 6.2/13 BAS 750 F: metabolic routes in livestock animals (food commodities)

Note, arrows indicate metabolic routes, not separate biochemical reactions. The group of M750F023, M750F024, and M750F025 are acyl-conjugates of M750F022, differing only in the fatty acid side chain, thus only one representative molecular structure is provided). Metabolites occurring only in non-edible commodities are not considered.



Metabolites occurring in edible livestock matrices in major amounts (>10% TRR) in minor amounts (<10% TRR) are listed in Table 6.2.5-6. This table groups the metabolites according to their chemical structure together with their corresponding conjugates. The non-conjugated metabolites that were identified in food commodities are highlighted (underlined). A detailed discussion of any possible relevance for the residue definitions, either for MRL enforcement or for dietary risk assessment, is provided in section CA6.7.1.

Table 6.2.5-6: Residue components identified in edible livestock commodities

group definition	(unconjugated) group metabolite members in LIVESTOCK	
	≥ 10% TRR	**< 10% TRR**
a) parent and conjugates		
	BAS 750 F M750F068	M750F072
b) "C-ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates		
	M750F016 M750F034	**M750F015** **M750F041** M750F063
c) cleavage products & downstream metabolites / conjugates		
Metabolites without the C-ring		**M750F003**
Metabolites without 1,2,4-T-ring	**M750F022** M750F023 M750F024 M750F025 **M750F038** M750F043 M750F064	-
1,2,4-triazole and triazole-derived metabolites (TDM)	**1,2,4-T**	-

CA 6.3 Magnitude of residues trials in plants

The present dossier supports the use of the representative formulated product BAS 750 01 F on the crops wheat and barley. In this section, the supporting residue data is summarized, for wheat in section CA 6.3.1 and for barley in section CA 6.3.2 are summarized.

CA 6.3.1 Wheat

In support of the representative use (formulated product BAS 750 01 F) in wheat, field trials were conducted in season 2013 and season 2014. Field trial data obtained with the formulated product BAS 750 01 F is available for the season 2014 (4 trials N-EU trials, 5 trials S-EU). In addition, in season 2013 field trial data is available for the formulated BAS 750 00 F (4 trials N-EU trials, 4 trials S-EU).
Generally, equivalent residue data is obtained with both formulated products as confirmed by BAS 750 01 F residue data (season 2014) generated in parallel with the formulated product BAS 750 00 F (see Table 6.3.1-15).

The cGAP is provided in Table 6.3.1-1. The residue trials were performed in various European Member States in N-EU and S-EU during the growing seasons 2013 and 2014 and thereby fulfill the requirements of seasonal and geographical distribution (see Table 6.3.1-2).

Table 6.3.1-1: Summary of the critical GAPs for the proposed uses in wheat

crop	outdoor/ protected	growth stage (BBCH)	maximum number of applications	minimum application interval (days)	maximum		minimum PHI (days)
					rate (kg as/ha)	water (L/ha)	
wheat	outdoor	49, 69	2	14	0.15	200	35

Table 6.3.1-2: Number of residue trials per geographical region and vegetation period

crop	season	number of trials					reference
		N-EU	country	S-EU	country	total	
wheat	2013	4	DE, NL, UK	4	FR, GR, IT, ES	8	6.3.1/1
wheat	2014	4	DE, FR, NL	5	FR, GR, IT, ES	9	6.3.1/2
total number of trials per region		**8**	**-**	**9**	**total number of trials**	**17**	

Table 6.3.1-3: Residues of BAS 750 F: overall summary from residue trials in wheat

crop	region	RAC	n	Residue data BAS 750 F (mg/kg)			
				2013	2014	HR	STMR
wheat	N-EU	grain	8	<0.01 (3x), 0.024	<0.01 (3x), 0.014	0.024	0.010
	S-EU		9	<0.01 (3x), 0.018	<0.01 (4x), 0.026	0.026	0.010
	N-EU	straw	8	2.3, 3.9, 5.5, 10	1.9, 2.6, 3.6, 4.4	10	3.75
	S-EU		9	0.5, 2.9, 3.8, 18	0.56, 1.6, 3.1, 4.6, 8.5	18	3.10

The study reports are summarized below.

Table 6.3.1-4: Wheat residue data: summary of maximum storage interval

crop	maximum storage interval *(days)* [1)]		confirmed storage stability [1)]				
	BAS 750 F analytics	TDM analytics	BAS 750 F *(days)*	1,2,4-T *(months)*	TA *(months)*	TAA *(months)*	TLA *(months)*
wheat grain	576	477	547 [2)]	54	54	26	48
wheat straw	554	390	551 [3)]	40	54	40	48

[1)] *from harvest until extraction as deep frozen samples, 1,2,4-T= 1,2,4-triazole, TA= triazole alanine, TAA=triazole acetic acid, TLA= triazole lactic acid,* [2)] *note, that storage stability study ongoing, results obtained so far show that after 547 days, stability is 93.0%, , and* [3)] *after 551 days, stability is 99.5%.*

Report:	CA 6.3.1/1 Erdmann H.-P., 2015 a Study on the residue behaviour of Reg.No. 5834378 (BAS 750 F) in wheat after application of EXP 5834378 F-AV (BAS 750 00 F) under field condition in Germany, The Netherlands, United Kingdom, Southern France, Greece, Italy and Spain, 2013 2014/1010809
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011)
GLP:	yes (certified by Land Brandenburg Ministerium fuer Umwelt, Gesundheit und Verbraucherschutz, Potsdam, Germany)

EXECUTIVE SUMMARY

During the growing season of 2013, eight independent field trials with wheat were conducted in Europe (N-EU and S-EU) in order to determine the magnitude of the BAS 750 F residues (analytes were parent, and the TDM with 1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) after application of the formulated product BAS 750 00 F. The EC formulation BAS 750 00 F (0.1 kg as/L) was applied two times (BBCH 49 and 69) at rates of 0.15 kg as/ha for BAS 750 F in a spray volume of 200 L/ha. Wheat specimens were collected as whole plant (no roots) shortly before the second application (untreated control) and directly after the second application. Depending on the maturity of the crop, sampling after 34-35 days, 41-43 days and 46-50 days, provided either ears and rest-of-plant (no root) or grain and straw. In one trial, the crop did not reach growth stage BBCH89 after 49 days, thus, an additional sampling was done at 54 days. Generally, samples were analysed within the time period of confirmed freezer storage stability for BAS 750 F and TDM in high starch matrices (grain), high water matrices (green plant) and straw. Recoveries (mean) were within the acceptable range of 70 – 110% for both BAS 750 F and TDM.

For BAS 750 F, no residues exceeding the LOQ were detected in untreated samples. For grain from treated samples, BAS 750 F residues were on average 0.01 mg/kg (n=8), with six trials below LOQ, one trial with 0.018 mg/kg and as the highest residue 0.024 mg/kg. For straw from treated samples, BAS 750 F residues showed high variability ranging from 0.5 to 18 mg/kg. Notably, for one S-EU trial (Spain, L130173) above-average residue level in plant parts (straw, green plant) were found. In straw at DALA43, residues amounted to 9.9 mg/kg, increasing further to 18 mg/kg at DALA49 (in the corresponding plant sample at DALA35 residues were 12 mg/kg). In addition, in one trial (L130169, N-EU) residues of 10 mg/kg were determined at DALA35, declining further to 6.2 mg/kg at DALA50. For the other six trials residue level were 0.5, 2.3, 2.9, 3.8, 3.9 and 5.5 mg/kg. Based on this data, average residues of 5.9 mg/kg are calculated with the highest residues at 10 mg/kg (L130169, N-EU) and at 18 mg/kg (L130173, S-EU). For plants harvested directly after application (DALA0), BAS 750 F residues were in the range of 2.2 – 3.9 mg/kg (7 trials) and the trial in Spain (L130173) with 6.3 mg/kg. Residues in plant parts (ears, roots removed) declined to levels of 1.6 – 2.7 mg/kg at DALA34-35 and for the trial in Spain (L130173) 12 mg/kg.

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in treated (untreated) samples amounted for TA up to 0.76 mg/kg (0.49 mg/kg), for TAA up to 0.20 mg (0.04 mg/kg) and for TLA up to 0.09 mg/kg (0.06 mg/kg). For straw residue levels in treated (untreated) samples amounted for TA up to 0.04 mg/kg (0.33 mg/kg), for TAA up to 0.01 mg (0.03 mg/kg) and for TLA up to 1.5 mg/kg (1.1 mg/kg). Note that, TDM data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

I. MATERIAL AND METHODS

A. MATERIALS

1.

Test Material:	BAS 750 00 F	
Description:	BAS 750 00 F: 100 g/L of BAS 750 F, EC formulation	
Lot/Batch #:	BAS 750 00 F: 370168	
Purity:	*not relevant*	
CAS#:	1417782-03-6	
Development code:	BAS 750 F	
Spiking levels:	BAS 750 F:	0.01, 0.1, 1.0, 10, 20 mg/kg
	1,2,4-triazole:	0.01, 1.0 mg/kg
	triazole alanine:	0.01, 1.0 mg/kg
	triazole acetic acid:	0.01, 1.0 mg/kg
	triazole lactic acid:	0.01, 1.0, 10 mg/kg

2.

Test Commodity:	cereal
Crop:	wheat
Type:	*Triticum aestivum* L.
Variety:	Asano, Smaragd, Premio, Solstice, Tiepolo, Trofeo, Palasio, Artur Nick
Crop parts(s) or processed Commodity:	whole plant (no root), ear, rest-of-plant (no root), grain, straw
Sample size:	at least 1.0 kg (for straw, at least 0.5 kg)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2013, eight field trials on wheat with the formulated product BAS 750 00 F (EC formulation) were conducted in Germany, the Netherlands, the United Kingdom, Southern France, Greece, Italy and Spain, in order to determine the magnitude of the residues of BAS 750 F and its metabolites 1,2,4-triazole, TA, TAA and TLA. The EC formulation was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (0.1 kg as/L) in a spray volume of 200 L/ha.

Sampling included the harvest of whole plant (root removed) both shortly before the second application (untreated control) as well as directly after the second application. Samples were also taken at three time points after the last application, namely after 34-35 days, 41-43 days and 46-50 days. In the case of unripe crop, samples consisted of ear and rest-of-plant (roots removed) while for the ripe crop grain and straw were taken. In case, BBCH growth stage 89 was not reached after 49 days, an additional sampling time point was added.

Table 6.3.1-5 Application and sampling details

region	no. of trials	no. of appl.	F, G, I[2]	method	test item	active substance	application		target timing	
							rate (kg a.s. /ha)	water volume (L/ha)	application (BBCH)	sampling (DALA)[1]
EU-N & EU-S	8	2	F	foliar spray	BAS 750 00 F (EC)	BAS 750 F	0.15	200	1st appl.: BBCH49 2nd appl.: BBCH69	0 34 - 35 41 - 43 46 – 50 54

1) days after last application
2) field, glasshouse or indoor

2. Description of analytical procedures

BAS 750 F residues were determined according to BASF method L0076/09. BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane. The final determination was performed by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg. Stability of BAS 750 F (deep frozen plant matrices) has been confirmed for a minimum of 547 days in high starch matrices (e.g. grain), a minimum of 551 days in high water matrices (e.g. green plant) and for a minimum of 551 days in plant straw (e.g. cereal straw).

Procedural recovery (average) were 94% for BAS 750 F using fortification levels of 0.01-20 mg/kg. The results are summarized in the following table.

Table 6.3.1-6 Summary of recoveries for BAS 750 F

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0076/09		**BAS 750 F**			
whole plant (no root)	0.01, 0.10, 10	3	95.8	2.1	2.2
ears	0.01, 0.10, 1.0, 10	6	96.0	5.3	5.6
rest of plant (no root)	0.01, 0.10, 1.0, 10, 20	7	92.9	6.4	6.9
grain	0.01, 0.10, 1.0	6	98.6	3.9	4.0
straw	0.01, 0.10, 1.0, 10, 20	7	88.7	8.6	9.7
overall		**29**	**94.0**	**6.8**	**7.2**

TDM residues, namely 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were determined according to method L0170/02 (see section CA 4.3). The analytes are extracted with methanol/water. An aliquot of the extract is filtered, concentrated and cleaned-up by dispersive C_{18}-SPE prior to analysis by LC/DMS-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

Procedural recovery (average) were 99% (1,2,4-triazole), 89% (TA), 85.5% (TAA) and 87.9% (TLA). The results are summarized in the following table.

Table 6.3.1-7 Summary of recoveries for 1,2,4-T, TA, TAA and TLA

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0170/02		**1,2,4-triazole (T)**			
whole plant (no root),	0.01, 1.0	12	99.4	10	10
ears	0.01, 1.0	6	94.1	8.5	9.0
grain	0.01, 1.0	6	100	18	18
straw	0.01, 1.0	6	103	12	12
overall		**30**	**99.3**	**12**	**12**
method L0170/02		**triazole alanine (TA)**			
whole plant (no root),	0.01, 1.0	12	90.3	12	14
ears	0.01, 1.0	6	91.5	11	12
grain	0.01, 1.0	6	86.3	13	15
straw	0.01, 1.0	6	86.9	20	23
overall		**30**	**89.0**	**13**	**15**
method L0170/02		**triazole acetic acid (TAA)**			
whole plant (no root),	0.01, 1.0	12	88.4	15	17
ears	0.01, 1.0	5	85.8	15	17
grain	0.01, 1.0	6	88.8	15	17
straw	0.01, 1.0	6	76.3	15	20
overall		**29**	**85.5**	**15**	**18**
method L0170/02		**triazole lactic acid (TLA)**			
whole plant (no root),	0.01, 1.0, 10	10	94.8	15	15
ears	0.01, 1.0	5	93.0	23	25
grain	0.01, 1.0	6	77.3	10	13
straw	0.01, 1.0	4	80.1	7	9
overall		**25**	**87.9**	**16**	**18**

II. RESULTS AND DISCUSSION

The residue level of BAS 750 F and the TDM determined in wheat samples is summarized in Table 6.3.1-8 (treated samples) and Table 6.3.1-9 (untreated samples). Detailed residue data is provided in Table 6.3.1-10 and Table 6.3.1-11. The maximum storage interval of deep frozen samples from harvest until extraction was 626 days for BAS 750 F and 477 days for TDM.

BAS 750 F

In untreated samples no residues of BAS 750 F exceeding the LOQ were detected.
For grain from treated samples, BAS 750 F residues were on average 0.01 mg/kg (n=8), with six trials below LOQ, one trial with 0.018 mg/kg and as the highest residue 0.024 mg/kg.
For straw from treated samples, BAS 750 F residues showed high variability ranging from 0.5 to 18 mg/kg. Notably, for one S-EU trial (Spain, L130173) above-average residue level in plant parts (straw, green plant) were found. In straw at DALA43, residues amounted to 9.9 mg/kg, increasing further to 18 mg/kg at DALA49 (in the corresponding plant sample at DALA35 residues were 12 mg/kg). In addition, in one trial (L130169, N-EU) residues of 10 mg/kg were determined at DALA35, declining further to 6.2 mg/kg at DALA50. For the other six trials residue level were 0.5, 2.3, 2.9, 3.8, 3.9 and 5.5 mg/kg. Based on this data, average residues of 5.9 mg/kg are calculated with the highest residues at 10 mg/kg (L130169, N-EU) and at 18 mg/kg (L130173, S-EU).
For plants directly harvested after application (DALA0), BAS 750 F residues were in the range of 2.2 – 3.9 mg/kg (7 trials) and the trial in Spain (L130173) with 6.3 mg/kg. Residues in plant parts (ears, roots removed) declined to levels of 1.6 – 2.7 mg/kg at DALA34-35 and for the trial in Spain (L130173) 12 mg/kg.

TDM

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples.
For grain residue levels in treated (untreated) samples amounted for TA up to 0.76 mg/kg (0.49 mg/kg), for TAA up to 0.20 mg (0.04 mg/kg) and for TLA up to 0.09 mg/kg (0.06 mg/kg).
For straw residue levels in treated (untreated) samples amounted for TA up to 0.04 mg/kg (0.33 mg/kg), for TAA up to 0.01 mg (0.03 mg/kg) and for TLA up to 1.5 mg/kg (1.1 mg/kg).
Note that data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment:
"*A large residues data package has been submitted to address residues of the TDMs in primary crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."

Note that concerning residue data for forage used as feed item, as a result of the application timing at late stages of crop cultivation (last application at BBCH69), residue data for green plant parts is only provided for late stages of crop development. In practice, forage for use as livestock feed item is typically harvested at earlier growth stages, prior to the second application. Importantly, the intended use does not include use on cereal forage (livestock feed item) and therefore corresponding residue data was not specifically provided. Consequently, residues determined in the present study for green plants are an overestimation when compared to realistically expected BAS 750 F residues in forage (resulting from the use supported in the present dossier).

Table 6.3.1-8: Summary of BAS 750 F and TDM residues in BAS 750 00 F treated wheat

region	matrix	DALA[1)]	BBCH	BAS 750 F *[mg/kg]*	1,2,4-T *[mg/kg]*	TA *[mg/kg]*	TAA *[mg/kg]*	TLA *[mg/kg]*
N-EU & S-EU	whole plant[2)]	0	69	2.2 – 6.3	<0.01	0.014 – 0.13	< 0.01 – 0.012	0.25 – 4.1
	ears	34-35	77-87	0.10 – 1.3	<0.01	0.096 – 0.46	< 0.01 – 0.045	0.064 – 0.29
	rest of plant[2)]			0.46 - 12	<0.01	< 0.01 – 0.024	< 0.01	0.35 – 1.6
	grain	35	87-89	0.017	<0.01	0.15	0.017	< 0.01
	straw			10	<0.01	0.014	< 0.01	0.31
	ears	41-43	83-89	0.15 – 0.71	<0.01	0.18 – 0.34	0.014 – 0.027	0.024 – 0.22
	rest of plant[2)]			0.58 – 4.8	<0.01	< 0.01 – 0.028	< 0.01	0.25 – 2.1
	grain	42-43	89	< 0.01 – 0.017	<0.01	0.072 – 0.51	< 0.01 – 0.023	< 0.01 – 0.031
	straw			3.9 – 9.9	<0.01	< 0.01 – 0.012	< 0.01 – 0.014	0.42 – 0.63
	ears	49	87	0.34	<0.01	0.20	0.023	0.071
	rest of plant[2)]			3.8	<0.01	< 0.01	0.010	1.2
	grain	46-50	89	< 0.01 – 0.024	<0.01	< 0.01 – 0.76	< 0.01 – 0.023	< 0.01 – 0.092
	straw			0.50 - 18	<0.01	< 0.01 – 0.035	< 0.01 – 0.013	0.091 – 0.67
	grain	54	89	< 0.01	<0.01	0.32	0.20	< 0.01
	straw			3.8	<0.01	< 0.01	< 0.01	1.5

[1)] DALA =days after last application; [2)] no root

Table 6.3.1-9: Summary of BAS 750 F and TDM residues in untreated wheat

region	matrix	DALA[1)]	BBCH	BAS 750 F *[mg/kg]*	1,2,4-T *[mg/kg]*	TA *[mg/kg]*	TAA *[mg/kg]*	TLA *[mg/kg]*
N-EU & S-EU	whole plant [2)]	0	69	< 0.01	< 0.01	< 0.01 – 0.051	< 0.01	0.13 – 4.2
	ears	34-49	77-89	< 0.01	< 0.01	< 0.01 – 0.28	< 0.01 – 0.028	0.013 – 0.19
	rest of plant[2)]			< 0.01	< 0.01	< 0.01 – 0.024	< 0.01 – 0.017	0.056 – 0.99
	grain	35–54	87-89	< 0.01	< 0.01	0.032 – 0.49	< 0.01 – 0.043	< 0.01 – 0.057
	straw			< 0.01	< 0.01	< 0.01 – 0.33	< 0.01 – 0.025	0.030 – 1.1

[1)] DALA = days after last application; [2)] no root;

Table 6.3.1-10: Residues of BAS 750 F and TDM in wheat (treated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433781 2014/1010809 74193 Stetten a. H. Germany (N) L130166	GC 0654 Wheat Asano	1. 01.11 2012 2. 27.05.-18.06.2013 3. 05.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] grain straw	0 34 34 43 43	3.6 0.16 2.1 <u>< 0.01</u> 3.9	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.047 0.46 0.014 0 51 0.012	< 0.01 0.017 < 0.01 0.023 0.014	0.68 0.064 1.2 0.031 0.63
433781 2014/1010809 16833 Lentzke Germany (N) L130167	GC 0654 Wheat Smaragd	1. 10.10 2012 2. 10.06.-18.06.2013 3. 06.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 43 43 49 49	3.9 0.38 2.7 0.46 4.8 <u>< 0.01</u> 5.5	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.022 0 33 < 0.01 0 30 < 0.01 0 21 0.025	< 0.01 0.011 < 0.01 0.019 < 0.01 0.019 < 0.01	0.53 0.080 0.69 0.033 0.40 < 0.01 0.33
433781 2014/1010809 6595 ME Ottersum The Netherlands (N) L130168	GC 0654 Wheat Premio	1. 05.11.2012 2. 05.06.-19.06.2013 3.06.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 41 41 49 49	2.2 0.42 1.6 0.42 1.6 <u>< 0.01</u> 2.3	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.022 0 32 < 0.01 0 29 < 0.01 0 11 0.011	< 0.01 < 0.01 < 0.01 0.015 < 0.01 0.022 < 0.01	0.25 0.064 0.48 0.024 0.25 < 0.01 0.15
433781 2014/1010809 CO112NF Lawford/Manningtree, United Kingdom (N) L130169	GC 0654 Wheat Solstice	1. 12.11.2012 2. 24.06-08.07.2013 3. 27.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 08.07.13	69	plant [1] grain straw grain straw grain straw	0 35 35 42 42 50 50	2.8 0.017 10 0.015 8.6 <u>0.024</u> 6.2	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0 13 0 15 0.014 0 11 < 0.01 0 18 0.027	< 0.01 0.017 < 0.01 0.021 < 0.01 0.016 < 0.01	0.31 < 0.01 0.31 < 0.01 0.44 < 0.01 0.091
433781 2014/1010809 32130 Cazaux-Saves France (S) L130170	GC 0654 Wheat Tiepolo	1. 04.11.2012 2. 22.05.-28.05.2013 3. 12.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 43 43 46 46	2.7 0.10 0.46 0.15 0.58 <u>< 0.01</u> 0.5	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.022 0 22 0.011 0 32 < 0.01 0 33 < 0.01	< 0.01 < 0.01 < 0.01 0.014 < 0.01 0.015 < 0.01	0.48 0.072 0.35 0.052 0.26 0.034 0.22

Table 6.3.1-10: Residues of BAS 750 F and TDM in wheat (treated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433781 2014/1010809 58300 Galatades, Greece (S) L130171	GC 0654 Wheat Trofeo	1. 09.11.2012 2. 20.04.-30.04.2013 3. 05.06.-15.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 26.04.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 49 49 54 54	3.6 0.25 1.3 0.35 2.2 0.34 3.8 <u>< 0.01</u> 3.8	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted] [redacted] < 0.01	0.046 0 21 < 0.01 0 18 < 0.01 0 20 < 0.01 0 32 < 0.01	0.012 0.045 < 0.01 0.18 < 0.01 0.023 0.010 0.20 < 0.01	4.1 0.29 0.51 0.22 2.1 0.071 1.2 < 0.01 1.5
433781 2014/1010809 40018 Bologna Italy (S) L130172	GC 0654 Wheat Palaiso	1. 09.10.2012 2. 09.05.-18.05.2013 3. 05.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 42 42 48 48	2.4 0.48 2.3 0.71 2.1 <u>< 0.01</u> 2.9	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.014 0 26 0.024 0 34 0.028 0.76 0.035	< 0.01 0.025 < 0.01 0.019 < 0.01 0.023 0.013	0.64 0.19 1.6 0.073 0.64 0.092 0.67
433781 2014/1010809 41710 Utrera Spain (S) L130173	GC 0654 Wheat Artur Nick	1. 28.12.2012 2. 17.04.-22.04.2013 3. 10.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.04.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 35 35 43 43 49 49	6.3 1.3 12 0.017 9.9 <u>0.018</u> 18	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.019 0.096 < 0.01 0.072 0.010 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.45 0.12 0.49 < 0.01 0.42 < 0.01 0.54

1) whole plant (no root) 2) rest of plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. The underlined values (e.g. <u>0.018</u>) are used for calculation MRL for grain.

Table 6.3.1-11: Residues of BAS 750 F and TDM in wheat (untreated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433781 2014/1010809 74193 Stetten a. H. Germany (N) L130166	GC 0654 Wheat Asano	1. 01.11 2012 2. 27.05.-18.06.2013 3. 05.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] grain straw	0 34 34 43 43	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.049 0 28 < 0.01 0 31 0.015	< 0.01 0.016 < 0.01 < 0.01 0.019	0.66 0.093 0.98 0.026 0.37
433781 2014/1010809 16833 Lentzke Germany (N) L130167	GC 0654 Wheat Smaragd	1. 10.10 2012 2. 10.06.-18.06.2013 3. 06.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 43 43 49 49	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.023 0 19 < 0.01 0 17 < 0.01 0 11 < 0.01	< 0.01 0.017 < 0.01 0.018 < 0.01 0.023 < 0.01	0.41 0.044 0.76 0.019 0.31 < 0.01 0.18
433781 2014/1010809 6595 ME Ottersum The Netherlands (N) L130168	GC 0654 Wheat Premio	1. 05.11.2012 2. 05.06.-19.06.2013 3.06.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.06.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 41 41 49 49	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.024 0 21 < 0.01 0 20 < 0.01 0 14 < 0.01	< 0.01 < 0.01 < 0.01 0.019 < 0.01 0.020 0.013	0.31 0.046 0.37 0.015 0.20 < 0.01 0.18
433781 2014/1010809 CO112NF Lawford/Manningtree, United Kingdom (N) L130169	GC 0654 Wheat Solstice	1. 12.11.2012 2. 24.06-08.07.2013 3. 27.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 08.07.13	69	plant [1] grain straw grain straw grain straw	0 35 35 42 42 50 50	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.044 0.062 < 0.01 0.032 < 0.01 0.033 0.013	< 0.01 0.014 < 0.01 0.013 < 0.01 0.012 < 0.01	0.13 < 0.01 0.16 < 0.01 0.11 < 0.01 0.030

Table 6.3.1-11: Residues of BAS 750 F and TDM in wheat (untreated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433781 2014/1010809 32130 Cazaux-Saves France (S) L130170	GC 0654 Wheat Tiepolo	1. 04.11.2012 2. 22.05.-28.05.2013 3. 12.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 43 43 46 46	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 0.034 < 0.01 0.047 < 0.01 0.056 0.014	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.26 0.020 0.061 0.013 0.056 < 0.01 0.099
433781 2014/1010809 58300 Galatades, Greece (S) L130171	GC 0654 Wheat Trofeo	1. 09.11.2012 2. 20.04.-30.04.2013 3. 05.06.-15.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 26.04.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 49 49 54 54	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted] [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted] [redacted] [redacted]	0.051 0 11 < 0.01 0.089 < 0.01 0 11 < 0.01 0 13 < 0.01	< 0.01 0.028 0.014 0.026 < 0.01 0.020 0.017 0.040 0.025	4.2 0.18 0.32 0.19 0.99 0.061 0.95 0.019 1.1
433781 2014/1010809 40018 Bologna Italy (S) L130172	GC 0654 Wheat Palaiso	1. 09.10.2012 2. 09.05.-18.05.2013 3. 05.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 18.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 42 42 48 48	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	0.027 0 21 0.024 0 26 0.023 0.49 0.033	< 0.01 0.017 < 0.01 0.015 < 0.01 0.015 < 0.01	0.43 0.14 0.83 0.13 0.41 0.057 0.42
433781 2014/1010809 41710 Utrera Spain (S) L130173	GC 0654 Wheat Artur Nick	1. 28.12.2012 2. 17.04.-22.04.2013 3. 10.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.04.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 35 35 43 43 49 49	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 [redacted] [redacted]	< 0.01 < 0.01 < 0.01 0 13 < 0.01 0 29 < 0.01	< 0.01 < 0.01 < 0.01 0.012 < 0.01 0.043 < 0.01	0.28 0.014 0.11 0.017 0.095 0.032 0.14

Note, for trial L130166 no sampling was done at the last time point DALA49, while for trial L130171 an additional sampling was done at DALA54.
1) whole plant (no root) 2) rest of plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. The underlined values (e.g. <u>0.018</u>) are used for calculation MRL for grain.

III. CONCLUSION

Residue data obtained in 8 independent field trials in wheat (conducted in both N-EU, S-EU with the formulated product BAS 750 00 F according to the critical GAP) show that average residues of BAS 750 F are <0.01 mg/kg in grain (highest residue 0.024 mg/kg) and 5.9 mg/kg in straw (highest residue 18 mg/kg).

Residue level for 1,2,4-triazole, TA, TAA and TLA resulting from the use of the formulated product BAS 750 00 F on wheat according to the critical GAP are comparable to the data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

Report:	CA 6.3.1/2 Ale E., 2015 a Residue study (Decline) with BAS 750 01 F, BAS 750 00 F and BAS 750 BU F applied to wheat in Northern and Southern Europe in 2014 2015/1099704
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, OECD 509 Crop Field Trial (2009), EEC 7525/VI/95 rev. 9 (March 2011), EEC 7029/VI/95 rev. 5 (July 22 1997)
GLP:	yes (certified by ENAC, Entidad Nacional de Acreditación, Madrid, Spain)

EXECUTIVE SUMMARY

During the growing season of 2014, nine independent field trials with wheat were conducted in Europe (N-EU and S-EU). The magnitude of the BAS 750 F residues (analytes were parent, and the TDM with 1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) was determined after application of either the formulated product BAS 750 01 F or alternatively, on a parallel plot, of the formulated product BAS 750 00 F. The EC formulation BAS 750 01 F (0.1 kg as/L) and the EC formulation BAS 750 00 F (0.1 kg as/L) was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F in a spray volume of 200 L/ha. Wheat specimens were collected as whole plant (no roots) shortly before the second application (untreated control) and directly after the second application. Depending on the maturity of the crop, sampling after 34-35 days, 41-43 days and 46-50 days, provided either ears and rest-of-plant (no root) or grain and straw. Generally, samples were analysed within the time period of confirmed freezer storage stability for BAS 750 F and TDM in high starch matrices (grain), high water matrices (green plant) and straw. Recoveries (mean) were within the acceptable range of 70 – 110% for both BAS 750 F and TDM.

For BAS 750 F, no residues exceeding the LOQ were detected in untreated samples. For grain from BAS 750 01 F-treated samples, average residues were at 0.01 mg/kg (n=9), with <LOQ (7x), 0.014 and the highest residue at 0.026 mg/kg. For straw from BAS 750 01 F-treated samples, the average residue was 3.4 mg/kg (n=9) with the highest residue at 8.5 mg/kg (individual values were 0.56, 1.6, 1.9, 2.6, 3.1, 3.6, 4.4, 4.6, and 8.5 mg/kg). For plants taken directly after the last application of BAS 750 01 F (DALA0), residues were in the range of 2.2 - 6.6 mg/kg (n=9).

With the formulation BAS 750 00 F highly similar level of BAS 750 F were obtained. For plants directly after application (DALA0) residues were in the range of 2.3 – 7.1 mg/kg (n=9) thus corresponding well with the range obtained with the formulation BAS 750 01 F (2.2 – 6.6 mg/kg). The same observation was made at harvest stage, for grain and straw. For grain, with residue level of <0.01 (5x), 0.011, 0.14, 0.16 and 0.025 mg/kg, the values for average (0.01 mg/kg, n=9) and highest residue (0.025 mg/kg) were very similar to the values obtained for BAS 750 01 F. Similarly for straw, with residue level of 0.5, 1.5, 1.6, 3.1, 3.4, 3.6, 4.6, 4.9 and 9.0 mg/kg both values the average residue (3.6 mg/kg) and highest residue (9.0 mg/kg) are in good accordance with data obtained with formulation BAS 750 01 F. This data confirms that both formulations do not result in any significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F (see section 6.3.1/1) can be used as supporting residue data for the formulated product BAS 750 01 F.

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a large extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 1.5 mg/kg (1.0 mg/kg), for TAA up to 0.23 mg (0.29 mg/kg) and for TLA <0.01 mg/kg (0.19 mg/kg). For straw residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.39 mg/kg (0.78 mg/kg), for TAA up to 0.14 mg (0.17 mg/kg) and for TLA up to 0.08 mg/kg (0.09 mg/kg). Note that, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

I. MATERIAL AND METHODS

A. MATERIALS

1.

Test Material:	BAS 750 01 F, BAS 750 00 F, BAS 750 BU F	
Description:	BAS 750 01 F: 100 g/L of BAS 750 F, EC formulation	
	BAS 750 00 F: 100 g/L of BAS 750 F, EC formulation	
Lot/Batch #:	BAS 750 01 F: FD-140113-0006	
	BAS 750 00 F: FD-130326-0002	
Purity:	*not relevant*	
CAS#:	1417782-03-6	
Development code:	BAS 750 F	
Spiking levels:	BAS 750 F:	0.01, 0.1, 1.0, 10, 20, 100 mg/kg
	1,2,4-triazole:	0.01, 1.0 mg/kg
	triazole alanine:	0.01, 1.0, 1.5 mg/kg
	triazole acetic acid:	0.01, 1.0 mg/kg
	triazole lactic acid:	0.01, 1.0, 1.5 mg/kg

2.

Test Commodity:	cereal
Crop:	wheat
Type:	*Triticum aestivum* L.
Variety:	Asano, Elixier, Altigo, Tabsco, Adagio, Aprilio, Trofeo, Avorio, Califa
Crop parts(s) or processed Commodity:	whole plant (no root), ear, rest of plant (no root), grain, straw
Sample size:	at least 1.0 kg (for straw, at least 0.5 kg)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2014, nine field trials on wheat, each with separate plots for two formulated products, BAS 750 01 F and BAS 750 00 F, were conducted in Germany, the Netherlands, Northern and Southern France, Greece, Italy and Spain, in order to determine the magnitude of the residues of BAS 750 F and its metabolites 1,2,4-triazole, TA, TAA and TLA. (Note that the trials included an additional plot treated with a third formulation (BAS 750 BU F) which is not further considered, and therefore not reported in the present dossier).

- The EC formulation BAS 750 01 F was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (1.5 L/ha of BAS 750 01 F) in a spray volume of 200 L/ha (designated plot 2).
- The EC formulation BAS 750 00 F was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (1.5 L/ha of BAS 750 00 F) in a spray volume of 200 L/ha (designated plot 3)

Sampling included the harvest of whole plant (root removed) both shortly before the second application (untreated control) as well directly after the second application. Samples were also taken at three time points after the last application, namely after 34-36 days, after 41-43 days and after 48-51 days. In the case of unripe crop, samples consisted of ear and rest-of-plant (root removed). While for ripe crop, grain and straw were taken. In case, BBCH growth stage 89 was not reached after 49 days, an additional sampling time point was added.

Table 6.3.1-12 Application and sampling details

region	no. of trials	no. of appl.	F, G, I[2]	method	test items	active substance	application		target timing	
							rate (kg a.s. /ha)	water volume (L/ha)	application (BBCH)	sampling (DALA)[1]
EU-N & EU-S	9	2	F	foliar spray	BAS 750 01 F (EC) BAS 750 00 F (EC) BAS 750 BU F (SC)	BAS 750 F	0.15	200	1st appl.: BBCH49 2nd appl.: BBCH69	0 34 - 36 41 - 43 48 - 51 51

1) days after last application, 2) field, glasshouse or indoor

2. Description of analytical procedures

BAS 750 F residues were determined according to BASF method L0076/09. BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane. The final determination was performed by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg. Stability of BAS 750 F (deep frozen plant matrices) has been confirmed for a minimum of 547 days in high starch matrices (e.g. grain), a minimum of 551 days in high water matrices (e.g. green plant) and for a minimum of 551 days in plant straw (e.g. cereal straw).

Procedural recovery (average) were 88% for BAS 750 F using fortification levels of 0.01-100 mg/kg. The results are summarized in the following table.

Table 6.3.1-13 Summary of recoveries for BAS 750 F

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0076/09		**BAS 750 F**			
whole plant (no root)	0.01, 10, 100	3	84.5	2.6	3.1
ears	0.01, 0.10, 1.0, 10	8	89.6	5.9	6.6
rest of plant (no root)	0.01, 1.0, 10, 20	9	84.2	8.2	9.8
grain	0.01, 0.10, 1.0	12	87.4	3.4	3.9
straw	0.01, 0.10, 1.0, 10, 20	13	91.1	9.7	11
overall		**45**	**88.1**	**7.4**	**7.4**

TDM residues, namely 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were determined according to method L0170/02 (see section CA 4.3). The analytes are extracted with methanol/water. An aliquot of the extract is filtered, concentrated and cleaned-up by dispersive C_{18}-SPE prior to analysis by LC/DMS-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

Procedural recovery (average) were 95% (1,2,4-triazole), 93% (TA), 96% (TAA) and 87% (TLA). The results are summarized in the following table.

Table 6.3.1-14 Summary of recoveries for T, TA, TAA and TLA

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0170/02		**1,2,4-triazole (T)**			
whole plant (no root)	0.01, 1.0	8	93.7	7.9	8.4
ears		8	97.3	14	14
rest of plant (no root)	0.01, 1.0	8	89.9	13	14
grain	0.01, 1.0	7	99.3	11	11
straw	0.01, 1.0	8	95.8	18	19
overall		**39**	**95.1**	**13**	**14**
method L0170/02		**TA**			
whole plant (no root)	0.01, 1.0	7	103	17	17
ears	0.01, 1.0, 1.5	8	89.3	19	21
rest of plant (no root)	0.01, 1.0	7	90.6	17	19
grain	0.01, 1.0, 1.5	8	90.0	15	17
straw	0.01, 1.0	8	92.0	18	20
overall		**38**	**93.0**	**17**	**19**
method L0170/02		**TAA**			
whole plant (no root)	0.01, 1.0	7	105	6.8	6.5
ears	0.01, 1.0	7	98.1	18	18
rest of plant (no root)	0.01, 1.0	6	89.6	16	18
grain	0.01, 1.0	7	88.3	16	19
straw	0.01, 1.0	8	95.8	15	15
overall		**35**	**95.5**	**15**	**16**
method L0170/02		**TLA**			
whole plant (no root)	0.01, 1.0	8	93.4	9.9	11
ears	0.01, 1.0	7	91.5	18	20
rest-of-plant (no root)	0.01, 1.0	8	79.4	13	16
grain	0.01, 1.0, 1.5	8	79.3	14	18
straw	0.01, 1.0	8	91.1	12	13
overall		**39**	**87.1**	**14**	**17**

II. RESULTS AND DISCUSSION

The formulated product supported in the present dossier is BAS 750 01 F and was used in the present study to investigate magnitude of residues resulting from the use on wheat. This formulated product is equivalent to BAS 750 00 F and thus similar residue data is expected for both formulated products. In order to provide convincing confirmation, each trial of the present study included side-by-side plots with BAS 750 01 F (plot 2) and BAS 750 00 F (plot 3). (Note, that a further formulation BAS 750 BU F, was included in the trials, which is not considered further, thus, the data is not provided in the present dossier). The residue level of BAS 750 F and the TDM is summarized in Table 6.3.1-15 (BAS 750 F, treated samples) and Table 6.3.1-16 (TDM, treated and untreated samples). Detailed residue date is provided in Table 6.3.1-17, in Table 6.3.1-18 and Table 6.3.1-19. Generally, the maximum storage interval of deep frozen samples from harvest until extraction was 258 days for BAS 750 F and 319 days for TDM.

BAS 750 F

In untreated samples, no residues of BAS 750 F exceeding the LOQ were detected. For grain of plots treated with BAS 750 01 F, median residues were at 0.01 mg/kg (n=9), with seven trials below LOQ, one trial at 0.014 mg/kg and as the highest residue 0.026 mg/kg. For straw of plots treated with BAS 750 01 F, average residues were at 3.4 mg/kg (n=9) with the highest residue at 8.5 mg/kg (individual values were 0.56, 1.6, 1.9, 2.6, 3.1, 3.6, 4.4, 4.6 and 8.5 mg/kg). For plants directly after application of BAS 750 01 F (DALA0), BAS 750 F residues were in the range of 2.2 – 6.6 mg/kg (n=9).

With the formulation BAS 750 00 F highly similar level of BAS 750 F were obtained. A comparison is provided in Table 6.3.1-18. For plants directly after application (DALA0) residues were in the range of 2.3 - 7.1 mg/kg (n=9) thus corresponding well with the range obtained with the formulation BAS 750 01 F (2.3 - 6.4 mg/kg). The same observation was made at harvest stage, for grain and straw. For grain, with residue level of <0.01 (5x), 0.011, 0.14, 0.16 and 0.025 mg/kg, the values for average (0.01 mg/kg, n=9) and highest residue (0.025 mg/kg) were almost identical to the values obtained for BAS 750 01 F. Similarly for straw, with residue level of 0.5, 1.5, 1.6, 3.1, 3.4, 3.6, 4.6, 4.9 and 9.0 mg/kg both values the average residue (3.6 mg/kg) and highest residue (9.0 mg/kg) are in good accordance with data obtained with formulation BAS 750 01 F. This data confirms that both formulations do not result in any significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F (see section 6.3.1/1) can be used as supporting residue data for the formulated product BAS 750 01 F.

TDM

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 1.5 mg/kg (1.0 mg/kg), for TAA up to 0.23 mg (0.29 mg/kg) and for TLA <0.01 mg/kg (0.19 mg/kg). For straw residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.39 mg/kg (0.78 mg/kg), for TAA up to 0.14 mg (0.17 mg/kg) and for TLA up to 0.08 mg/kg (0.09 mg/kg). Note that, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment: "*A large residues data package has been submitted to address residues of the TDMs in primary crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns.*"

Note that concerning residue data for forage used as feed item, as a result of the application timing at late stages of crop cultivation (last application at BBCH69), residue data for green plant parts is only provided for late stages of crop development. In practice, forage for use as livestock feed item is typically harvested at earlier growth stages, prior to the second application. Importantly, the intended use does not include use on cereal forage (livestock feed item) and therefor corresponding residue data was not specifically provided. Consequently, residues determined in the present study for green plants therefor are an overestimation when compared to realistically expected BAS 750 F residues in forage (resulting from the use supported in the present dossier).

Table 6.3.1-15: Summary of BAS 750 F in wheat after treatment with the formulated products BAS 750 01 F and BAS 750 00 F

region	matrix	DALA [1)]	BBCH	n	BAS 750 F [mg/kg]	
					BAS 750 01 F	BAS 750 00 F
N-EU & S-EU	plant [2)]	0	69	9	2.2 – 6.6	2.3 – 7.1
	ears	34 - 36	79 - 87	9	0.063 – 2.8	0.073 – 3.5
	rest-of-plant [2)]			9	0.29 – 10	0.26 – 7.9
	ears	42 - 43	85 - 89	5	0.053 – 2.3	0.061 – 2.6
	rest-of-plant [2)]			5	0.37 – 7.2	0.60 – 8.8
	grain	41 - 42	87 - 89	4	< 0.01 – 0.012	< 0.01 – 0.014
	straw			4	1.9 – 4.6	1.3 – 5.0
	ears	49	89	1	1.2	0.93
	rest-of-plant [2)]			1	6.6	5.3
	grain	48 - 51	89	8	< 0.01 – 0.014	< 0.01 – 0.016
	straw			8	0.52 – 4.4	0.48 – 4.6
	grain	51	89	1	0.026	0.025
	straw			1	8.6	8.8

[1)] DALA = days after last application; [2)] no root

Table 6.3.1-16: Summary of TDM in untreated wheat (plot 1) as well as after treatment with BAS 750 01 F (plot2) or BAS 750 00 F (plot 3)

region	matrix	DALA [1)]	BBCH	plot	1,2,4-triazole [mg/kg]	TA [mg/kg]	TAA [mg/kg]	TLA [mg/kg]
N-EU & S-EU	plant [2)]	0	69	1	<0.01	0.10 - 0.24	<0.01 - 0.085	<0.01 - 0.42
				2	<0.01	0.043 - 0.17	<0.01 - 0.051	<0.01 - 0.29
				3	<0.01	0.043 - 0.19	<0.01 - 0.074	0.017 - 0.39
	ears	34-36	79/83-87	1	<0.01	0.019 - 0.18	<0.01	0.055 - 1.1
	rest-of-plant [2)]				<0.01	0.042 - 0.22	0.032 - 0.11	0.053 - 0.23
	ears			2	<0.01	0.030 - 0.27	<0.01	0.095 - 1.2
	rest-of-plant [2)]				<0.01	0.019 - 0.12	0.022 - 0.077	0.044 - 0.10
	ears			3	<0.01	0.038 - 0.32	<0.01	0.12 - 1.0
	rest-of-plant [2)]				<0.01	0.014 - 0.12	0.018 - 0.075	<0.01 - 0.10
	ears	42-43	85/87-87/89	1	<0.01	0.020 - 0.30	<0.01	0.042 - 0.27
	rest-of-plant [2)]				<0.01	0.036 - 0.17	0.010 - 0.24	0.086 - 0.26
	grain	41-42	87/89-89		<0.01	0.072 - 0.19	<0.01	0.14 - 0.96
	straw				<0.01	0.026 - 0.076	<0.01	0.22 - 0.72
	ears	42-43	85/87-87/89	2	<0.01	0.080 - 0.12	<0.01	0.12 - 0.30
	rest-of-plant [2)]				<0.01	0.030 - 0.070	0.019 - 0.078	0.027 - 0.19
	grain	41-42	87/89-89		<0.01	0.085 - 0.23	<0.01	0.18 - 1.3
	straw				<0.01	0.058 - 0.085	<0.01 - 0.075	<0.01 - 0.28
	ears	42-43	85/87-87/89	3	<0.01	0.017 - 0.087	<0.01	0.016 - 0.28
	rest-of-plant [2)]				<0.01	0.024 - 0.051	0.021 - 0.075	<0.01 - 0.099
	grain	41-42	87/89-89		<0.01	0.067 - 0.36	<0.01	0.21 - 1.2
	straw				<0.01	0.065 - 0.14	0.020 - 0.13	0.014 - 0.079
	ears	49	89	1	<0.01	0.023	<0.01	0.065
	rest-of-plant [2)]				<0.01	0.098	<0.01	0.062
	grain	48-50	89		<0.01	0.019 - 0.29	<0.01	0.064 - 1.0
	straw				<0.01	<0.01 - 0.15	<0.01 - 0.029	0.099 - 0.77
	ears	49	89	2	<0.01	0.062	<0.01	0.087
	rest-of-plant [2)]				<0.01	0.044	0.04	0.017
	grain	48-50	89		<0.01	0.043 - 0.20	<0.01	0.19 - 1.2
	straw				<0.01	<0.01 - 0.14	<0.01 - 0.077	0.090 - 0.43
	ears	49	89	3	<0.01	0.043	<0.01	0.060
	rest-of-plant [2)]				<0.01	0.025	0.027	0.018
	grain	48-50	89		<0.01	0.046 - 0.42	<0.01	0.17 - 1.2
	straw				<0.01	0.032 - 0.16	<0.01 - 0.051	0.020 - 0.11
	grain	51	89	1	<0.01	0.014 - 0.21	<0.01	0.11 - 0.19
	straw				<0.01	<0.01 - 0.10	<0.01	0.088 - 0.095
	grain	51	89	2	<0.01	0.068 - 0.16	<0.01	0.049 - 0.19
	straw				<0.01	0.029 - 0.030	0.017 - 0.037	<0.01 - 0.10
	grain	51	89	3	<0.01	0.060 - 0.083	<0.01	0.12
	straw				<0.01	0.024 - 0.025	<0.01	0.017 - 0.029

[1)] DALA = days after last application; [2)] no root

Table 6.3.1-17: Residues of BAS 750 F and TDM in wheat (BAS 750 01 F, treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783 2015/1099704 74193 Stetten a. H. Germany (N) L140168	GC 0654 Wheat Asano	1. 01.11.2013 2. 21.05.-02.06.2014 3. 23.07.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 02.06 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 51 51	2.8 0.46 2.8 0.45 4.2 <0.01 3.6 (3.6)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.071 0 17 0.047 0 12 0 19 0 19 0 10	0.085 0.089 0.064 0.12 0.055 0.16 0.029	0.020 <0.01 0.022 <0.01 0.019 <0.01 0.017
433783 2015/1099704 47589 Uedem Germany (N) L140169	GC 0654 Wheat Elixier	1. 18.11.2013 2. 04.06.-16.06.2014 3. 04.08.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 16.06 14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 36 36 42 42 49 49	2.2 0.38 1.5 <0.01 1.9 <0.01 1.7	<0.01 <0.01 <0.01 <0.01 <0.01 <0.0[redacted] <0.0[redacted]	<0.01 0 34 0.077 0 27 0 15 0 25 0 10	0.057 0.10 0.098 0.085 0.081 0.047 <0.01	0.020 <0.01 0.044 <0.01 0.033 <0.01 0.052
433783 2015/1099704 37360 Rouzières de Touraine France (N) L140170	GC 0654 Wheat Altigo	1. 01.10.2013 2. 20.05.-05.06.2014 3. 24.07.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.06 14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 35 35 42 42 49 49	2.6 0.42 2.6 0.012 2.6 0.014 2.3	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0 29 1.3 (1.1) 0.071 1.5 (1.2) 0.31 (0.24) 1 2 0.33 (0.41)	0.17 0.28 (0.26) 0.12 0.22 0.085 0.20 0.14	0.051 <0.01 0.070 <0.01 0.055 <0.01 0.073
433783 2015/1099704 6595 ME Ottersum, The Netherlands (N) L140171	GC 0654 Wheat Tabasco	1. 22.11.2013 2. 03.06-16.06.2014 3. 04.08.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 16.06 14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 36 36 42 42 49 49	2.2 0.70 2.5 <0.01 4.3 <0.01 4.4	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.064 0 20 0.066 0 18 0.099 0 25 0.39 (0.47)	0.051 0.12 0.084 0.11 0.071 0.10 0.072	0.024 <0.01 0.028 <0.01 0.036 <0.01 0.041

Table 6.3.1-17: Residues of BAS 750 F and TDM in wheat (BAS 750 01 F, treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783 2015/1099704 16220 Quintanar Del Rey Spain (S) L140173	GC 0654 Wheat Adagio	1. 08.11.2013 2. 25.04.-10.05.2014 3. 25.06.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.05 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 49 49 51 51	4.8 2.8 10 2.3 7.2 1.2 6.8 0.026 8.5 (8.5)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.041 0.095 0 10 0 13 0.096 0.087 0.017 0.049 <0.01	0.077 0.065 0.037 0.080 0.070 0.062 0.044 0.068 0.030	0.023 <0.01 0.058 <0.01 0.058 <0.01 0.040 <0.01 0.037
433783 2015/1099704 32220 St. Soulan France (S) L140174	GC 0654 Wheat Aprilio	1. 29.11.2013 2. 23.05.-29.05.2014 3. 15.07.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.06 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 42 42 49 49	3.2 0.19 1.0 0.18 1.3 <0.01 1.6 (1.6)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.054 0 29 0.080 0 30 0 11 0 33 0 16	0.055 0.095 0.039 0.11 0.058 0.061 0.060	0.022 <0.01 0.043 <0.01 0.026 <0.01 0.038
433783 2015/1099704 58500 Agios Greece (S) L140175	GC 0654 Wheat Trofeo	1. 10.11.2013 2. 15.04.-26.04.2014 3. 13.06.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.04 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 43 43 50 50	2.6 0.063 0.29 0.053 0.37 <0.01 0.53 (0.56)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.034 0 16 0.044 0 15 0.027 0 19 0 13	0.043 0.030 0.081 0.081 0.046 0.091 0.031	<0.01 <0.01 0.026 <0.01 0.024 <0.01 0.024
433783 2015/1099704 20060 S Martino Italy (S) L140176	GC 0654 Wheat Aprilio	1. 20.10.2013 2. 10.05.-17.05.2014 3. 02.07.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 15.05 14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 34 34 41 41 48 48	6.6 0.42 3.2 <0.01 4.6 <0.01 4.2	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.083 0 27 0.096 0 36 0 10 0 28 0.087	0.066 0.14 0.043 0.23 0.058 0.11 0.024	0.045 <0.01 0.077 <0.01 0.075 <0.01 0.077

Table 6.3.1-17: Residues of BAS 750 F and TDM in wheat (BAS 750 01 F, treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783	GC 0654	1. 17.01.2014	Foliar application	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 21.05 14	69	plant [1]	0	3.9	<0.01	0.086	0.053	<0.01
2015/1099704	Wheat	2. 10.05.-25.05.2014								ears	35	0.14	<0.01	0 15	0.066	<0.01
02110 La Gineta	Califa	3. 09.07.2014								rest of pl. [2]	35	1.9	<0.01	0.059	0.019	0.040
Spain (S)										ears	42	0.19	<0.01	0 22	0.10	<0.01
L140177										rest of pl. [2]	42	2.6	<0.01	0.067	0.030	0.078
										grain	49	<u>≤0.01</u>	<0.01	0 25	0.043	<0.01
										straw	49	3.1 (3.0)	<0.01	0 18	0.020	0.065

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. A number in parenthesis indicates re-analysis to confirm analytical results. The highest both values is selected. The underlined values (e.g. <u>0.018)</u> are used for calculation MRL for grain.

Table 6.3.1-18: Residues of BAS 750 F and TDM in wheat (BAS 750 00 F, treated samples, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783 2015/1099704 74193 Stetten a. H. Germany (N) L140168	GC 0654 Wheat Asano	1. 01.11.2013 2. 21.05.-02.06.2014 3. 23.07.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 02.06 14	69	plant [1]	0	2.6	<0.01	0.092	0.068	0.012
										ears	35	0.48	<0.01	0.13	0.072	<0.01
										rest of pl. [2]	35	4.1	<0.01	0.034	0.041	0.027
										ears	42	0.41	<0.01	0.12	0.086	<0.01
										rest of pl. [2]	42	4.2	<0.01	0.069	0.051	0.021
										grain	51	0.011	<0.01	0.12	0.083	<0.01
										straw	51	3.6 (3.4)	<0.01	0.029	0.025	<0.01
433783 2015/1099704 47589 Uedem Germany (N) L140169	GC 0654 Wheat Elixier	1. 18.11.2013 2. 04.06.-16.06.2014 3. 04.08.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 16.06 14	69	plant [1]	0	2.5	<0.01	0.043	0.069	0.028
										ears	36	0.46	<0.01	0.18	0.060	<0.01
										rest of pl. [2]	36	0.78	<0.01	0.016	0.064	0.024
										grain	42	<0.01	<0.01	0.30	0.067	<0.01
										straw	42	1.3	<0.01	0.035	0.066	0.020
										grain	49	<0.01	<0.[redacted]	0.20	0.063	<0.01
										straw	49	1.6	<0.[redacted]	0.030	0.088	<0.01
433783 2015/1099704 37360 Rouzières de Touraine France (N) L140170	GC 0654 Wheat Altigo	1. 01.10.2013 2. 20.05.-05.06.2014 3. 24.07.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.06 14	69	plant [1]	0	2.3	<0.01	0.39	0.19	0.074
										ears	35	0.60	<0.01	1.1 (0.91)	0.30 (0.34)	<0.01
										rest of pl. [2]	35	3.0	<0.01	0.10	0.12	0.059
										grain	42	0.012	<0.01	1.2	0.36	<0.01
										straw	42	3.4	<0.01	0.079	0.14	0.050
										grain	49	0.016	<0.01	1.2	0.42	<0.01
										straw	49	3.1	<0.01	0.11	0.16	0.045

Table 6.3.1-18: Residues of BAS 750 F and TDM in wheat (BAS 750 00 F, treated samples, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783 2015/1099704 6595 ME Ottersum, The Netherlands (N) L140171	GC 0654 Wheat Tabasco	1. 22.11.2013 2. 03.06-16.06.2014 3. 04.08.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 16.06 14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 36 36 42 42 49 49	2.4 <0.01 3.5 0.014 4.9 <0.01 4.6	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.072 0.17 0.056 0.21 0.014 0.18 0.021	0.061 0.11 0.061 0.099 0.086 0.10 0.086	0.026 <0.01 0.035 <0.01 0.030 <0.01 0.027
433783 2015/1099704 16220 Quintanar Del Rey Spain (S) L140173	GC 0654 Wheat Adagio	1. 08.11.2013 2. 25.04.-10.05.2014 3. 25.06.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.05 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 49 49 51 51	5.4 3.5 7.9 2.6 8.8 0.93 5.3 0.025 9.0 (8.6)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.024 0.14 0.027 0.081 <0.01 0.060 0.018 0.12 0.017	0.052 0.054 0.053 0.059 0.041 0.043 0.025 0.060 0.024	0.018 <0.01 0.054 <0.01 0.056 <0.01 0.027 <0.01 <0.01
433783 2015/1099704 32220 St. Soulan France (S) L140174	GC 0654 Wheat Aprilio	1. 29.11.2013 2. 23.05.-29.05.2014 3. 15.07.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.06 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 34 34 42 42 49 49	2.7 0.16 0.91 0.16 1.1 <0.01 1.5 (1.5)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.050 0.29 0.054 0.28 0.080 0.36 0.061	0.047 0.10 0.033 0.087 0.048 0.081 0.043	0.016 <0.01 0.049 <0.01 0.028 <0.01 <0.01
433783 2015/1099704 58500 Agios Greece (S) L140175	GC 0654 Wheat Trofeo	1. 10.11.2013 2. 15.04.-26.04.2014 3. 13.06.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.04 14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 43 43 50 50	2.5 0.073 0.26 0.061 0.61 <0.01 0.46 (0.45)	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.017 0.12 <0.01 0.10 <0.01 0.22 0.023	0.054 0.038 0.044 0.046 0.033 0.050 0.041	<0.01 <0.01 0.018 <0.01 0.023 <0.01 0.027

Table 6.3.1-18: Residues of BAS 750 F and TDM in wheat (BAS 750 00 F, treated samples, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783	GC 0654	1. 20.10.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 15.05 14	69	plant [1]	0	7.1	<0.01	0.072	0.067	0.035
2015/1099704	Wheat	2. 10.05.-17.05.2014								ears	34	0.40	<0.01	0.23	0.11	<0.01
20060 S Martino	Aprilio	3. 02.07.2014								rest of pl. [2]	34	4.0	<0.01	0.022	0.051	0.075
Italy (S)										grain	41	<0.01	<0.01	0.29	0.082	<0.01
L140176										straw	41	3.7	<0.01	0.031	0.065	0.13
										grain	48	<0.01	<0.01	0.31	0.11	<0.01
										straw	48	4.6	<0.01	0.020	0.054	0.051
433783	GC 0654	1. 17.01.2014	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 21.05 14	69	plant [1]	0	4.3	<0.01	0.11	0.043	<0.01
2015/1099704	Wheat	2. 10.05.-25.05.2014								ears	35	0.22	<0.01	0.18	0.043	<0.01
02110 La Gineta	Califa	3. 09.07.2014								rest of pl. [2]	35	1.7	<0.01	0.045	0.014	0.024
Spain (S)										ears	42	0.45	<0.01	0.016	0.017	<0.01
L140177										rest of pl. [2]	42	2.9	<0.01	0.099	0.024	0.075
										grain	49	<0.01	<0.01	0.17	0.046	<0.01
										straw	49	3.1 (3.0)	<0.01	0.014	0.032	0.022

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. A number in parenthesis indicates re-analysis to confirm analytical results.

Table 6.3.1-19: Level of BAS 750 F and TDM in untreated wheat (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783 2015/1099704 74193 Stetten a. H. Germany (N) L140168	GC 0654 Wheat Asano	1. 01.11.2013 2. 21.05.-02.06.2014 3. 23.07.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 35 35 42 42 51 51	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0 12 0 16 0 12 0 27 0 13 0 19 0.088	0.24 0.15 0.11 0.27 (0.26) 0.17 0.21 0.10	0.057 <0.01 0.036 <0.01 0.035 <0.01 <0.01
433783 2015/1099704 47589 Uedem Germany (N) L140169	GC 0654 Wheat Elixier	1. 18.11.2013 2. 04.06.-16.06.2014 3. 04.08.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] grain straw grain straw	0 36 36 42 42 49 49	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.0[redacted] <0.0[redacted]	0.053 0 19 0 20 0 14 0.78 (0.66) 0 11 0.70 (0.83)	0.13 0.11 0.16 0.077 0.061 0.088 0.071	0.049 <0.01 0.067 <0.01 <0.01 <0.01 <0.01
433783 2015/1099704 37360 Rouzières de Touraine France (N) L140170	GC 0654 Wheat Altigo	1. 01.10.2013 2. 20.05.-05.06.2014 3. 24.07.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] grain straw grain straw	0 35 35 42 42 49 49	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.42 1.3 (0.94) 0 23 0 96 0.25 (0.20) 1.0 0.34 (0.41)	0.15 0.18 0.13 0.19 0.17 0.18 0.11	0.071 <0.01 0.055 <0.01 <0.01 <0.01 0.016
433783 2015/1099704 6595 ME Ottersum, The Netherlands (N) L140171	GC 0654 Wheat Tabasco	1. 22.11.2013 2. 03.06-16.06.2014 3. 04.08.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] grain straw grain straw	0 36 36 42 42 49 49	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.036 0 29 0.064 0 16 0.24 (0.22) 0 22 0.25 (0.23)	0.10 0.071 0.081 0.072 0.026 0.043 0.055	0.023 <0.01 0.032 <0.01 <0.01 <0.01 <0.01

Table 6.3.1-19: Level of BAS 750 F and TDM in untreated wheat (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783	GC 0654	1. 08.11.2013	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.015	0.17	<0.01
2015/1099704	Wheat	2. 25.04.-10.05.2014								ears	35	<0.01	<0.01	0.055	0.032	<0.01
16220 Quintanar Del Rey	Adagio	3. 25.06.2014								rest of pl. [2]	35	<0.01	<0.01	0 14	0.22	0.015
Spain (S)										ears	42	<0.01	<0.01	0.052	0.027	<0.01
L140173										rest of pl. [2]	42	<0.01	<0.01	0 15	0.16	0.016
										ears	49	<0.01	<0.01	0.065	0.023	<0.01
										rest of pl. [2]	49	<0.01	<0.01	0.062	0.098	<0.01
										grain	51	<0.01	<0.01	0 11	0.014	<0.01
										straw	51	<0.01	<0.01	0.095	<0.01	<0.01
433783	GC 0654	1. 29.11.2013	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0 14	0.21	0.085
2015/1099704	Wheat	2. 23.05.-29.05.2014								ears	34	<0.01	<0.01	0.48	0.17	<0.01
32220 St. Soulan	Aprilio	3. 15.07.2014								rest of pl. [2]	34	<0.01	<0.01	0 16	0.15	0.098
France (S)										ears	42	<0.01	<0.01	0 20	0.31 (0.28)	<0.01
L140174										rest of pl. [2]	42	<0.01	<0.01	0 26	0.14	0.081
										grain	49	<0.01	<0.01	0.75	0.29	<0.01
										straw	49	<0.01	<0.01	0 10	0.15	0.029
433783	GC 0654	1. 10.11.2013	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	<0.01	0.11	<0.01
2015/1099704	Wheat	2. 15.04.-26.04.2014								ears	35	<0.01	<0.01	0 11	0.024	<0.01
58500 Agios	Trofeo	3. 13.06.2014								rest of pl. [2]	35	<0.01	<0.01	0.053	0.15	0.013
Greece (S)										ears	43	<0.01	<0.01	0 11	0.020	<0.01
L140175										rest of pl. [2]	43	<0.01	<0.01	0 15	0.12	0.010
										grain	50	<0.01	<0.01	0.069	0.030	<0.01
										straw	50	<0.01	<0.01	0.23 (0.13)	<0.01	<0.01
433783	GC 0654	1. 20.10.2013	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.061	0.11	0.034
2015/1099704	Wheat	2. 10.05.-17.05.2014								ears	34	<0.01	<0.01	0 23	0.060	<0.01
20060 S Martino	Aprilio	3. 02.07.2014								rest of pl. [2]	34	<0.01	<0.01	0.095	0.084	0.047
Italy (S)										grain	41	<0.01	<0.01	0 18	0.13	<0.01
L140176										straw	41	<0.01	<0.01	0.26 (0.24)	0.076	<0.01
										grain	48	<0.01	<0.01	0 16	0.097	<0.01
										straw	48	<0.01	<0.01	0 16	0.028	<0.01

Table 6.3.1-19: Level of BAS 750 F and TDM in untreated wheat (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433783	GC 0654	1. 17.01.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.044	0.10	<0.01
2015/1099704	Wheat	2. 10.05.-25.05.2014								ears	35	<0.01	<0.01	0 20	0.019	<0.01
02110 La Gineta	Califa	3. 09.07.2014								rest of pl. [2]	35	<0.01	<0.01	0 16	0.042	0.11
Spain (S)										ears	42	<0.01	<0.01	0.042	0.023	<0.01
L140177										rest of pl. [2]	42	<0.01	<0.01	0.086	0.036	0.24
										grain	49	<0.01	<0.01	0.064	0.019	<0.01
										straw	49	<0.01	<0.01	0.099	0.012	<0.01

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. A number in parenthesis indicates re-analysis to confirm analytical results.

III. CONCLUSION

Residue data obtained in 9 independent field trials in wheat (conducted in both N-EU, S-EU with the formulated product BAS 750 01 F according to the critical GAP) show that average residues of BAS 750 F are 0.01 mg/kg in grain (highest residue 0.026 mg/kg) and 3.4 mg/kg in straw (highest residue 8.5 mg/kg). With the formulated product BAS 750 00 F highly similar data is obtained in side-by-side plots within the same trial, both considering the median residue and the highest residue. In grain average residues were 0.01 mg/kg, highest residue 0.025 mg/kg, for straw average residues were 3.6 mg/kg, highest residue was 9.0 mg/kg. This comparative investigation of BAS 750 01 F and BAS 750 00 F confirms that both formulations do not result in significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F (see section 6.3.1/1) can be used as supporting residue data for the formulated product BAS 750 01 F (see section CA 6.7).

Residue level for 1,2,4-triazole, TA, TAA and TLA resulting from the use of the formulated product BAS 750 00 F on wheat according to the critical GAP are comparable to the data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

CA 6.3.2 Barley

In support of the representative use (formulated product BAS 750 01 F) in barley, field trials were conducted in season 2013 and season 2014. Field trial data obtained with the formulated product BAS 750 01 F is available for the season 2014 (4 trials N-EU trials, 5 trials S-EU). In addition, for season 2013, field trial data is available for the formulated BAS 750 00 F (4 trials N-EU trials, 4 trials S-EU). Generally, equivalent residue data is obtained with both formulated products as confirmed by BAS 750 00 F residue data (season 2014) generated in parallel with the formulated product BAS 750 01 F (see Table 6.3.2-15).

The cGAP is given in Table 6.3.2-1. The residue trials were performed in various European Member States in N-EU and S-EU during the growing seasons 2013 and 2014. They, considering trials with BAS 750 01 F and trials with BAS 750 00 F together, thereby fulfill the requirements of seasonal and geographical distribution (see Table 6.3.2-2).

Table 6.3.2-1: Summary of the critical GAPs for the proposed uses in barley

crop	outdoor/ protected	growth stage (BBCH)	maximum number of applications	minimum application interval (days)	maximum		minimum PHI (days)
					rate (kg as/ha)	water (L/ha)	
barley	outdoor	49, 69	2	14	0.15	200	35

Table 6.3.2-2: Number of residue trials per geographical region and vegetation period

crop	season	number of trials					reference
		N-EU	country	S-EU	country	total	
barley	2013	4	DE (2x), NL, UK	4	ES, FR, GR, IT	8	6.3.2/1
barley	2014	5	DE (2x), FR, NL, UK	5	ES (2x), FR, GR, IT	10	6.3.2/2
total number of trials per region		**9**		**9**	**total number of trials**	**18**	

Table 6.3.2-3: Overall summary of residue data for BAS 750 F from barley residue trials

crop	region	RAC	n	Residue data BAS 750 F (mg/kg)			
				2013	2014	HR	STMR
barley	N-EU	grain	9	0.014, 0.071, 0.15, 0.19	0.06, 0.085, 0.087, 0.11, 0.28	0.28	0.087
	S-EU		9	0.070, 0.10, 0.16, 0.41	0.018, 0.033, 0.058, 0.088, 0.14	0.14	0.088
	N-EU	straw	9	1.0, 3.9, 5.6, 15	1.7, 3.1, 3.6, 4.3, 6.8	15	3.9
	S-EU		9	0.39, 4.2, 6.4, 11	2.1, 2.2, 2.4, 4.6, 18	18	4.2

The study reports are summarized below.

Table 6.3.2-4: Barley residue data: summary of maximum storage interval

crop	maximum storage interval *(days)* [1]		confirmed storage stability [1]				
	BAS 750 F analytics	TDM analytics	BAS 750 F *(days)*	1,2,4-T *(months)*	TA *(months)*	TAA *(months)*	TLA *(months)*
barley grain	662	389	547 [2]	54	36	36	48
barley straw	736	389	551 [3]	54	36	36	48

[1] from harvest until extraction as deep frozen samples, 1,2,4-T= 1,2,4-triazole, TA= triazole alanine, TAA=triazole acetic acid, TLA= triazole lactic acid, [2] note, that storage stability study ongoing, results obtained so far show that after 547 days, stability is 93.0%, , and [3] after 551 days, stability is 99.5%.

Report:	CA 6.3.2/1 Erdmann H.-P., 2015 b Study on the residue behaviour of Reg.No. 5834378 (BAS 750 F) in barley after application of EXP 5834378 F-AV (BAS 750 00 F) under field condition in Germany, The Netherlands, United Kingdom, Southern France, Greece, Italy and Spain, 2013 2014/1010808
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011)
GLP:	yes (certified by Land Brandenburg Ministerium fuer Umwelt, Gesundheit und Verbraucherschutz, Potsdam, Germany)

EXECUTIVE SUMMARY

During the growing season of 2013, eight independent field trials with barley were conducted in Europe (N-EU and S-EU) in order to determine the magnitude of the BAS 750 F residues (analytes were parent, and the TDM with 1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) after application of the formulated product BAS 750 00 F. The EC formulation BAS 750 00 F (0.1 kg as/L) was applied two times (BBCH 49 and 69) at rates of 0.15 kg as/ha for BAS 750 F in a spray volume of 200 L/ha. Barley specimens were collected as whole plant (no roots) shortly before the second application (untreated control) and directly after the second application. Depending on the maturity of the crop, sampling after 28±1 days, 35±1 days and 42±1 days, provided either ears and rest-of-plant (no root) or grain and straw. In three trials, the crop did not reach growth stage BBCH 89 after 49 days, thus, an additional sampling was done at 54-55 days. Generally, samples were analysed within the time period of confirmed freezer storage stability for BAS 750 F and TDM in high starch matrices (grain), high water matrices (green plant) and straw. Recoveries (mean) were within the acceptable range of 70 – 110% for both BAS 750 F and TDM.

For BAS 750 F, no residues exceeding the LOQ were detected in untreated samples. For grain from treated samples, BAS 750 F residues were on average 0.15 mg/kg (n=8), with individual residues of 0.014, 0.070, 0.071, 0.10, 0.15, 0.16, 0.19 and as the highest residue 0.41 mg/kg.

For straw from treated samples, BAS 750 F residues showed high variability ranging from 0.39 to 15 mg/kg. Residue level were 0.39, 1.0, 3.9, 4.2, 5.6, 6.4, 11 and as the highest residues 15 mg/kg. Based on this data, average residues of 5.9 mg/kg. For plants directly after application (DALA0), BAS 750 F residues were in the range of 2.3 – 7.4 mg/kg (8 trials).

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in treated (untreated) samples amounted for TA up to 2.6 mg/kg (1.9 mg/kg), for TAA up to 0.37 mg (0.36 mg/kg) and for TLA up to 1.2 mg/kg (1.1 mg/kg). For straw residue levels in treated (untreated) samples amounted for TA up to 0.67 mg/kg (0.34 mg/kg), for TAA up to 0.20 mg (0.18 mg/kg) and for TLA up to 11 mg/kg (10 mg/kg).

Note that, TDM data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

I. MATERIAL AND METHODS

A. MATERIALS

1.

Test Material:	BAS 750 00 F	
Description:	BAS 750 00 F: 100 g/L of BAS 750 F, EC formulation	
Lot/Batch #:	BAS 750 00 F: 370168	
Purity:	*not relevant*	
CAS#:	1417782-03-6	
Development code:	BAS 750 F	
Spiking levels:	BAS 750 F:	0.01, 0.1, 1, 10, 20 mg/kg
	1,2,4-triazole:	0.01, 1.0 mg/kg
	triazole alanine:	0.01, 1.0, 20 mg/kg
	triazole acetic acid:	0.01, 1.0 mg/kg
	triazole lactic acid:	0.01, 1.0, 20 mg/kg

2.

Test Commodity:	cereal
Crop:	barley
Type:	*Hordeum vulgare* L.,
Variety:	Propino, Sandra, Sequel, Cassata, Bamboo, Moutso, Cometa, Prestige
Crop parts(s) or processed Commodity:	whole plant (no root) ear, rest-of-plant (no root) grain, straw
Sample size:	at least 1.0 kg (for straw, at least 0.5 kg)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2013, eight field trials on barley with the formulated product BAS 750 00 F (EC formulation) were conducted in Germany, the Netherlands, the United Kingdom, Southern France, Greece, Italy and Spain, in order to determine the magnitude of residues of BAS 750 F and its metabolites 1,2,4-triazole, TA, TAA and TLA. The EC formulation BAS 750 00 F was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (0.1 kg/L of BAS 750 00 F) in a spray volume of 200 L/ha.

Sampling included the harvest of whole plant (root removed) both shortly before second application (untreated control) and directly after the second application. Samples were also taken at three time points after the last application, namely after 27-29 days, 34-36 days and 41-42 days. In the case of unripe crop, samples consisted of ear and rest-of-plant (root removed) while for the ripe crop, grain and straw were taken. In case, growth stage BBCH89 was not reached after 41-42 days, an additional sampling timepoint was added (48-55 days).

Table 6.3.2-5 Application and sampling details

region	no. of trials	no. of appl.	F, G, I[2]	method	test item	active substance	application		targeted timing	
							rate (kg a.s. /ha)	water volume (L/ha)	application (BBCH)	sampling (DALA)[1]
N-EU & S-EU	8	2	F	foliar spray	BAS 750 00 F (EC)	BAS 750 F	0.15	200	1st appl.: BBCH 49 2nd appl.: BBCH 69	0 28 ± 1 35 ± 1 42 ± 1 48 - 55

1) days after last application, 2) field, glasshouse or indoor

2. Description of analytical procedures

BAS 750 F residues were determined according to BASF method L0076/09. BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane. The final determination was performed by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg. Stability of BAS 750 F (deep frozen plant matrices) has been confirmed for a minimum of 547 days in high starch matrices (e.g. grain), a minimum of 551 days in high water matrices (e.g. green plant) and for a minimum of 551 days in plant straw (e.g. cereal straw).

Procedural recovery (average) were 92% for BAS 750 F using fortification levels of 0.01-20 mg/kg. The results are summarized in Table 6.3.2-6.

Table 6.3.2-6: Summary of recoveries

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0076/09		**BAS 750 F**			
whole plant (no root)	0.01, 1.0, 20	3	99.7	4.0	4.0
ears	0.01, 0.10, 1.0, 10	7	96.7	9.0	9.3
rest of plant (no root)	0.01, 1.0, 10, 20	13	88.7	15	16
grain	0.01, 0.10, 1.0	6	94.0	12	12
straw	0.01, 0.02, 1.0, 10, 20	10	91.2	15	16
overall		**39**	**92.4**	**13**	**14**

TDM residues, namely 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were determined according to method L0170/02 (see section CA 4.3). The analytes are extracted with methanol/water. An aliquot of the extract is filtered, concentrated and cleaned-up by dispersive C_{18}-SPE prior to analysis by LC/DMS-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

Procedural recovery (average) were at 94.5% (1,2,4-triazole), 84.8% (TA), 85.3% (TAA) and 97.5% (TLA). The results are summarized in the following Table 6.3.2-7.

Table 6.3.2-7 Summary of recoveries for T, TA, TAA and TLA

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0170/02		**1,2,4-triazole**			
whole plant (no root) rest-of-plant (no root)	0.01, 1.0	10	92.5		10
ears	0.01, 1.0	5	95.4		6.3
grain	0.01, 1.0	8	95.8		16
straw	0.01, 1.0	8	95.0		16
overall		**31**	**94.5**		**13**
method L0170/02		**TA**			
whole plant (no root) rest-of-plant (no root)	0.01, 1.0, 20	9	87.6		20
ears	0.01, 1.0, 20	7	91.4		13
grain	0.01, 1.0, 20	9	77.3		16
straw	0.01, 1.0, 20	8	84.2		22
overall		**33**	**84.8**		**18**
method L0170/02		**TAA**			
whole plant (no root) rest-of-plant (no root)	0.01, 1.0	10	91.4		16
ears	0.01, 1.0	6	90.0		13
grain	0.01, 1.0	8	75.9		13
straw	0.01, 1.0	8	83.7		15
overall		**32**	**85.3**		**16**
method L0170/02		**TLA**			
whole plant (no root) rest-of-plant (no root)	0.01, 1.0, 20	9	95.2		20
ears	0.01, 1.0, 20	7	103		11
grain	0.01, 1.0, 20	9	96.8		18
straw	0.01, 1.0, 20	9	96.2		7.1
overall		**34**	**97.5**		**14**

II. RESULTS AND DISCUSSION

The residue level of BAS 750 F and the TDM determined in barley samples is summarized in Table 6.3.2-8 (treated samples) and Table 6.3.2-9 (untreated samples). Detailed residue data is provided in Table 6.3.2-10 and Table 6.3.2-11. The maximum storage interval of deep frozen samples from harvest until extraction was 736 days for BAS 750 F (grain 675 days) and 389 days for TDM.

BAS 750 F

In untreated samples, no residues of BAS 750 F exceeding the LOQ were detected. For grain from treated samples, average residues were at 0.15 mg/kg (n=8), with individual values of 0.014, 0.070, 0.071, 0.10, 0.15, 0.16, 0.19, and a highest residue of 0.41 mg/kg. For straw from treated samples, median residues were 5.9 mg/kg (n=8) with individual values of 0.39, 1.0, 3.9, 4.2, 5.6, 6.4, 11 and a highest residue of 15 mg/kg. For plants directly after application (DALA0), BAS 750 F residues were in the range of 2.3 – 7.4 mg/kg (n=8).

TDM

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extent treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in treated (untreated) samples amounted for TA up to 2.6 mg/kg (1.9 mg/kg), for TAA up to 0.37 mg (0.36 mg/kg) and for TLA up to 1.2 mg/kg (1.1 mg/kg). For straw residue levels in treated (untreated) samples amounted for TA up to 0.67 mg/kg (0.34 mg/kg), for TAA up to 0.20 mg (0.11 mg/kg) and for TLA up to 11 mg/kg (10 mg/kg).

Notably, the TLA residue data obtained in one trial (L130176) appears to be treatment-unrelated. While residue data for BAS 750 F is comparable to the data of other residue trials (7 additional trials in 2013, 10 trials in 2014), the residue levels of TLA by far exceed the levels seen in any of the other 17 trials. Both in inflorescence (unripe ears and mature grain with 1.0-13 mg/kg) as well as plant part (plant DALA0, rest-of-plant, straw with 8-11 mg/kg) TLA residues are extremely high. Compared of the overall data package (2013, 2014 in total 18 trials in barley), higher levels are also seen in this trial for other triazole derivative metabolites, TA (e.g. inflorescence 1.4-2.0 mg/kg) and TAA (e.g. inflorescence 0.3-0.4mg/kg). It has to be assumed that these atypical residue levels are result of a non-documented "triazole-releasing treatment", such as a fertilizer treatment, specifically at this trial site since the control samples (untreated plot) did contain residues in the same order of magnitude. Importantly, the residue behavior of BAS 750 F appears to be not affected. Therefore, data for the analyte BAS 750 F, obtained in this trial is valid.

Note that data obtained in the present field study with BAS 750 F is with one exception (trial L130176, see above) comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment: "*A large residues data package has been submitted to address residues of the TDMs in primary crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."

Note that concerning residue data for forage used as feed item, as a result of the application timing at late stages of crop cultivation (last application at BBCH69), residue data for green plant parts is only provided for late stages of crop development. In practice, forage for use as livestock feed item is typically harvested at earlier growth stages, prior to the second application.

Importantly, the intended use does not include use on cereal forage (livestock feed item) and therefor corresponding residue data was not specifically provided. Consequently, residues determined in the present study for green plants therefor are an overestimation when compared to realistically expected BAS 750 F residues in forage (resulting from the use supported in the present dossier).

Table 6.3.2-8: Summary of BAS 750 F and TDM residues in BAS 750 00 F treated barley

region	matrix	DALA[1)]	BBCH	BAS 750 F [mg/kg]	1,2,4-T [mg/kg]	TA [mg/kg]	TAA [mg/kg]	TLA [mg/kg]
N-EU & S-EU	plant [2)]	0	69	2.3 – 7.4	<0.01	< 0.01 - 0.40	< 0.01 - 0.041	0.26 - 9.6
	ears	27 - 28	75-87	0.082 – 1.2	<0.01	0.095 – 1.4	< 0.01 – 0.35	0.070 – 1.3
	rest-of-plant[3)]			0.49 – 12	<0.01	< 0.01 – 0.13	< 0.01 – 0.086	0.18 – 7.9
	grain	28 - 29	85-89	0.14 – 0.42	<0.01	0.13 – 0.22	0.022 – 0.029	0.087 – 0.16
	straw			9.3 – 11	<0.01	< 0.01	< 0.01	1.5 – 2.2
	ears	34 - 35	77-87	0.058 – 1.0	<0.01	0.074 – 0.24	< 0.01 – 0.028	0.081 – 0.20
	rest-of-plant[3)]			0.20 – 13	<0.01	< 0.01 – 0.015	< 0.01	0.15 – 1.1
	grain	34 - 36	87-89	0.056 – 0.29	<0.01	0.061 – 2.0	0.025 – 0.29	0.076 – 0.96
	straw			2.9 – 15	<0.01	< 0.01 – 0.16	< 0.01 – 0.11	1.2 - 11
	ears	41 - 42	83-87	0.065 – 1.7	<0.01	0.090 – 0.79	< 0.01 – 0.023	0.099 – 0.25
	rest-of-plant[3)]			0.18 - 13	<0.01	< 0.01 – 0.064	< 0.01	0.20 – 1.0
	grain	41 - 42	89	0.071 - 0.41	<0.01	0.054 – 2.6	0.019 – 0.37	0.17 – 1.2
	straw			3.9 – 11	<0.01	< 0.01 – 0.67	< 0.01 – 0.20	0.83 – 10
	grain	48 - 55	89	0.014 – 0.16	<0.01	0.052 – 1.1	< 0.01 – 0.021	0.074 – 0.12
	straw			0.39 – 6.4	<0.01	< 0.01 – 0.11	< 0.01	0.53 – 4.4

[1)] DALA = days after last application; [2)] whole plant (no roots); [3)] no roots

Table 6.3.2-9: Summary of BAS 750 F and TDM in untreated barley

region	matrix	DALA[1)]	BBCH	BAS 750 F [mg/kg]	1,2,4-T [mg/kg]	TA [mg/kg]	TAA [mg/kg]	TLA [mg/kg]
N-EU & S-EU	plant [2)]	0	69	<0.01	<0.01	< 0.01 - 0.41	< 0.01 - 0.058	0.20 - 7.3
	ears	27 - 28	75-87	<0.01	<0.01	0.016 – 1.1	<0.01 – 0.34	0.060 – 1.3
	rest-of-plant[3)]			<0.01	<0.01	<0.01 – 0.11	<0.01 – 0.078	0.14 – 6.8
	grain	28 - 29	85-89	<0.01	<0.01	0.096 – 0.11	0.021 – 0.034	0.037 – 0.11
	straw			<0.01	<0.01	<0.01	<0.01 – 0.18	1.3 – 3.4
	ears	34 - 35	77-87	<0.01	<0.01	< 0.01 – 0.13	< 0.01	0.012 – 0.082
	rest-of-plant[3)]			<0.01	<0.01	< 0.01 – 0.011	< 0.01	0.12 – 0.49
	grain	34 - 36	87-89	<0.01	<0.01	0.045 – 1.9	0.015 – 0.28	0.11 – 0.88
	straw			<0.01	<0.01	< 0.01 – 0.15	< 0.01 – 0.11	1.2 - 10
	ears	41 - 42	83-87	<0.01	<0.01	0.023 – 0.43	< 0.01	0.043 – 0.096
	rest-of-plant[3)]			<0.01	<0.01	<0.01 – 0.028	< 0.01	0.14 – 0.62
	grain	41 - 42	89	<0.01	<0.01	0.050 – 1.5	0.017 – 0.36	0.10 – 1.1
	straw			<0.01	<0.01	< 0.01 - 0.34	< 0.01 – 0.11	0.67 - 10
	grain	48 - 55	89	<0.01	<0.01	0.014 – 0.49	< 0.01 – 0.011	0.044 – 0.052
	straw			<0.01	<0.01	< 0.01 – 0.030	< 0.01	0.18 – 0.48

[1)] DALA = days after last application; [2)] whole plant (no roots); [3)] no roots

Table 6.3.2-10: Residues of BAS 750 F and TDM in barley (treated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 67294 Mauchenheim Germany (N) L130174	GC 0640 Barley Propino	1. 30.03 2013 2. 21.06.-28.06.2013 3. 02.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.06.13	69	plant [1] grain straw grain straw grain straw	0 28 28 35 35 41 41	7.4 0.14 9.3 <u>0.15</u> 15 0.13 11	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.028 0 22 < 0.01 0.061 < 0.01 0.054 < 0.01	< 0.01 0.022 < 0.01 0.025 0.025 0.019 0.025	0.68 0.087 1.5 0.60 1.2 0.46 0.93
433782 2014/1010808 16833 Lentzke Germany (N) L130175	GC 0640 Barley Sandra	1. 12.09 2012 2. 20.05.-24.05.2013 3. 16.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 27 27 34 34 42 42 53 53	4.9 0.082 0.84 0.058 0.52 0.065 0.61 <u>0.014</u> 1.0	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.055 0 21 0.022 0 24 0.015 0.79 0.064 1 1 0.097	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 0.011 < 0.01 0.021 < 0.01	0.34 0.070 0.41 0.081 0.31 0.13 0.56 0.086 0.80
433782 2014/1010808 6595 ME Ottersum The Netherlands (N) L130176	GC 0640 Barley Sequel	1. 02.10.2012 2. 29.05.-12.06.2013 3. 22.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 11.06.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 34 34 41 41	2.3 1.2 2.4 0.13 5.3 <u>0.19</u> 5.6	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.40 1.4 0 13 2.0 0 16 1.4 0.67	0.041 0.35 0.086 0.29 0.11 0.37 0.20	9.6 1.3 7.9 0.96 11 1.2 10
433782 2014/1010808 CO112NF Mannigtree, United Kingdom (N) L130177	GC 0640 Barley Cassata	1. 16.10.2012 2. 11.06.-18.06.2013 3. 26.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 14.06.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 35 35 41 41	3.2 0.71 1.9 0.056 2.9 <u>0.071</u> 3.9	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.029 0 19 0.017 0 33 0.030 2.6 0.071	< 0.01 0.027 < 0.01 0.030 0.038 0.34 0.027	0.50 0.32 1.1 0.076 1.7 1.2 1.1

Table 6.3.2-10: Residues of BAS 750 F and TDM in barley (treated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 32130 Cazaux-Saves France (S) L130178	GC 0640 Barley Bamboo	1. 20.10.2012 2. 08.05.-17.05.2013 3. 09.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 17.05.13	69	plant [1]	0	3.4	< 0.01	0.023	< 0.01	0.75
										ears	28	0.36	< 0.01	0 13	< 0.01	0.23
										rest of pl. [2]	28	0.49	< 0.01	< 0.01	< 0.01	0.27
										ears	34	0.13	< 0.01	0 17	< 0.01	0.11
										rest of pl. [2]	34	0.20	< 0.01	< 0.01	< 0.01	0.15
										ears	41	0.16	< 0.01	0 17	< 0.01	0.099
										rest of pl. [2]	41	0.18	< 0.01	< 0.01	< 0.01	0.20
										grain	55	0.070	< 0.01	0.81	< 0.01	0.11
										straw	55	0.39	< 0.01	0.078	< 0.01	0.61
433782 2014/1010808 58300 Galatades Greece (S) L130179	GC 0640 Barley Moutso	1. 09.12.2012 2. 08.04.-18.04.2013 3. 30.05.-05.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 12.04.13	69	plant [1]	0	7.2	< 0.01	0.011	< 0.01	0.44
										ears	28	0.86	< 0.01	0 15	0.033	0.35
										rest of pl. [2]	28	12	< 0.01	< 0.01	< 0.01	1.2
										ears	35	1.0	< 0.01	0.074	0.028	0.20
										rest of pl. [2]	35	12	< 0.01	< 0.01	< 0.01	1.1
										ears	42	1.7	< 0.01	0.090	0.023	0.25
										rest of pl. [2]	42	13	< 0.01	< 0.01	< 0.01	1.0
										grain	54	0.16	< 0.01	0.052	< 0.01	0.074
										straw	54	6.4	< 0.01	< 0.01	< 0.01	0.53
433782 2014/1010808 12050 Castagnito d´Alba Italy (S) L130180	GC 0640 Barley Corneta Delicious	1. 16.10.2012 2. 01.05.-10.05.2013 3. 26.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.13	69	plant [1]	0	3.9	< 0.01	< 0.01	< 0.01	0.26
										ears	27	0.40	< 0.01	0.095	< 0.01	0.11
										rest of pl. [2]	27	1.8	< 0.01	< 0.01	< 0.01	0.18
										ears	34	0.31	< 0.01	0.096	< 0.01	0.12
										rest of pl. [2]	34	13	< 0.01	< 0.01	< 0.01	0.28
										ears	42	0.35	< 0.01	0.098	< 0.01	0.11
										rest of pl. [2]	42	1.9	< 0.01	< 0.01	< 0.01	0.26
										grain	48	0.10	< 0.01	0 12	< 0.01	0.12
										straw	48	4.2	< 0.01	0 11	< 0.01	4.4

Table 6.3.2-10: Residues of BAS 750 F and TDM in barley (treated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 41720 Los Palacios Spain (S) L130181	GC 0640 Barley Prestige	1. 15.12.2012 2. 17.04.-22.04.2013 3. 03.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.04.13	69	plant [1]	0	5.7	< 0.01	0.055	< 0.01	1.2
										grain	29	0.42	< 0.01	0 13	0.029	0.16
										straw	29	11	< 0.01	< 0.01	< 0.01	2.2
										grain	36	0.29	< 0.01	0 13	0.028	0.16
										straw	36	11	< 0.01	< 0.01	< 0.01	2.6
										grain	42	<u>0.41</u>	< 0.01	0 10	0.020	0.17
										straw	42	11	< 0.01	0.012	< 0.01	0.83

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. A number in parenthesis indicates re-analysis to confirm analytical results. The highest both values is selected. The underlined values (e.g. <u>0.018</u>) are used for calculation MRL for grain.

Table 6.3.2-11: Level of BAS 750 F and TDM in barley (untreated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 67294 Mauchenheim Germany (N) L130174	GC 0640 Barley Propino	1. 30.03 2013 2. 21.06.-28.06.2013 3. 02.08.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 27.06.13	69	plant [1] grain straw grain straw grain straw	0 28 28 35 35 41 41	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.022 0 11 < 0.01 0.045 < 0.01 0.050 < 0.01	< 0.01 0.021 < 0.01 0.015 < 0.01 0.019 0.017	0.72 0.037 1.3 0.58 1.2 0.54 0.67
433782 2014/1010808 16833 Lentzke Germany (N) L130175	GC 0640 Barley Sandra	1. 12.09 2012 2. 20.05.-24.05.2013 3. 16.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.05.13	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 27 27 34 34 42 42 53 53	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.023 0 16 0.020 0 13 0.011 0.43 0.028 0.49 0.028	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 0.011 < 0.01	0.31 0.12 0.38 0.053 0.27 0.096 0.23 0.052 0.48
433782 2014/1010808 6595 ME Ottersum The Netherlands (N) L130176	GC 0640 Barley Sequel	1. 02.10.2012 2. 29.05.-12.06.2013 3. 22.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 11.06.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 34 34 41 41	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.41 1.07 0 11 1 9 0 15 1 5 0 34	0.058 0.34 0.078 0.28 0.11 0.36 0.11	7.3 1.3 6.8 0.88 10 1.1 10
433782 2014/1010808 CO112NF Mannigtree, United Kingdom (N) L130177	GC 0640 Barley Cassata	1. 16.10.2012 2. 11.06.-18.06.2013 3. 26.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 14.06.13	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 35 35 41 41	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01 < 0.01	0.013 0 19 0.015 0 22 0.016 0 28 0.061	< 0.01 0.019 < 0.01 0.24 0.014 0.029 0.022	0.20 0.38 0.94 0.11 1.2 0.10 1.1

Table 6.3.2-11: Level of BAS 750 F and TDM in barley (untreated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 32130 Cazaux-Saves France (S) L130178	GC 0640 Barley Bamboo	1. 20.10.2012 2. 08.05.-17.05.2013 3. 09.07.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 17.05.13	69	plant [1]	0	< 0.01	< 0.01	0.012	< 0.01	0.49
										ears	28	< 0.01	< 0.01	0.042	< 0.01	0.10
										rest of pl. [2]	28	< 0.01	< 0.01	< 0.01	< 0.01	0.16
										ears	34	< 0.01	< 0.01	0.056	< 0.01	0.071
										rest of pl. [2]	34	< 0.01	< 0.01	< 0.01	< 0.01	0.12
										ears	41	< 0.01	< 0.01	0.071	< 0.01	0.074
										rest of pl. [2]	41	< 0.01	< 0.01	< 0.01	< 0.01	0.14
										grain	55	< 0.01	< 0.01	0 37	< 0.01	0.047
										straw	55	< 0.01	< 0.01	0.030	< 0.01	0.18
433782 2014/1010808 58300 Galatades Greece (S) L130179	GC 0640 Barley Moutso	1. 09.12.2012 2. 08.04.-18.04.2013 3. 30.05.-05.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 12.04.13	69	plant [1]	0	< 0.01	< 0.01	< 0.01	< 0.01	0.36
										ears	28	< 0.01	< 0.01	0.028	< 0.01	0.061
										rest of pl. [2]	28	< 0.01	< 0.01	< 0.01	< 0.01	0.55
										ears	35	< 0.01	< 0.01	0.027	< 0.01	0.082
										rest of pl. [2]	35	< 0.01	< 0.01	< 0.01	< 0.01	0.49
										ears	42	< 0.01	< 0.01	0.026	< 0.01	0.093
										rest of pl. [2]	42	< 0.01	< 0.01	< 0.01	< 0.01	0.62
										grain	54	< 0.01	< 0.01	0.014	< 0.01	0.044
										straw	54	< 0.01	< 0.01	0.017	< 0.01	0.24
433782 2014/1010808 12050 Castagnito d´Alba Italy (S) L130180	GC 0640 Barley Corneta Delicious	1. 16.10.2012 2. 01.05.-10.05.2013 3. 26.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.13	69	plant [1]	0	< 0.01	< 0.01	< 0.01	< 0.01	0.36
										ears	27	< 0.01	< 0.01	0.016	< 0.01	0.060
										rest of pl. [2]	27	< 0.01	< 0.01	< 0.01	< 0.01	0.14
										ears	34	< 0.01	< 0.01	< 0.01	< 0.01	0.012
										rest of pl. [2]	34	< 0.01	< 0.01	< 0.01	< 0.01	0.20
										ears	42	< 0.01	< 0.01	0.023	< 0.01	0.043
										rest of pl. [2]	42	< 0.01	< 0.01	< 0.01	< 0.01	0.22
										grain	48	< 0.01	< 0.01	0.034	< 0.01	0.044
										straw	48	< 0.01	< 0.01	< 0.01	< 0.01	0.25

Table 6.3.2-11: Level of BAS 750 F and TDM in barley (untreated samples)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433782 2014/1010808 41720 Los Palacios Spain (S) L130181	GC 0640 Barley Prestige	1. 15.12.2012 2. 17.04.-22.04.2013 3. 03.06.2013	Foliar application	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.04.13	69	plant [1]	0	< 0.01	< 0.01	0.023	< 0.01	0.95
										grain	29	< 0.01	< 0.01	0.096	0.034	0.11
										straw	29	< 0.01	< 0.01	< 0.01	0.18	3.4
										grain	36	< 0.01	< 0.01	0.075	0.045	0.17
										straw	36	< 0.01	< 0.01	< 0.01	< 0.01	3.1
										grain	42	< 0.01	< 0.01	0.060	0.017	0.11
										straw	42	< 0.01	< 0.01	< 0.01	< 0.01	0.87

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid.

III. CONCLUSION

Residue data obtained in 8 independent field trials in barley (conducted in both N-EU, S-EU with the formulated product BAS 750 00 F according to the critical GAP) show that average residues of BAS 750 F are 0.15 mg/kg in grain (highest residue 0.41 mg/kg) and 5.9 mg/kg in straw (highest residue 15 mg/kg).
Concerning residue data for forage used as feed item, as a result of the application timing at late stages of crop cultivation (last application at BBCH69), residue data for green plant parts is only provided for late stages of crop development. In practice, forage for use as livestock feed item is typically harvested at earlier growth stages, prior to the second application. Note that the intended use does not include use on cereal forage (livestock feed item) and therefore corresponding residue data was not specifically provided. Importantly, residues determined in the present study for green plants therefor are an overestimation compared to BAS 750 F residues in forage resulting from the use supported in the present dossier.
Residue level for 1,2,4-triazole, TA, TAA and TLA resulting from the use of the formulated product BAS 750 00 F on barley according to the critical GAP are comparable to the data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

Report:	CA 6.3.2/2 Ale E., 2015 b Residue study (Decline) with BAS 750 01 F, BAS 750 00 F and BAS 750 BU F applied to barley in Northern and Southern Europe in 2014 2015/1099703
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, OECD 509 Crop Field Trial (2009), EEC 7525/VI/95 rev. 9 (March 2011), EEC 7029/VI/95 rev. 5 (July 22 1997)
GLP:	yes (certified by ENAC, Entidad Nacional de Acreditación, Madrid, Spain)

EXECUTIVE SUMMARY

During the growing season of 2014, ten independent field trials with barley were conducted in Europe (N-EU and S-EU) in order to determine the magnitude of the BAS 750 F residues (analytes were parent, and the TDM with 1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) after application of either the formulated product BAS 750 01 F or alternatively, on a parallel plot, of the formulated product BAS 750 00 F. The EC formulation BAS 750 01 F (0.1 kg as/L) and the EC formulation BAS 750 00 F (0.1 kg as/L) were applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F in a spray volume of 200 L/ha. Barley specimens were collected as whole plant (no roots) shortly before the second application (untreated control) and directly after the second application. Depending on the maturity of the crop, sampling after 28±1 days, 35±1 days and 42±1 days, provided either ears and rest-of-plant (no root) or grain and straw. In one trial, the crop did not reach growth stage BBCH 89 after 49 days, thus, an additional sampling was done after 48 days. Generally, samples were analysed within the time period of confirmed freezer storage stability for BAS 750 F and TDM in high starch matrices (grain), high water matrices (green plant) and straw. Recoveries (mean) were within the acceptable range of 70 – 110% for both BAS 750 F and TDM.

In untreated samples, no residues of BAS 750 F exceeding the LOQ were detected. For grain of plots treated with BAS 750 01 F, average residues were at 0.096 mg/kg (n=10), with individual values of 0.018, 0.033, 0.058, 0.060, 0.085, 0.087, 0.088, 0.11, 0.14 and as the highest residue 0.28 mg/kg. For straw of plots treated with BAS 750 01 F, average residues were at 4.88 mg/kg (n=10) with the highest residue at 18 mg/kg (individual values were 1.7, 2.1, 2.2, 2.4, 3.1, 3.6, 4.3, 4.6, 6.8, 18 mg/kg).

With the formulation BAS 750 00 F highly similar level of BAS 750 F were obtained. For plants taken directly after application (DALA0) residues were in the range of 2.5 - 8.1 mg/kg (n=10) thus corresponding well with the range obtained with the formulation BAS 750 01 F (2.6 - 9.2 mg/kg). The same observation was made at harvest stage, for grain and straw. For grain, with residue level of 0.029, 0.03, 0.05, 0.057, 0.10 (2x), 0.14, 0.15, 0.26 and 0.29 mg/kg, the values for average (0.12 mg/kg, n=10) and highest residue (0.029 mg/kg) were almost identical to the values obtained for BAS 750 01 F. Similarly for straw, with residue level of 0.99, 1.9 (2x), 2.5, 3.1 (2x), 3.3, 4.3, 5.9, and 16 mg/kg both values, the average residue (4.3 mg/kg) and highest residue (16 mg/kg) are in good accordance with data obtained with formulation BAS 750 01 F. This data confirms that both formulations do not result in any significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F can be used as supporting residue data for the formulated product BAS 750 01 F.

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.97 mg/kg (1.1 mg/kg), for TAA up to 0.55 mg (0.44 mg/kg) and for TLA 0.22 mg/kg (0.10 mg/kg). For straw residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.11 mg/kg (0.11 mg/kg), for TAA up to 0.33 mg/kg (0.19 mg/kg) and for TLA up to 0.20 mg/kg (0.58 mg/kg). Note that, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

I. MATERIAL AND METHODS

A. MATERIALS

1.

Test Material:	BAS 750 01 F, BAS 750 00 F, BAS 750 BU F	
Description:	BAS 750 01 F: 100 g/L of BAS 750 F, EC formulation	
	BAS 750 00 F: 100 g/L of BAS 750 F, EC formulation	
Lot/Batch #:	BAS 750 01 F: FD-140113-0006;	
	BAS 750 00 F: FD-130326-0002;	
Purity:	*not relevant*	
CAS#:	1417782-03-6	
Development code:	BAS 750 F	
Spiking levels:	BAS 750 F:	0.01, 0.1, 1, 5, 10, 20, 100 mg/kg
	1,2,4-triazole :	0.01, 1.0 mg/kg
	triazole alanine:	0.01, 1.0 mg/kg
	triazole acetic acid:	0.01, 1.0 mg/kg
	triazole lactic acid:	0.01, 1.0 mg/kg

2.

Test Commodity:	cereal
Crop:	barley
Type:	*Hordeum vulgare* L.,
Variety:	Propino, Meridian, Sequel, Flagon, Sandra, Ketos, Chill, Atomo, Acapulco, Hispanic
Crop parts(s) or processed Commodity:	whole plant (no root), ear, rest-of-plant (no root), grain, straw
Sample size:	for grain, at least 1.0 kg (12 plants), for straw, at least 0.5 kg (12 plants)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2014, ten field trials on barley, each with separate plots for two formulated products BAS 750 01 F and BAS 750 00 F, were conducted in Germany, the Netherlands, Northern and Southern France, Greece, Italy and Spain, in order to determine the magnitude of the residues of BAS 750 F and its metabolites 1,2,4-triazole, TA, TAA and TLA. (Note, that the trials included an additional plot treated with a third formulation (BAS 750 BU F) which is not further considered, and therefore not reported in the present dossier).

- The EC formulation BAS 750 01 F was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (1.5 L/ha of BAS 750 01 F) in a spray volume of 200 L/ha (designated plot 2).
- The EC formulation BAS 750 00 F was applied two times (BBCH 49 and 69) at a rate of 0.15 kg as/ha for BAS 750 F (1.5 L/ha of BAS 750 00 F) in a spray volume of 200 L/ha (designated plot 3).

Sampling included the harvest of whole plant (root removed) directly after the second application as soon as the spray deposit had dried. Samples were also taken at three time points after the last application, namely at DALA28±1, DALA 35±1, and DALA 42±1. In the case of unripe crop, samples consisted of ear and rest-of-plant (root removed). While for ripe crop, grain and straw were taken. For one trial (Spain, L140167), BBCH growth stage 89 was not reached at DALA42, thus, an additional sampling timepoint at DALA48 was added to obtain a grain and straw sample from this trial.

Table 6.3.2-12 Application and sampling details

region	no. of trials	no. of appl.	F, G, I[2]	method	test item	active substance	application		timing target	
							rate (kg a.s. /ha)	water vol. (L/ha)	application (BBCH)	sampling (DALA)[1]
EU North & South	10	2	F	-	BAS 750 01 F (EC) BAS 750 00 F (EC)	BAS 750 F	0.15	200	1st appl.: BBCH 49 2nd appl.: BBCH 69	0 28 ± 1 35 ± 1 42 ± 1 48

1) days after last application, 2) field, glasshouse or indoor

2. Description of analytical procedures

BAS 750 F residues were determined according to BASF method L0076/09. BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane. The final determination was performed by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg. Stability of BAS 750 F (deep frozen plant matrices) has been confirmed for a minimum of 547 days in high starch matrices (e.g. grain), a minimum of 551 days in high water matrices (e.g. green plant) and for a minimum of 551 days in plant straw (e.g. cereal straw).

Procedural recovery (average) were at 95.2% for BAS 750 F using fortification levels of 0.01-100 mg/kg. The results are summarized in the following Table 6.3.2-13.

Table 6.3.2-13 Summary of recoveries for BAS 750 F

matrix	fortification level (mg/kg)	BAS 750 F			
		n	mean (%)	SD (+/-)	RSD (%)
method L0076/09					
whole plant (no root)	0.01, 1.0, 10, 20, 100	10	96.2	3.5	3.6
ear	0.01, 0,1, 1,0, 5.0, 10	12	92.0	3.9	4.2
rest-of-plant (no root)	0.01, 1.0, 20	14	98.6	7.3	7.4
grain	0.01, 0.1, 1.0	11	96.1	10	11
straw	0.01, 0,1, 1.0, 20	14	93.3	7.7	8.3
overall		**61**	**95.2**	**7.3**	**7.7**

TDM residues, namely 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were determined according to method L0170/02 (see section CA 4.3). The analytes are extracted with methanol/water. An aliquot of the extract is filtered, concentrated and cleaned-up by dispersive C_{18}-SPE prior to analysis by LC/DMS-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

Procedural recovery (average) were at 90.5% (1,2,4-triazole), at 89.2% (TA), at 95.7% (TAA) and at 89.0% (TLA). The results are summarized in the following Table 6.3.2-14.

Table 6.3.2-14 Summary of recoveries for T, TA, TAA and TLA

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0170/02		**1,2,4-triazole (T)**			
whole plant (no root)	0.01, 1.0	10	97.3	11	11
ear	0.01, 1.0	12	89.3	12	13
rest-of-plant (no root)	0.01, 1.0	16	93.9	8.5	9.0
grain	0.01, 1.0	8	87.0	9.2	11
straw	0.01, 1.0	8	80.4	9.4	12
overall		**54**	**90.5**	**11**	**12**
method L0170/02		**TA**			
whole plant (no root)	0.01, 1.0	8	93.0	16	17
ear	0.01, 1.0	12	86.2	19	22
rest-of-plant (no root)	0.01, 1.0	8	89.8	18	21
grain	0.01, 1.0	13	90.9	22	24
straw	0.01, 1.0	11	87.6	18	21
overall		**52**	**89.2**	**19**	**21**
method L0170/02		**TAA**			
whole plant (no root)	0.01, 1.0	10	93.6	11	11
ear	0.01, 1.0	12	98.5	13	14
rest-of-plant (no root)	0.01, 1.0	16	103	17	16
grain	0.01, 1.0	8	86.6	1	15
straw	0.01, 1.0	7	86.9	19	21
overall		**53**	**95.7**	**16**	**17**
method L0170/02		**TLA**			
whole plant (no root)	0.01, 1.0	8	103	10	10
ear	0.01, 1.0	12	89.3	17	19
rest-of-plant (no root)	0.01, 1.0	10	91.4	13	15
grain	0.01, 1.0	13	78.2	8.8	11
straw	0.01, 1.0	12	89.0	19	21
overall		**55**	**89.0**	**16**	**18**

II. RESULTS AND DISCUSSION

The formulated product supported in the present dossier is BAS 750 01 F and was used in the present study to investigate magnitude of residues resulting from the use on barley. This formulated product is equivalent to BAS 750 00 F and thus similar residue data is expected for both formulated products. In order to provide convincing confirmation, each trial of the present study included side-by-side plots with BAS 750 01 F (plot 2) and BAS 750 00 F (plot 3). Note, that a further formulation BAS 750 BU F, was included in the trials, which is not considered further. The residue level of BAS 750 F and the TDM is summarized in Table 6.3.2-15 (BAS 750 F, treated samples) and Table 6.3.2-16 (TDM, treated and untreated samples). Detailed residue date is provided in Table 6.3.2-17, Table 6.3.2-18 and Table 6.3.2-19. Generally, the maximum storage interval of deep frozen samples from harvest until extraction was 374 days for BAS 750 F and 354 days for TDM.

BAS 750 F

In untreated samples, no residues of BAS 750 F exceeding the LOQ were detected. For grain of plots treated with BAS 750 01 F, average residues were at 0.096 mg/kg (n=10), with individual values of 0.018, 0.033, 0.058, 0.060, 0.085, 0.087, 0.088, 0.11, 0.14 and as the highest residue 0.28 mg/kg. For straw of plots treated with BAS 750 01 F, average residues were at 4.88 mg/kg (n=10) with the highest residue at 18 mg/kg (individual values were 1.7, 2.1, 2.2, 2.4, 3.1, 3.6, 4.3, 4.6, 6.8, 18 mg/kg). For plants directly after application of BAS 750 01 F (DALA0), BAS 750 F residues were in the range of 2.6 - 9.2 mg/kg (n=10).

With the formulation BAS 750 00 F highly similar level of BAS 750 F were obtained. A comparison is provided in Table 6.3.2-18. For plants taken directly after application (DALA0) residues were in the range of 2.5 - 8.1 mg/kg (n=10) thus corresponding well with the range obtained with the formulation BAS 750 01 F (2.6 - 9.2 mg/kg). The same observation was made at harvest stage, for grain and straw. For grain, with residue level of 0.029, 0.03, 0.05, 0.057, 0.10 (2x), 0.14, 0.15, 0.26 and 0.29 mg/kg, the values for average (0.12 mg/kg, n=10) and highest residue (0.029 mg/kg) were almost identical to the values obtained for BAS 750 01 F. Similarly for straw, with residue level of 0.99, 1.9 (2x), 2.5, 3.1 (2x), 3.3, 4.3, 5.9, and 16 mg/kg both values, the average residue (4.3 mg/kg) and highest residue (16 mg/kg) are in good accordance with data obtained with formulation BAS 750 01 F. This data confirms that both formulations do not result in any significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F (see section 6.3.2/1) can be used as supporting residue data for the formulated product BAS 750 01 F.

TDM

For 1,2,4-triazole, no residues exceeding the LOQ were detected in any sample, both for treated samples and untreated samples. For TA, TAA, and TLA, residue levels above LOQ were determined. These residues are to a certain extend treatment-unrelated as confirmed by determination of residues above LOQ in untreated samples. For grain residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.97 mg/kg (1.1 mg/kg), for TAA up to 0.55 mg (0.44 mg/kg) and for TLA 0.22 mg/kg (0.10 mg/kg). For straw residue levels in BAS 750 01 F-treated (untreated) samples amounted for TA up to 0.11 mg/kg (0.11 mg/kg), for TAA up to 0.33 mg/kg (0.19 mg/kg) and for TLA up to 0.20 mg/kg (0.58 mg/kg). Note that, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment: "*A large residues data package has been submitted to address residues of the TDMs in primary crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."

Note that concerning residue data for forage used as feed item, as a result of the application timing at late stages of crop cultivation (last application at BBCH69), residue data for green plant parts is only provided for late stages of crop development. In practice, forage for use as livestock feed item is typically harvested at earlier growth stages, prior to the second application. Importantly, the intended use does not include use on cereal forage (livestock feed item) and therefor corresponding residue data was not specifically provided. Consequently, residues determined in the present study for green plants therefor are an overestimation compared to BAS 750 F residues in forage resulting from the use supported in the present dossier.

Table 6.3.2-15: Summary of BAS 750 F residues in barley treated with the formulated products BAS 750 01 F (plot 2), or BAS 750 00 F (plot 3)

region	matrix	DALA [1)]	BBCH	n	BAS 750 F *[mg/kg]*	
					BAS 750 01 F	BAS 750 00 F
N-EU & S-EU	plant [2)]	0	69	10	2.0 – 9.2	2.5 – 8.1
	ears	27 - 29	73 - 87	10	0.094 – 5.7	0.14 – 3.9
	rest-of-plant [3)]			10	1.0 - 21	0.72 - 20
	ears	34 - 36	83 - 87	5	0.26 – 6.0	0.19 – 5.3
	rest-of-plant [3]			5	1.5 - 21	0.86 - 16
	grain	34 - 36	87 – 89	5	0.085 – 0.28	0.057 – 0.22
	straw			5	2.2 – 6.8	1.6 – 5.9
	ears	41	87 - 89	1	0.40	0.33
	rest-of-plant [3]			1	1.6	1.4
	grain	41 - 43	89	9	0.018 – 0.25	0.030 – 0.29
	straw			9	1.7 - 18	0.99 - 16
	grain	48	89	1	0.033	0.029
	straw			1	2.2	1.9

[1)] DALA = days after last application; [2)] no roots; [3)] without roots

Table 6.3.2-16: Summary of TDM residues in barley treated with the formulated products BAS 750 01 F (plot 2), or BAS 750 00 F (plot 3)

region	matrix	DALA [1)]	BBCH	Plot	1,2,4-triazole *[mg/kg]*	TA *[mg/kg]*	TAA *[mg/kg]*	TLA *[mg/kg]*
N-EU & S-EU	plant [2)]	0	69	1	<0.01	0.029 - 0.11	0.012 - 0.18	<0.01 - 0.25
				2	<0.01	0.020 - 0.098	<0.01 - 0.21	0.012 - 0.27
				3	<0.01	0.013 - 0.14	0.084 - 0.41	<0.01 - 0.36
	ears	27-29	73-87	1	<0.01	0.019 - 0.64	<0.01 - 0.095	<0.01 - 1.0
	rest-of-plant [3]				<0.01	<0.01 - 0.090	<0.01 - 0.15	<0.01 - 0.091
	ears			2	<0.01	0.032 - 0.48	<0.01 - 0.028	0.016 - 0.84
	rest-of-plant [3]				<0.01	0.017 - 0.11	<0.01 - 0.21	<0.01 - 0.039
	ears			3	<0.01	0.028 - 0.38	<0.01 - 0.060	0.050 - 0.77
	rest-of-plant [3]				<0.01	0.016 - 0.11	<0.01 - 0.68	<0.01 - 0.19
	ears	34-36	83-87/89	1	<0.01	0.061 - 0.16	<0.01 - 0.075	<0.01 - 0.44
	rest-of-plant [3]				<0.01	0.036 - 0.062	0.011 - 0.087	<0.01 - 0.026
	grain				<0.01	0.019 - 0.46	0.023 - 0.047	0.015 - 0.87
	straw				<0.01	0.027 - 0.28	0.044 - 0.30	<0.01 - 0.30
	ears			2	<0.01	0.080 - 0.23	<0.01 - 0.023	0.058 - 0.20
	rest-of-plant [3]				<0.01	0.029 - 0.10	0.027 - 0.11	<0.01 - 0.023
	grain				<0.01	0.041 - 0.55	<0.01 - 0.015	0.035 - 0.97
	straw				<0.01	0.022 - 0.28	<0.01 - 0.20	0.016 - 0.38
	ears			3	<0.01	0.080 - 0.27	<0.01 - 0.043	0.015 - 0.61
	rest-of-plant [3]				<0.01	0.010 - 0.092	<0.01 - 1.1	<0.01 - 0.10
	grain				<0.01	0.032 - 0.40	0.055 - 0.082	0.037 - 0.74
	straw				<0.01	0.033 - 0.21	0.15 - 0.59	<0.01 - 0.090
	ears	41-43	87-89/89	1	<0.01	0.053	0.014	0.026
	rest-of-plant [3)]				<0.01	0.025	0.019	<0.01
	grain				<0.01	0.023 - 0.59	<0.01 - 0.080	<0.01 - 1.1
	straw				<0.01	0.022 - 0.17	0.038 - 0.62	<0.01 - 0.062
	ears			2	<0.01	0.085	<0.01	0.11
	rest-of-plant [3)]				<0.01	0.032	0.019	0.030
	grain				<0.01	0.017 - 0.50	<0.01 - 0.22	0.039 - 0.82
	straw				<0.01	0.019 - 0.33	<0.01 - 0.18	0.014 - 0.12
	ears			3	<0.01	0.087	0.016	0.060
	rest-of-plant [3]				<0.01	0.020	0.21	<0.01
	grain				<0.01	0.025 - 0.44	<0.01 - 0.10	0.059 - 1.1
	straw				<0.01	0.026 - 0.19	0.15 - 0.58	<0.01 - 0.11
	grain	48	89	1	<0.01	0.088	<0.01	0.035
	straw				<0.01	0.045	0.059	0.011
	grain			2	<0.01	0.091	<0.01	0.078
	straw				<0.01	0.035	0.031	0.013
	grain			3	<0.01	0.079	<0.01	0.050
	straw				<0.01	0.024	0.40	<0.01

[1)] DALA = days after last application; [2)] no roots; [3)] without roots

Table 6.3.2-17: Residues of BAS 750 F and TDM in barley (BAS 750 01 F treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 67294 Mauchenhein, Germany (N) L140158	GC 0640 Barley Propino	1. 06.03 2014 2. 30.05.-10.06.2014 3. 23.07.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 10.06.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 29 29 35 35 43 43	3.6 0.89 3.0 0.087 6.8 0.061 5 3	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.013 0.050 <0.01 0.035 0.14 0.11 0.12	0.045 0.080 0.019 0.069 0.031 0.096 0.035	0.023 0.016 <0.01 <0.01 <0.01 <0.01 <0.01
433784 2015/1099703 47589 Uedem, Germany (N) L140159	GC 0640 Barley Meridian	1. 01.10 2013 2. 08.-22.05.2014 3. 02.07.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 27 27 36 36 41 41	2.6 0.42 1 2 0.085 2 2 0.071 3 1	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.054 0.21 <0.01 0.19 0.38 0.058 0.093	0.058 0.11 0.060 0.15 0.12 0.11 0.12	0.069 0.011 0.095 <0.01 0.10 <0.01 0.049
433784 2015/1099703 6595 ME Ottersum, The Netherlands (N) L140160	GC 0640 Barley Sequel	1. 27.09.2013 2. 08.-21.05.2014 3.01.07.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 21.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 35 35 41 41	2.7 0.53 2 1 0.11 2 2 0.10 3.6	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.27 0.86 (0.82) 0.039 0.97 0.11 0.82 0.078	0.098 0.47 (0.49) 0.10 0.55 0.28 0.50 0.33	0.21 0.028 0.21 0.015 0.20 0.22 0.18
433784 2015/1099703 CM22 6JD Ugley Green, United Kingdom (N) L140161	GC 0640 Barley Flagon	1. 25.10.2013 2. 10.-28.06.2014 3. 05.08.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.06.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 29 29 35 35 42 42	6 1 1 3 2.7 0.28 4 3 0.25 3.7	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.033 0.016 0.012 0.040 0.016 0.039 0.018	0.027 0.032 0.021 0.041 0.022 0.017 0.019	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01

Table 6.3.2-17: Residues of BAS 750 F and TDM in barley (BAS 750 01 F treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 72500 Saint Pierre de chevillé, France (N) L140162	GC 0640 Barley Sandra	1. 07.10.2013 2. 02.-15.05.2014 3. 25.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 15.05.14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 27 27 34 34 41 41	2.0 0.20 1.0 0.26 1 5 0.060 1.7	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.048 0.22 <0.01 0.14 <0.01 0.21 0.021	0.050 0.17 0.041 0.23 0.039 0.21 0.099	0.050 0.016 0.060 0.011 0.064 <0.01 0.080
433784 2015/1099703 32380 Tournecoupe, France (S) L140163	GC 0640 Barley Ketos	1. 27.10.2013 2. 05.-09.05.2014 3. 20.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 28 28 35 35 42 42	4 5 0.43 2.0 0.51 2.8 0.088 2.4	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.21 0.24 0.024 0.20 0.023 0.54 0.042	0.042 0.088 0.033 0.18 0.053 0.20 0.050	0.075 0.015 0.083 0.023 0.10 0.011 0.089
433784 2015/1099703 57011 Prochoma, Greece (S) L140164	GC 0640 Barley Chill	1. 03.11.2013 2. 10.-23.04.2014 3. 05.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 23.04.14	69	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 28 28 36 36 43 43	5 3 0.094 2.0 0.26 2.8 0.018 2 1	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.012 0.062 <0.01 0.058 <0.01 0.11 0.014	0.020 0.087 0.017 0.12 0.034 0.081 0.045	0.028 <0.01 0.022 <0.01 0.035 <0.01 0.028
433784 2015/1099703 20062 Cassano d´Adda, Italy (S) L140165	GC 0640 Barley Atomo	1. 15.10.2013 2. 01.-10.05.2014 3. 19.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 27 27 34 34 41 41	4 9 0.36 2 2 0.12 2.8 0.14 4.6	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.043 0.058 <0.01 0.12 0.025 0.14 0.024	0.057 0.071 0.041 0.12 0.10 0.18 0.091	0.049 <0.01 0.028 <0.01 0.053 <0.01 0.055

Table 6.3.2-17: Residues of BAS 750 F and TDM in barley (BAS 750 01 F treated samples, plot 2)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 16220 Quintanar del Rey, Spain (S) L140166	GC 0640 Barley Acapulco	1. 21.11.2013 2. 20.04.-05.05.2014 3. 16.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.05.14	69	plant [1]	0	9 2	<0.01	0.029	0.076	0.028
										ears	28	5.7	<0.01	0.092	0.12	<0.01
										rest of pl. [2]	28	21	<0.01	<0.01	0.11	0.083
										ears	35	6.0	<0.01	0.070	0.11	<0.01
										rest of pl. [2]	35	21	<0.01	<0.01	0.090 (0.11)	0.11
										grain	42	<u>0.058</u>	<0.01	0.088	0.11	<0.01
										straw	42	18	<0.01	0.047	0.11	0.076
433784 2015/1099703 02110 La Gineta, Spain (S) L140167	GC 0640 Barley Hispanic	1. 17.12.2013 2. 25.04.-05.05.2014 3. 19.06.2014	Boom sprayer	BAS 750 01 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 02.05.14	69	plant [1]	0	4.6	<0.01	0.032	0.049	0.019
										ears	28	0.23	<0.01	0.068	0.055	<0.01
										rest of pl. [2]	28	1 3	<0.01	<0.01	0.025	0.028
										ears	35	0.34	<0.01	0.069	0.080	<0.01
										rest of pl. [2]	35	2.0	<0.01	<0.01	0.029	0.027
										ears	41	0.40	<0.01	0.11	0.085	<0.01
										rest of pl. [2]	41	1.6	<0.01	0.030	0.032	0.019
										grain	48	<u>0.033</u>	<0.01	0.078	0.091	<0.01
										straw	48	2 2	<0.01	0.013	0.035	0.031

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. The highest both values is selected. The underlined values (e.g. <u>0.018)</u> are used for calculation MRL for grain. Note, for trial L140167 an additional sampling was done at DALA48.

Table 6.3.2-18: Residues of BAS 750 F and TDM in barley (BAS 750 00 F, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 67294 Mauchenhein, Germany (N) L140158	GC 0640 Barley Propino	1. 06.03 2014 2. 30.05.-10.06.2014 3. 23.07.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 10.06.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 29 29 35 35 43 43	3.6 0.71 3.6 <u>0.057</u> 5.9 0.048 5.6	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.034 0.021 <0.01 0.059 <0.01 0.078 <0.01	0.048 0.060 0.025 0.069 0.033 0.053 0.030	0.084 <0.01 0.018 0.059 0.15 0.077 0.15
433784 2015/1099703 47589 Uedem, Germany (N) L140159	GC 0640 Barley Meridian	1. 01.10 2013 2. 08.-22.05.2014 3. 02.07.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 22.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 27 27 36 36 41 41	2.7 0.33 1.3 <u>0.10</u> 2.4 0.077 2.5	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.13 0.29 <0.01 0.16 <0.01 0.35 <0.01	0.059 0.12 0.058 0.098 0.11 0.099 0.084	0.37 0.019 0.081 0.060 0.37 0.047 0.33
433784 2015/1099703 6595 ME Ottersum, The Netherlands (N) L140160	GC 0640 Barley Sequel	1. 27.09.2013 2. 08.-21.05.2014 3.01.07.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 21.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 28 28 35 35 41 41	2.9 0.53 1.6 <u>0.15</u> 1.6 0.10 4.3	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.36 0.77 <0.01 0.74 0.090 1.1 <0.01	0.14 0.38 0.11 0.40 0.21 0.44 0.19	0.29 0.060 0.16 0.082 0.53 0.064 0.38
433784 2015/1099703 CM22 6JD Ugley Green, United Kingdom (N) L140161	GC 0640 Barley Flagon	1. 25.10.2013 2. 10.-28.06.2014 3. 05.08.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 24.06.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 29 29 35 35 42 42	6.0 0.93 3.0 0.22 3.1 <u>0.26</u> 2.7	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.061 0.055 0.022 0.037 0.023 0.059 <0.01	0.027 0.028 0.027 0.032 0.037 0.025 0.026	0.087 <0.01 0.014 0.067 0.51 0.10 0.58

Table 6.3.2-18: Residues of BAS 750 F and TDM in barley (BAS 750 00 F, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 72500 Saint Pierre de chevillé, France (N) L140162	GC 0640 Barley Sandra	1. 07.10.2013 2. 02.-15.05.2014 3. 25.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 15.05.14	69	plant [1] ears rest of pl. 2 ears rest of pl. 2 grain straw	0 27 27 34 34 41 41	2.5 0.17 0.72 0.19 0.86 <u>0.050</u> 0.99	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.30 0.58 0.19 0.61 0.10 0.83 0.11	0.055 0.23 0.034 0.27 0.036 0.30 0.11	0.21 0.045 0.11 0.043 0.15 0.014 0.46
433784 2015/1099703 32380 Tournecoupe, France (S) L140163	GC 0640 Barley Ketos	1. 27.10.2013 2. 05.-09.05.2014 3. 20.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.14	69	plant [1] ears rest of pl. 2 ears rest of pl. 2 grain straw	0 28 28 35 35 42 42	4.3 0.46 2.4 0.65 3.3 <u>0.10</u> 3.3	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.11 0.51 <0.01 0.49 0.032 0.69 0.095	0.059 0.14 0.043 0.21 0.050 0.14 0.049	0.12 0.024 0.10 0.040 0.16 <0.01 0.37
433784 2015/1099703 57011 Prochoma, Greece (S) L140164	GC 0640 Barley Chill	1. 03.11.2013 2. 10.-23.04.2014 3. 05.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 23.04.14	69	plant [1] ears rest of pl. 2 ears rest of pl. 2 grain straw	0 28 28 36 36 43 43	5.6 0.14 2.8 0.31 2.8 <u>0.030</u> 1.9	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 0.050 0.016 0.088 0.017 0.11 <0.01	0.013 0.074 0.016 0.11 0.010 0.081 0.034	0.41 0.010 0.52 0.033 <0.01 <0.01 0.57
433784 2015/1099703 20062 Cassano d´Adda, Italy (S) L140165	GC 0640 Barley Atomo	1. 15.10.2013 2. 01.-10.05.2014 3. 19.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 09.05.14	69	plant [1] ears rest of pl. [2] grain straw grain straw	0 27 27 34 34 41 41	4.4 0.52 2.3 0.10 2.5 <u>0.14</u> 3.1	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.048 0.11 <0.01 0.10 0.027 0.085 0.021	0.043 0.13 0.044 0.11 0.053 0.13 0.074	0.13 0.017 <0.01 0.055 0.59 0.093 0.52

Table 6.3.2-18: Residues of BAS 750 F and TDM in barley (BAS 750 00 F, plot 3)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 16220 Quintanar del Rey, Spain (S) L140166	GC 0640 Barley Acapulco	1. 21.11.2013 2. 20.04.-05.05.2014 3. 16.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 05.05.14	69	plant [1]	0	8.1	<0.01	0.017	0.046	0.14
										ears	28	3.9	<0.01	0.064	0.10	<0.01
										rest of pl. 2	28	20	<0.01	0.041	0.062	0.68
										ears	35	5.3	<0.01	0.015	0.11	<0.01
										rest of pl. 2	35	16	<0.01	0.046	0.092	1.1
										grain	42	<u>0.29</u>	<0.01	0.090	0.083	<0.01
										straw	42	16	<0.01	<0.01	0.075	0.20
433784 2015/1099703 02110 La Gineta, Spain (S) L140167	GC 0640 Barley Hispanic	1. 17.12.2013 2. 25.04.-05.05.2014 3. 19.06.2014	Boom sprayer	BAS 750 00 F (EC) 100 g/L BAS 750 F	0.075	200	0.15	2 02.05.14	69	plant [1]	0	4.3	<0.01	0.038	0.036	0.11
										ears	28	0.29	<0.01	0.087	0.063	<0.01
										rest of pl. 2	28	1.5	<0.01	<0.01	0.020	<0.01
										ears	35	0.43	<0.01	0.12	0.080	<0.01
										rest of pl. 2	35	1.6	<0.01	<0.01	0.028	0.054
										ears	41	0.33	<0.01	0.060	0.087	0.016
										rest of pl. 2	41	1.4	<0.01	<0.01	0.020	0.21
										grain	48	<u>0.029</u>	<0.01	0.050	0.079	<0.01
										straw	48	1.9	<0.01	<0.01	0.024	0.40

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid. The underlined values (e.g. <u>0.018</u>) are used for calculation MRL for grain.

Table 6.3.2-19: Level of BAS 750 F and TDM in untreated barley (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 67294 Mauchenhein, Germany (N) L140158	GC 0640 Barley Propino	1. 06.03 2014 2. 30.05.-10.06.2014 3. 23.07.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.028	0.063	0.042
										ears	29	<0.01	<0.01	0.090	0.074	<0.01
										rest of pl. [2]	29	<0.01	<0.01	0.058	0.034	0.065
										grain	35	<0.01	<0.01	0.095	0.11	0.047
										straw	35	<0.01	<0.01	<0.01	0.062	0.057
										grain	43	<0.01	<0.01	0.11	0.066	0.028
										straw	43	<0.01	<0.01	0.017	0.033	0.11
433784 2015/1099703 47589 Uedem, Germany (N) L140159	GC 0640 Barley Meridian	1. 01.10 2013 2. 08.-22.05.2014 3. 02.07.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.049	0.079	0.082
										ears	27	<0.01	<0.01	0.13	0.10	0.071
										rest of pl. [2]	27	<0.01	<0.01	0.013	0.058	0.067
										grain	36	<0.01	<0.01	0.12	0.15	0.023
										straw	36	<0.01	<0.01	0.029	0.14	0.19
										grain	41	<0.01	<0.01	0.094	0.11	<0.01
										straw	41	<0.01	<0.01	<0.01	0.080	0.39
433784 2015/1099703 6595 ME Ottersum, The Netherlands (N) L140160	GC 0640 Barley Sequel	1. 27.09.2013 2. 08.-21.05.2014 3.01.07.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.25	0.11	0.18
										ears	28	<0.01	<0.01	1.3 (0.76)	0.60 (0.68)	0.095
										rest of pl. [2]	28	<0.01	<0.01	0.019	0.071	0.15
										grain	35	<0.01	<0.01	0.87	0.46	0.045
										straw	35	<0.01	<0.01	0.41 (0 19)	0.28	0.30
										grain	41	<0.01	<0.01	1.1	0.59	0.042
										straw	41	<0.01	<0.01	0.061	0.17	0.19
433784 2015/1099703 CM22 6JD Ugley Green, United Kingdom (N) L140161	GC 0640 Barley Flagon	1. 25.10.2013 2. 10.-28.06.2014 3. 05.08.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.010	0.038	0.012
										ears	29	<0.01	<0.01	<0.01	0.019	<0.01
										rest of pl. [2]	29	<0.01	<0.01	<0.01	<0.01	<0.01
										grain	35	<0.01	<0.01	0.015	0.019	0.027
										straw	35	<0.01	<0.01	0.046	0.027	0.044
										grain	42	<0.01	<0.01	0.034	0.023	0.033
										straw	42	<0.01	<0.01	<0.01	0.024	0.038

Table 6.3.2-19: Level of BAS 750 F and TDM in untreated barley (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 72500 Saint Pierre de chevillé, France (N) L140162	GC 0640 Barley Sandra	1. 07.10.2013 2. 02.-15.05.2014 3. 25.06.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 27 27 34 34 41 41	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.051 0.24 <0.01 0.12 <0.01 0.18 <0.01	0.060 0.085 0.046 0.16 0.057 0.14 0.098	0.042 0.01 0.042 0.075 0.047 0.016 0.11
433784 2015/1099703 32380 Tournecoupe, France (S) L140163	GC 0640 Barley Ketos	1. 27.10.2013 2. 05.-09.05.2014 3. 20.06.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 28 28 35 35 42 42	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.14 0.42 0.091 0.44 0.023 0.092 0.062	0.038 0.14 0.028 0.15 0.062 0.18 0.027	0.070 0.024 0.069 0.034 0.087 0.080 0.11
433784 2015/1099703 57011 Prochoma, Greece (S) L140164	GC 0640 Barley Chill	1. 03.11.2013 2. 10.-23.04.2014 3. 05.06.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] ears rest of pl. [2] grain straw	0 28 28 36 36 43 43	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.029 0.043 0.028 0.061 0.036 0.031 0.022	0.012 <0.01 <0.01 <0.01 0.011 0.074 0.11
433784 2015/1099703 20062 Cassano d´Adda, Italy (S) L140165	GC 0640 Barley Atomo	1. 15.10.2013 2. 01.-10.05.2014 3. 19.06.2014	-	-	-	-	-	-	-	plant [1] ears rest of pl. [2] grain straw grain straw	0 27 27 34 34 41 41	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01 <0.01 <0.01 <0.01	0.029 0.039 <0.01 0.15 0.033 0.12 0.017	0.056 0.083 0.029 0.091 0.071 0.11 0.057	0.034 0.065 0.026 0.028 0.071 0.018 0.11

Table 6.3.2-19: Level of BAS 750 F and TDM in untreated barley (plot 1)

Report No. Location (EU-region) trial No	Commodity / Variety	Date of 1. Sowing or planting 2. Flowering 3. Harvest	Method of treatment	Formulation	Application rate per treatment			No. of treat-ments and last date	Growth stage at last date	Portion analysed	PHI (days)	Residues (mg/kg)				
					kg as/hL	Water L/ha	kg as/ha					BAS 750 F	1,2,4-T	TA	TAA	TLA
433784 2015/1099703 16220 Quintanar del Rey, Spain (S) L140166	GC 0640 Barley Acapulco	1. 21.11.2013 2. 20.04.-05.05.2014 3. 16.06.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	<0.01	0.065	0.017
										ears	28	<0.01	<0.01	0.039	0.048	<0.01
										rest of pl. [2]	28	<0.01	<0.01	0.013	0.090	0.053
										ears	35	<0.01	<0.01	0.054	0.084	0.028
										rest of pl. [2]	35	<0.01	<0.01	0.026	0.062	0.083
										grain	42	<0.01	<0.01	<0.01	0.050	0.078
										straw	42	<0.01	<0.01	0.035	0.063	0.62
433784 2015/1099703 02110 La Gineta, Spain (S) L140167	GC 0640 Barley Hispanic	1. 17.12.2013 2. 25.04.-05.05.2014 3. 19.06.2014	-	-	-	-	-	-	-	plant [1]	0	<0.01	<0.01	0.037	0.053	0.034
										ears	28	<0.01	<0.01	0.023	0.074	<0.01
										rest of pl. [2]	28	<0.01	<0.01	0.011	0.062	0.032
										ears	35	<0.01	<0.01	0.038	0.08	<0.01
										rest of pl. [2]	35	<0.01	<0.01	<0.01	0.038	0.027
										ears	41	<0.01	<0.01	0.026	0.053	0.014
										rest of pl. [2]	41	<0.01	<0.01	<0.01	0.025	0.019
										grain	48	<0.01	<0.01	0.035	0.088	<0.01
										straw	48	<0.01	<0.01	0.011	0.045	0.059

1) whole plant (no root) 2) rest-of-plant (no root), 1,2,4-T =1,2,4-triazole , TA = triazole alanine, TAA= triazole acetic acid, TLA=triazole lactic acid.

III. CONCLUSION

Residue data obtained in ten independent field trials in barley (conducted in both N-EU, S-EU with the formulated product BAS 750 01 F according to the critical GAP) show that average residues of BAS 750 F are 0.096 mg/kg in grain (highest residue 0.28 mg/kg) and 3.4 mg/kg in straw (highest residue 18 mg/kg). With the formulated product BAS 750 00 F highly similar data is obtained in side-by-side plots within the same trial, both considering the median residue and the highest residue. In grain average residues were 0.12 mg/kg, highest residue 0.29 mg/kg, for straw average residues were 3.1 mg/kg, highest residue was 16 mg/kg. This comparative investigation of BAS 750 01 F and BAS 750 00 F confirms that both formulations do not result in significantly different residue data. In conclusion, residue trials conducted with the formulated product BAS 750 00 F (see section 6.3.2/1) can be used as supporting residue data for the formulated product BAS 750 01 F (see section CA 6.7).

Residue level for 1,2,4-triazole, TA, TAA and TLA resulting from the use of the formulated product BAS 750 00 F on wheat according to the critical GAP are comparable to the data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7).

CA 6.4 Feeding studies

To investigate transfer of BAS 750 F residues to commodities of animal origin, feeding studies were conducted with BAS 750 F in hen and cow. No feeding study is required for pig and for fish.

CA 6.4.1 Poultry

Report:	CA 6.4.1/1 [redacted] 2015 a Magnitude of residues in tissues and eggs of laying hens following multiple oral administrations of BAS 750 F 2015/1106667
Guidelines:	Commission of the European Communities KOM(2005) 221 2005/0099 (CNS) (30 May 2005), OECD 505 (Jan. 2007), EPA 860.1480, EPA 860.1340, EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), SANCO/3029/99 rev. 4 (11 July 2000), SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2013)8 Guidance Document on Residues in Livestock No. 73
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

EXECUTIVE SUMMARY

A residue transfer study with BAS 750 F was conducted in laying hens with nominal doses of 0.15 mg/kg, 1.5 mg/kg, 4.5 mg/kg and 15.0 mg/kg feed. Actual doses were 0.18 mg/kg, 1.74 mg/kg, 5.12 mg/kg, 17.25 mg/kg and 17.19 mg/kg feed (DM). This corresponds doses of 0.01 mg/kg bw/day, 0.10 mg/kg bw/day, 0.30 mg/kg bw/day and 0.98 mg/kg bw/day. Doses were applied in capsules for a period of 33 days followed by a depuration period of 2, 7 or 14 days. Animals of the control group received empty capsules only. Residues of parent BAS 750 F, M750F022 and the TDM (triazole derivative metabolites) 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA), were analysed in samples of whole egg (including egg sample representative of the plateau phase, separated into egg white and yolk) as well as tissues (muscle, liver, fat, skin with fat).

Generally, the residue data obtained indicates a linear relationship between feed intake (BAS 750 F dose level) and the residue level in animal commodities for the analytes parent BAS 750 F, M750F022 and 1,2,4-triazole with absence of absence of BAS 750 F-specific residues in untreated controls (BAS 750 F, M750F022). TA, if detected, was apparently treatment-unrelated, TAA and TLA were not found in level above LOQ.

Residue levels in egg did reach a plateau level within 10 days (BAS 750 F, M750F022) and within 5 days (TDM). The plateau levels found for the highest dose group (given as group mean, highest individual given in parenthesis) were for BAS 750 F at 0.030 mg/kg (0.042 mg/kg), for M750F022 at 0.066 mg/kg (0.095 mg/kg), and for 1,2,4-triazole at 0.080 mg/kg (0.099 mg/kg). Upon withdrawal residues did decline rapidly to levels below the LOQ demonstrating that residues do not accumulate in egg (BAS 750 F latest after 7 days, M750F022 latest after 14 days, 1,2,4-triazole latest after 7 days).

In egg of the highest dose group, residues were localized in yolk for BAS 750 F (0.076 mg/kg) and M750F022 (0.017 mg/kg) while for 1,2,4-trizole a higher level was seen in egg white (0.083 mg/kg) compared with egg yolk (0.047 mg/kg).

Residue levels in tissues were higher for BAS 750 F in fat and liver than in muscle and skin with fat. For the highest dose group, group mean level (highest individual in parenthesis) in fat were 0.17 mg/kg (0.25 mg/kg), in liver 0.10 mg/kg (0.20 mg/kg), in muscle 0.016 mg/kg (0.027 mg/kg) and in skin with fat 0.10 mg/kg (0.15 mg/kg).

A similar tissue distribution was found for M750F022 with slightly higher residue levels compared to parent. For the highest dose group, group mean level (highest individual in parenthesis) in fat were 0.31 mg/kg (0.36 mg/kg), in liver 0.15 mg/kg (0.20 mg/kg), in muscle 0.03 mg/kg (0.04 mg/kg) and in skin with fat 0.18 mg/kg (0.19 mg/kg). Generally, roughly similar levels were found for parent BAS 750 F and its metabolite M750F022 (except for the matrix skin with fat).

A different tissue distribution was found for 1,2,4-triazole with residue level in liver, muscle and skin with fat exceeding the level in fat. For the highest dose group, group mean level (highest individual) in liver were 0.10 mg/kg (0.12 mg/kg), in muscle 0.10 mg/kg (0.11 mg/kg), in skin with fat 0.039 mg/kg (0.044 mg/kg), while in fat below the LOQ of 0.01 mg/kg.

Upon withdrawal, residues in tissues did rapidly decline to levels below the LOQ, demonstrating absence of accumulation for BAS 750 F (in muscle, liver, and fat latest after 2 days of depuration), for M750F022 (in muscle, latest after 2 days, in liver, latest after 7 days, in fat, latest after 14 days) and for 1,2,4-triazole (in muscle and liver, latest after 7 days, in fat latest after 2 days).

The relative amounts of BAS 750 F and its metabolites (M750F022 and TDM) are seen most clearly in the highest dose group (see Table 6.4.1-16), comparable relative amounts are seen in the lower dose groups as a result of the observed linear dose-dependency for all three analytes. However, these residue levels are obtained with highly overdosed feed residue intake, with a realistic feed burden, residues expected in practice are much lower (see section CA6.7).

Table 6.4.1-1: Overview tissue distribution in dose group E (highly overdosed)[3)]

	BAS 750 F		M750F022		1,2,4-T	
dose group E (15 mg/kg)	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*
egg [1)]	**0.03**	*0.04*	**0.06**	*0.10*	**0.08**	*0.10*
yolk [2)]	**0.08**	*0.09*	**0.02**	*0.02*	**0.05**	*0.05*
white [2)]	**<LOQ**	*<LOQ*	**<LOQ**	*<LOQ*	**0.08**	*0.09*
muscle	**0.02**	*0.03*	**0.03**	*0.04*	**0.03**	*0.08*
liver	**0.10**	*0.20*	**0.15**	*0.20*	**0.10**	*0.12*
fat	**0.17**	*0.25*	**0.31**	*0.36*	**<LOQ**	*<LOQ*
skin with fat	**0.01**	*0.15*	**0.18**	*0.19*	**0.04**	*0.04*

[1)] plateau level [2)] taken during plateau phase [3)] Note, detailed data is given in section CA 6.4.1.4

Overall conclusion, BAS 750 F residues present in feed items are transferred to poultry commodities showing a linear dose-residue-dependency. Residues in eggs reach plateau level within 10 days indicating absence of accumulation for parent BAS 750 F, its metabolite M750F022 and 1,2,4-triazole. Residues in eggs and tissues decline rapidly after withdrawal of the dose.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

Description: BAS 750 F (TGAI)
Lot/Batch #: COD-001800
CAS Number: 40487-42-1
Purity: BAS 750 F: 99.61%
Development code: BAS 750 F
Spiking levels: 0.01-0.1 mg/kg

2. Test Animals: laying hens
Species: ISA brown
Gender: female
Age: approximately 22 weeks
Weight at dosing: 1.3 - 2.4 kg (at begin of study)
Number of animals: 69
Acclimation period: 21 days
Diet: commercially available non-medicated diet (Pondeuse Farine, SCAR scrl), *ad libitum*
Water: tap water, *ad libitum*
Housing: individually (in clean cages, holding area 0.75 m^2)

3. Environmental conditions:

Temperature: mean housing temperatures were 19-26 °C
(box A: 19-22°C, box B 20-24°C, box C: 19-23 °C, box D: 15-26°C, box E: 13-24°C)

Humidity: relative humidity was 18-68% (box A: 18-64%, box B: 16-64%, box C: 18-64%, box D: 22-68%, box E: 34-72%)

Air change: air conditioned (no further details)

Photoperiod: 16 h light / 8 h dark

B. STUDY DESIGN

1. Dosing regime:

Oral: Amount of dose: BAS 750 F doses were:
Group A (hens 01-09) 0 mg /kg feed, control
Group B (hens 10-21) 0.15 mg /kg feed
Group C (hens 22-33) 1.5 mg /kg feed
Group D (hens 34-45) 4.5 mg /kg feed
Group E (hens 46-57) 15.0 mg /kg feed
Group F (hens 58-69) 15.0 mg /kg feed
three subgroups for depuration with withdrawal of 0 days(F1), of 7 days (F2) and of 14 days (F3)

Food consumption: recorded daily

Vehicle: gelatine capsules (administered by hand, weekly preparation, storage at 2-8C until administration, accuracy and stability of dose confirmed by analysis)

Timing: once daily

Duration: 34 consecutive days

Withdrawal: 2, 7 or 14 days

Observations: daily (appearance and behavior)

2. Sample collection

Egg collection: twice daily (for each sub-group pooling of afternoon and morning eggs)
eggs for analysis sampled on days:
-1, 1, 3, 5, 7, 10, 14, 17, 21, 24, 28, 33
For depuration and on study day 35, 40 and 47 (depuration period control animals)
Study days: 35, 40, 47 (depuration period groups F1, F2, F3)

Interval from last dose to sacrifice:

<3 hours (subgroup A1, group B, group C)
<5 hours (group D, group E)
2 days (subgroup A2, subgroup F1)
7 days (subgroup F2)
14 days (subgroup A3, subgroup F3)

Samples collected and analyzed:
eggs, abdominal fat, liver, muscle, skin with fat

3. **Storage of samples:**
tissue samples were immediately taken after sacrifice, all tissue samples and eggs (eggs were pooled before as described above) were stored deep frozen until analysis

4. **Extraction and characterization-**
Analytical method & type:
BASF method L0272/01: BAS 750 F
BASF m ethod L0309/01: M750F022
BASF m ethod L0263/01: 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA), triazole lactic acid (TLA)

Analysis of BAS 750 F in eggs and tissues was carried out with BASF method L0272/01. The metabolite M750F022 was analysed with the BASF method L0309/01. The metabolites 1,2,4-T, TA, TAA and TLA were determined in eggs and tissues using the modified BASF method L0263/01.

4.1 Principle of the methods

BASF method L0272/01

The limit of quantification (LOQ) of the method is 0.01 mg/kg for all matrices.
Egg, liver and muscle: BAS 750 F was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned twice at alkaline conditions against cyclohexane. The final determination of BAS 750 F is performed by LC-MS/MS.
Fat and skin: BAS 750 F was extracted with a mixture of acetonitrile and iso-hexane. An aliquot of the extract was centrifuged and partitioned twice against iso-hexane. An aliquot of the acetonitrile phase is concentrated to dryness and re-dissolved in a mixture of methanol and water. The final determination of BAS 750 F is performed by LC-MS/MS.

BASF method L0309/01

The limit of quantification (LOQ) of the method is 0.01 mg/kg for all matrices.
Egg: M750F022 was extracted from protein-rich matrices with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned twice at alkaline conditions against dichloromethane. After evaporating to dryness and re-dissolving in methanol/water, a SPE cleanup was performed. Residues of the clean-up were re-dissolved in acetone and AP mix was added prior to analysis by GC-MS.

Muscle and liver: M750F022 was extracted with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned twice at alkaline conditions against cyclohexane. After evaporating the cyclohexane phase to dryness and re-dissolving in methanol/water the SPE clean-up was performed. Residues of the clean-up were re-dissolved in acetone and AP mix was added prior to analysis by GC-MS.
Fat and skin: M750F022 was extracted with a mixture of acetonitrile and iso-hexane. An aliquot of the acetonitrile phase was centrifuged and partitioned twice against iso-hexane. After evaporating an aliquot of the acetonitrile phase to dryness and re-dissolving in methanol/water the SPE clean-up was performed. Residues of the clean-up were re-dissolved in acetone and AP mix was added prior to analysis by GC-MS.

BASF method L0263/01

The limit of quantification (LOQ) of the method is 0.01 mg/kg for all matrices.
Egg, fat, skin, muscle, liver: The analytes were extracted from specimens with methanol/water (4/1, v/v). After filtration and clean-up, extracts were analysed with LC-MS/MS for 1,2,4-T, TA, TAA and TLA. For quantification, stable isotope labelled internal standards were used, which compensate for any matrix effects.

4.2 Recoveries

Average recoveries were in the required range of 70-100% with RSDs ≤ 20% for all analytes, BAS 750 F, M750F022 and TDM.

Concurrent recoveries obtained from control samples spiked with BAS 750 F, M750F022, and TDM (1,2,4-T, TA, TAA and TLA) were analysed to demonstrate the validity of the methods L0272/01, L0309/01 and L0263/01.

Table 6.4.1-2: Method L0272/01 and L0309/01 : accuracy and precision (mean procedural recoveries)

matrix	fortification level [mg/kg]	L0272/01 mean recovery BAS 750 F [%]	RSD [%] BAS 750 F	L0309/01 mean recovery M750F022 [%]	RSD [%] M750F022
egg	0.01 / 0.02 / 0.05 / 0.1	97.8	3.9 (n=30)	82.0	12 (n=54)
muscle	0.01 / 0.05 / 0.1	102	1.5 (n=5)	74.1	6.1 (n=4)
liver	0.01 / 0.05 / 0.1	93.7	4.4 (n=8)	79.1	5.7 (n=4)
fat	0.01 / 0.05 / 0.1	84.5	2.2(n=5)	89.7	6.4 (n=5)
skin with fat	0.01 / 0.05 / 0.1	84.7	9.0(n=7)	85.0	8.8 (n=5)

RSD = relative standard deviation,

Table 6.4.1-3: Method L0263/01: accuracy and precision (mean procedural recoveries)

matrix	fortification level [mg/kg]	mean recovery 1,2,4-T [%]	RSD [%] 1,2,4-T	mean recovery TA [%]	RSD [%] TA
egg	0.01 / 0.05 / 0.1	84.9	3.9 (n=36)	97.7	7.0 (n=33)
muscle	0.01 / 0.05 / 0.1	86.0	7.1 (n=3)	93.2	6.5 (n=3)
liver	0.01 / 0.05 / 0.1	81.7	6.0 (n=3)	86.0	6.2 (n=3)
fat	0.01 / 0.05 / 0.1	86.0	3.1(n=3)	92.0	12 (n=3)
skin with fat	0.01 / 0.05 / 0.1	92.0	9.7(n=3)	84.7	8.7 (n=3)
matrix	**fortification level [mg/kg]**	**mean recovery TAA [%]**	**RSD [%] TAA**	**mean recovery TLA [%]**	**RSD [%] TLA**
egg	0.01 / 0.05 / 0.1	91.8	6.1 (n=33)	91.2	5.8 (n=33)
muscle	0.01 / 0.05 / 0.1	84.3	10 (n=3)	108	2.1 (n=3)
liver	0.01 / 0.05 / 0.1	97.7	2.1 (n=3)	95.7	5.4 (n=3)
fat	0.01 / 0.05 / 0.1	98.0	5.4 (n=3)	97.7	3.9 (n=3)
skin with fat	0.01 / 0.05 / 0.1	99.3	7.3 (n=3)	93.7	4.3 (n=3)

RSD = relative standard deviation

II. RESULTS AND DISCUSSION

1. Dose level

The dietary burden for poultry was estimated based on preliminary information on residue levels available at the time of study start. Estimation were done according to current EFSA and OECD procedures (EFSA: Appendix G: Lundehn document 7031/VI/95 rev.4, July 1996, OECD: OECD Guidance document (ENV/JM/MONO(2013)8, vers Sept 04th, 2013) resulting in estimated residue intake for *EU Laying hen* (EFSA) of 0.33 mg/kg feed (0.021 mg/kg bw/d). Note the highest residue intake obtained with the OECD procedure was 8.11 mg/kg feed (0.56 mg/kg bw/d). In order to accommodate estimations obtained with both calculation procedures as well as increased residue intake as a result of future uses, the study was conducted with four dose levels ranging from below the EFSA estimated feed intake (0.15 mg/kg feed) including dose levels 1.5 and 4.5 mg/kg feed, up to 15 mg/kg feed. The actual dose levels achieved in the study expressed as mg/kg feed (dry matter) and mg/kg bodyweight/day are shown in Table 6.4.1-4.

Table 6.4.1-4: Dose levels (actual mean)

group	dose group	nominal dose *(mg/kg feed)*	actual concentration *(mg/kg dry feed)*	actual concentration (mg/kg bw/d) [1)]
1 (control)	A (0 X)	-	-	-
2	B (0.1X)	0.15	0.176	0.010
3	C (1 X)	1.5	1.743	0.096
4	D (3 X)	4.5	5.124	0.296
5	E (10 X)	15	17.247	0.984
6	F (10 X)	15	17.194	0.978

[1)] *the mean bodyweight from week -1 to 5 for each subgroup was used for calculation*

2. Animal health

All birds remained healthy for the duration of the study (confirmed by weekly veterinary inspection). Absence of any treatment related health effects was confirmed by daily observations.

3. Feed intake, bodyweight, egg production

Feed intake was determined daily (by comparing weight of evening feed given and of feed remaining the subsequent morning). The average feed consumption was 93-109 g dry matter per day (during dosing phase). Bodyweight was determined every fifth day (from study start until slaughtering). No significant treatment related effect on bodyweight was observed. The average number of eggs was 6-7 egg per hen. No significant treatment related effects on egg production were observed.

4. Residues

Residue level of parent BAS 750 F, metabolite M750F022 as well as the four metabolites of the TDM group, 1,2,4-triazole (1,2,4-T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were analysed in eggs (part 4.1) as well as tissues (part 4.2) namely muscle, liver, fat and skin with fat.

4. 1.Residues in eggs

The following tables provide a summary of the residues in eggs of

4.1.1 BAS 750 F
4.1.2 M750F022
4.1.3 TDM

Residue data is provided as the both the “group mean residue” as well as the “maximum individual residue” value (given in parenthesis) in Table 6.4.1-5 (BAS 750 F), Table 6.4.1-6 (M750F022), Table 6.4.1-7 (1,2,4-T), Table 6.4.1-8 (TA), Table 6.4.1-9 (TAA), and Table 6.4.1-10 (TLA). The data includes residue levels occurring prior to treatment (day-1), during the treatment phase (days 1-33) as well as during the subsequent depuration period (days 35, 40 and 47). For one sampling data representative of the plateau phase (i.e. eggs of day 24), residue data is provided separately for yolk and egg white.

4.1.1 BAS 750 F in egg

In the highest dose group (15 mg/kg feed) BAS 750 F residues above LOQ were found. The group mean reached a plateau level around 0.030 mg/kg (starting day 10). The highest group mean value was 0.035 mg/kg. The highest individual residue value was 0.042 mg/kg (day 14). In the samples of the depuration group (dose of 15 mg/kg feed) residues were below the LOQ after 7 days of depuration (after 2 days depuration the group mean was 0.023 mg/kg). Analysis of samples representative for the plateau phase (day 24) showed that residues are predominantly present in egg yolk (group mean 0.076 mg/kg, maximal individual 0.091 mg/kg) while residues in egg white were below the LOQ. In the dose groups D (4.5 mg/kg feed), C (1.5 mg/kg feed) and A (control 0 mg/kg feed) residues were below the LOQ. Consequently, dose group B (0.15 mg/kg feed) can be expected to show no residues above LOQ and therefore was not analysed.

Table 6.4.1-5: Residues of BAS 750 F in egg (including yolk and egg white)

study day	BAS 750 F : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	-	**<0.01**	**<0.01**	**0.01** *(0.011)*	**<0.01**
5	**<0.01**	-	**<0.01**	**<0.01**	**0.017** *(0.024)*	**0.015** *(0.019)*
7	**<0.01**	-	**<0.01**	**<0.01**	**0.028** *(0.038)*	**0.020** *(0.023)*
10	**<0.01**	-	**<0.01**	**<0.01**	**0.030** *(0.041)*	**0.026** *(0.028)*
14	**<0.01**	-	**<0.01**	**<0.01**	**0.035** *(0.042)*	**0.026** *(0.030)*
17	**<0.01**	-	**<0.01**	**<0.01**	**0.031** *(0.035)*	**0.024** *(0.029)*
21	**<0.01**	-	**<0.01**	**<0.01**	**0.024** *(0.030)*	**0.024** *(0.030)*
24 [1)]	-	-	**<0.01**	**<0.01**	-	**0.027** *(0.032)*
28	**<0.01**	-	**<0.01**	**<0.01**	**0.030** *(0.037)*	**0.028** *(0.029)*
33	**<0.01**	-	**<0.01**	**<0.01**	**0.030** *(0.036)*	**0.025** *(0.037)*
35	**<0.01**	-	-	-	-	**0.023** *(0.029)*
40	**<0.01**	-	-	-	-	**<0.01**
47	**<0.01**	-	-	-	-	**<0.01**
mean (day 14-33)	**<0.01**	-	**<0.01**	**<0.01**	**0.03**	**0.03**
yolk (day 24)	**<0.01**	-	-	-	**0.076** *(0.091)*	-
white (day 24)	**<0.01**	-	-	-	**<0.01**	-

[1)] on day 24, eggs from group A and E were separated into egg white and yolk
<0.01 denotes less than the LOQ. For calculation of mean < 0.01 was set to 0.01 mg/kg.

4.1.2 M750F022 in egg

In the highest dose group (15 mg/kg feed) residues above LOQ were found. The group mean residue reached a plateau level around 0.066 mg/kg (starting day 10). The highest group mean value was 0.083 mg/kg. The highest individual residue value was 0.094 mg/kg (day 14).

Table 6.4.1-6: Residues of M750F022 in egg

study day	M750F022 : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.011** *(0.012)*	**<0.01**
5	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.022** *(0.032)*	**0.012** *(0.013)*
7	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.026** *(0.035)*	**0.038** *(0.047)*
10	**<0.01**	**<0.01**	**<0.01**	**0.012** *(0.013)*	**0.046** *(0.059)*	**0.047** *(0.051)*
14	**<0.01**	**<0.01**	**<0.01**	**0.015** *(0.015)*	**0.079** *(0.094)*	**0.062** *(0.070)*
17	**<0.01**	**<0.01**	**<0.01**	**0.015** *(0.016)*	**0.061** *(0.064)*	**0.054** *(0.062)*
21	**<0.01**	**<0.01**	**<0.01**	**0.019** *(0.020)*	**0.059** *(0.063)*	**0.051** *(0.054)*
24 [1)]	-	**<0.01**	**<0.01**	**<0.01**	-	**0.060** *(0.066)*
28	**<0.01**	**<0.01**	**<0.01**	**0.012** *(0.012)*	**0.056** *(0.063)*	**0.055** *(0.065)*
33	**<0.01**	**<0.01**	**<0.01**	**0.016** *(0.016)*	**0.071** *(0.076)*	**0.059** *(0.076)*
35	**<0.01**	-	-	-	-	**0.064** *(0.069)*
40	**<0.01**	-	-	-	-	**0.014** *(0.014)*
47	**<0.01**	-	-	-	-	**<0.01**
mean (day 14-33)	**<0.01**	**<0.01**	**<0.01**	**0.015**	**0.066**	**0.075**
yolk (day 24)	**<0.01**	-	-	-	**0.017** *(0.021)*	-
white (day 24)	**<0.01**	-	-	-	**<0.01**	-

[1)] On day 24, eggs from group A and E were separated into egg white and yolk. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg

In the samples of the depuration group (dose of 15 mg/kg feed) residues were below the LOQ after 14 days of depuration (having declined from 0.064 mg/kg and 0.014 mg/kg after 2 and 7 days of depuration). Analysis of samples representative for the plateau phase (day 24) showed that residues are predominantly present in egg yolk (group mean 0.017 mg/kg, maximal individual 0.021 mg/kg), while residues in egg white were below the LOQ.
In the dose group D (4.5 mg/kg feed), residue levels were below or close to LOQ, only transiently exceeding the LOQ (during day 10 to day 21 group mean was 0.012 – 0.19 mg/kg). For plateau phase, the difference between the residue level (group E: 0.066 and group D: 0.015 mg/kg) corresponds to the difference in dose level (group E: 15 mg/kg feed and group D: 4.5 mg/kg feed) indicating a linear dose-dependency. In dose groups C (1.5 mg/kg feed), B (0.15 mg/kg feed) and A (control 0 mg/kg feed) residues were < LOQ.

4.1.3 TDM in egg

In eggs, 1,2,4-triazole was found at levels above LOQ, while residues of TA, TAA and TLA were generally below the LOQ (except for one treated sample: separately analysed yolk of the highest dose group, day 24, see below).
In whole eggs, detectable residues of 1,2,4-triazole were seen in the highest dose group (15 mg/kg feed) while for TA, TAA and TLA residues were <LOQ. The group mean residue reached a plateau level around 0.080 mg/kg (starting day 5). The highest group mean value was 0.088 mg/kg (day 14). The highest individual residue value was 0.099 mg/kg (day 7). In the samples of the depuration group (dose of 15 mg/kg feed) residues were below the LOQ no later than 7 days of depuration.

Analysis of a samples representative of the plateau phase (day 24) showed that 1,2,4-triazole residues are distributed between egg yolk (group mean residue 0.047 mg/kg, maximal individual residue 0.050 mg/kg) and egg white (group mean residue 0.083 mg/kg, highest individual residue 0.090 mg/kg). Egg yolk did contain TA level of 0.021 mg/kg (group average) which is considered largely treatment-unrelated as for the corresponding control sample with 0.016 mg/kg similar TA level were determined (comparable observation for tissues, see chapter 4.2.3 below). Residues of TA and TLA in egg yolk were below the LOQ. In egg white, for all three analytes, TA, TAA and TLA, residue level were below LOQ.

In the dose group D (4.5 mg/kg feed), 1,2,4-triazole residue levels were present at detectable amounts reaching a plateau around 0.022 mg/kg at day 5. The difference between the residues (group mean E: 0.080 mg/kg and D: 0.022 mg/kg) corresponds to the difference in dose level (E: 15 mg/kg feed and D: 4.5 mg/kg feed) indicating a linear dose-dependency. In dose groups C (1.5 mg/kg feed), 1,2,4-triazole levels were near or below the LOQ (highest individual 0.012 mg/kg at day 33). In dose groups B (0.15 mg/kg feed) and A (control 0 mg/kg feed) residues were below the LOQ.

In the dose groups D (4.5 mg/kg feed), C (1.5 mg/kg feed) and A (control 0 mg/kg feed) residues of TA, TAA and TLA were below the LOQ. Consequently, dose group B (0.15 mg/kg) can be expected to show no residues above LOQ and therefore was not analysed.

Table 6.4.1-7: Residues of 1,2,4-T in eggs

study day	TDM : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	**<0.01**	**<0.01**	**0.015** *(0.017)*	**0.059** *(0.063)*	**0.056** *(0.063)*
5	**<0.01**	**<0.01**	**0.01** *(0.01)*	**0.022** *(0.024)*	**0.081** *(0.089)*	**0.069** *(0.072)*
7	**<0.01**	**<0.01**	**0.01** *(0.011)*	**0.022** *(0.026)*	**0.086** *(0.099)*	**0.068** *(0.072)*
10	**<0.01**	**<0.01**	**<0.01**	**0.022** *(0.025)*	**0.081** *(0.090)*	**0.080** *(0.083)*
14	**<0.01**	**<0.01**	**<0.01**	**0.023** *(0.028)*	**0.088** *(0.090)*	**0.083** *(0.094)*
17	**<0.01**	**<0.01**	**<0.01**	**0.021** *(0.025)*	**0.084** *(0.091)*	**0.073** *(0.080)*
21	**<0.01**	**<0.01**	**<0.01**	**0.022** *(0.027)*	**0.076** *(0.082)*	**0.066** *(0.068)*
24[1)]	-	**<0.01**	**<0.01**	*0.022* *(0.025)*	-	**0.065** *(0.072)*
28	**<0.01**	**<0.01**	**<0.01**	**0.021** *(0.025)*	**0.075** *(0.086)*	**0.071** *(0.074)*
33	**<0.01**	**<0.01**	**0.011** *(0.012)*	**0.021** *(0.024)*	**0.078** *(0.083)*	**0.069** *(0.072)*
35	**<0.01**	-	**<0.01** *(0.011)*	-	-	**0.066** *(0.068)*
40	**<0.01**	-	-	-	-	**<0.01**
47	**<0.01**	-	-	-	-	**<0.01**
mean (day 14-33)	**<0.01**	**<0.01**	**<0.01**	**0.022**	**0.080**	**0.071**
yolk (day 24)[1)]	**<0.01**	-	-	-	**0.047** *(0.050)*	-
white (day 24)[1)]	**<0.01**	-	-	-	**0.083** *(0.090)*	-

[1)] On day 24, eggs from group A and E were separated into egg white and yolk. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of the mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

Table 6.4.1-8: Residues of TA in eggs

study day	TDM : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
5	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
7	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
10	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
14	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
17	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
21	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
24[1)]	-	-	**<0.01**	**<0.01**	-	**<0.01**
28	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
33	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
35	**<0.01**	-	-	-	-	**<0.01**
40	**<0.01**	-	-	-	-	**<0.01**
47	**<0.01**	-	-	-	-	**<0.01**
yolk (day 24)[1)]	**0.016** *(0.019)*	-	-	-	**0.021** *(0.025)*	-
white (day 24)[1)]	**<0.01**	-	-	-	**<0.01**	-

[1)] On day 24, eggs from group A and E were separated into egg white and yolk. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

Table 6.4.1-9: Residues of TAA in eggs

study day	TDM : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
5	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
7	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
10	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
14	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
17	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
21	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
24 [1)]	-	-	**<0.01**	**<0.01**	-	**<0.01**
28	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
33	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
35	**<0.01**	-	-	-	-	**<0.01**
40	**<0.01**	-	-	-	-	**<0.01**
47	**<0.01**	-	-	-	-	**<0.01**
yolk (day 24)[1)]	**<0.01**	-	-	-	**<0.01**	-
white (day 24)[1)]	**<0.01**	-	-	-	**<0.01**	-

[1)] On day 24, eggs from group A and E were separated into egg white and yolk. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

Table 6.4.1-10: Residues of TLA in eggs

study day	TDM : group mean residue (highest individual) in mg/kg					
	Group A *(0 mg/kg)*	**Group B** *(0.15 mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F** *(15 mg/kg)*
-1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
1	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
3	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
5	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
7	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
10	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
14	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
17	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
21	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
24 [1)]	-	-	**<0.01**	**<0.01**	-	**<0.01**
28	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
33	**<0.01**	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**
35	**<0.01**	-	-	-	-	**<0.01**
40	**<0.01**	-	-	-	-	**<0.01**
47	**<0.01**	-	-	-	-	**<0.01**
yolk (day 24)[1)]	**<0.01**	-	-	-	**<0.01**	-
white (day 24)[1)]	**<0.01**	-	-	-	**<0.01**	-

[1)] On day 24, eggs from group A and E were separated into egg white and yolk. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg

4.2 Residues in tissues (muscle, liver, fat, skin with fat)

The following tables provide a summary of the residues in tissues of

4.2.1 BAS 750 F
4.2.2 M750F022
4.2.3 TDM.

Residue data is provided as both the group mean as well as the maximum individual residue value (given in parenthesis) in Table 6.4.1-11 (BAS 750 F), Table 6.4.1-12 (M750F022) and Table 6.4.1-13 (1,2,4-triazole), Table 6.4.1-14 (TA), Table 6.4.1-15 (TAA), and Table 6.4.1-16 (TLA). The data includes residue levels occurring prior to treatment (day -1), during the treatment phase (days 1-33) as well as during the subsequent depuration period (day 35, 40 and 47).

4.2.1 BAS 750 F in tissue

For the highest dose group (15 mg/kg feed), the group mean residues were in muscle at 0.016 mg/kg (highest individual 0.027 mg/kg), in liver at 0.097 (highest individual 0.20 mg/kg), in fat at 0.17 (highest individual 0.25 mg/kg), in skin with fat 0.10 mg/kg (highest individual 0.15 mg/kg). In the samples of the depuration group (dose of 15 mg/kg feed) residues in tissues were below the LOQ latest 2 days after start of depuration.
Residues in other dose groups were lower, if present at quantifiable levels, group mean levels did indicate a linear dose-dependency, namely for liver (groups E-D-C), in fat (groups E-D) and for skin with fat (groups E-D).

Table 6.4.1-11: Residues of BAS 750 F in tissues

tissue	BAS 750 F : group mean residue (highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.016** *(0.027)*	**<0.01**	**<0.01**	**<0.01**
liver	**<0.01**	**<0.01**	**0.013** *(0.017)*	**0.015** *(0.021)*	**0.097** *(0.20)*	**<0.01**	**<0.01**	**<0.01**
fat	**<0.01**	**<0.01**	**<0.01**	**0.022** *(0.025)*	**0.17** *(0.25)*	**<0.01**	**<0.01**	**<0.01**
skin with fat	**<0.01**	**<0.01**	**<0.01**	**0.011** *(0.011)*	**0.10** *(0.15)*	**<0.01**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal, Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg

4.2.2 M750F022 in tissue

For the highest dose group (15 mg/kg feed), the group mean residues were in muscle at 0.033 mg/kg (highest individual 0.037 mg/kg), in liver at 0.15 (highest individual 0.20 mg/kg), in fat at 0.31 (highest individual 0.36 mg/kg), in skin with fat 0.18 mg/kg (highest individual 0.19 mg/kg).
In the samples of the depuration group (dose of 15 mg/kg) residues were below the LOQ in muscle at least 2 days after start of depuration, in liver and skin with fat at least after 7 days, in fat at least after 14 days.
Residues in other dose groups were lower. In muscle, residues were below the LOQ. Levels in liver indicate a linear dose-dependency, namely for liver (groups E-D-C), for fat (groups E-D-C) and for skin with fat (groups E-D-C-B).

Table 6.4.1-12: Residues of M750F022 in tissues

tissue	M750F022 : group mean residues (and highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.033** *(0.037)*	**<0.01**	**<0.01**	**<0.01**
liver	**<0.01**	**<0.01**	**0.018** *(0.019)*	**0.028** *(0.033)*	**0.15** *(0.20)*	**0.015**	**<0.01**	**<0.01**
fat	**<0.01**	**<0.01**	**0.033** *(0.044)*	**0.069** *(0.071)*	**0.31** *(0.36)*	**0.061**	**0.013** *(0.013)*	**<0.01**
skin with fat	**<0.01**	**0.012** *(0.015)*	**0.017** *(0.021)*	**0.037** *(0.041)*	**0.18** *(0.19)*	**0.037**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal, Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg

4.2.3 TDM in tissue

In tissues, TDM were present above the LOQ, with 1,2,4-triazole as the predominant component, while residues of TA, TAA, TLA generally were below LOQ. Residues of TA in muscle (around 0.020 mg/kg) and liver (0.024 mg/kg) can be considered treatment-unrelated as they were similar in all dose groups including the untreated control group (comparable observation for egg yolk, see above).

For the highest dose group (15 mg/kg feed), the group mean residues of 1,2,4-triazole were in muscle at 0.10 mg/kg (highest individual 0.11 mg/kg), in liver at 0.099 (highest individual 0.12 mg/kg), in fat below LOQ, in skin with fat 0.039 mg/kg (highest individual 0.044 mg/kg).

In the samples of the depuration group (dose of 15 mg/kg feed) residues were below the LOQ in muscle and liver at latest after 7 days of depuration.

Residues in other dose groups were lower. Levels in muscle indicate a linear dose-dependency (group E: 0.10 mg/kg, group D: 0.030 mg/kg, group C: 0.012 mg/kg) as do the levels in liver (group E: 0.099 mg/kg, group D: 0.027 mg/kg, group C: 0.012 mg/kg) and skin with fat (group E: 0.039 mg/kg, group D: 0.012 mg/kg).

Table 6.4.1-13: Residues of 1,2,4-triazole in tissues

tissue	1,2,4-T : group mean residue (highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**<0.01**	**<0.01**	**0.012** *(0.014)*	**0.030** *(0.035)*	**0.10** *(0.110)*	**0.024**	**<0.01**	**<0.01**
liver	**<0.01**	**<0.01**	**0.012** *(0.015)*	**0.027** *(0.029)*	**0.099** *(0.12)*	**0.023**	**<0.01**	**<0.01**
fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
skin with fat	**<0.01**	**<0.01**	**<0.01**	**0.012** *(0.015)*	**0.039** *(0.044)*	**<0.01**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

Table 6.4.1-14: Residues of TA in tissues

tissue	TA : group mean residue (highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**0.019** *(0.014)*	**0.017** *(0.019)*	**0.017** *(0.018)*	**0.017** *(0.018)*	**0.018** *(0.020)*	**0.020**	**0.014**	**0.017**
liver	**0.023** *(0.024)*	**0.023** *(0.026)*	**0.021** *(0.028)*	**0.025** *(0.029)*	**0.024** *(0.030)*	**0.028**	**0.024**	**0.024**
fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
skin with fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

Table 6.4.1-15: Residues of TAA in tissues

tissue	TA : group mean residues (highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
liver	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
skin with fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg

Table 6.4.1-16: Residues of TLA in tissues

tissue	TA : group mean residues (highest individual) in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(0.15mg/kg)*	**Group C** *(1.5 mg/kg)*	**Group D** *(4.5 mg/kg)*	**Group E** *(15 mg/kg)*	**Group F**[1] *(15 mg/kg)*	**Group F**[2] *(15 mg/kg)*	**Group F**[3] *(15 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
liver	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
skin with fat	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

[1] 2 days withdrawal; [2] 7 days withdrawal; [3] 14 days withdrawal. Residues below the LOQ are denoted by <0.01 mg/kg. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg.

CONCLUSION

The residue data obtained indicates generally a linear relationship between feed intake (BAS 750 F dose level) and the residue level in animal commodities for the analytes parent BAS 750 F, M750F022 and 1,2,4-triazole with absence of BAS 750 F-specific residues in untreated controls (BAS 750 F, M750F022). TA, if detected, was apparently treatment-unrelated, TAA and TLA were not found in level above LOQ.

Residue levels in egg did reach a plateau level within 10 days (BAS 750 F, M750F022) and within 5 days (TDM). The plateau levels found for the highest dose group (given as group mean, highest individual given in parenthesis) were for BAS 750 F at 0.030 mg/kg (0.042 mg/kg), for M750F022 at 0.066 mg/kg (0.095 mg/kg), and for 1,2,4-triazole at 0.080 mg/kg (0.099 mg/kg). Upon withdrawal residues did decline rapidly to levels below the LOQ demonstrating that residues do not accumulate in egg (BAS 750 F latest after 7 days, M750F022 latest after 14 days, 1,2,4-triazole latest after 7 days).

In egg of the highest dose group, residues were localized in yolk for BAS 750 F (0.076 mg/kg) and M750F022 (0.017 mg/kg) while for 1,2,4-trizole a higher level was seen in egg white (0.083 mg/kg) compared with egg yolk (0.047 mg/kg).

Residue levels in tissues were higher for BAS 750 F in fat and liver than in muscle and skin with fat. For the highest dose group, group mean level (highest individual in parenthesis) in fat were 0.17 mg/kg (0.25 mg/kg), in liver 0.10 mg/kg (0.20 mg/kg), in muscle 0.016 mg/kg (0.027 mg/kg) and in skin with fat 0.10 mg/kg (0.15 mg/kg).

A similar tissue distribution was found for M750F022 with slightly higher residue levels compared to parent. For the highest dose group, group mean level (highest individual in parenthesis) in fat were 0.31 mg/kg (0.36 mg/kg), in liver 0.15 mg/kg (0.20 mg/kg), in muscle 0.03 mg/kg (0.04 mg/kg) and in skin with fat 0.18 mg/kg (0.19 mg/kg). Generally, roughly similar level were found for parent BAS 750 F and its metabolite M750F022 (except for the matrix skin with fat).

A different tissue distribution was found for 1,2,4-triazole with residue level in liver, muscle and skin with fat exceeding the level in fat. For the highest dose group, group mean level (highest individual) in liver were 0.10 mg/kg (0.12 mg/kg), in muscle 0.10 mg/kg (0.11 mg/kg), in skin with fat 0.039 mg/kg (0.044 mg/kg), while in fat below the LOQ of 0.01 mg/kg.

Upon withdrawal residues in tissues did rapidly decline to levels below the LOQ demonstrating absence of accumulation for BAS 750 F (in muscle, liver, and fat latest after 2 days of depuration), for M750F022 (in muscle, latest after 2 days, in liver, latest after 7 days, in fat, latest after 14 days) and for 1,2,4-triazole (in muscle and liver, latest after 7 days, in fat latest after 2 days).

The relative amounts of BAS 750 F and its metabolites (M750F022 and TDM) are seen most clearly in the highest dose group (see Table 6.4.1-17) comparable relative amounts are seen in the lower dose groups as a result of the observed linear dose-dependency for all three analytes. However, these residue levels are obtained with highly overdosed feed residue intake, with a realistic feed burden, residues expected in practice are much lower (see section CA 6.7).

Table 6.4.1-17: Overview tissue distribution in dose group E (highly overdosed)[3)]

	BAS 750 F		**M750F022**		**1,2,4-T**	
dose group E (15 mg/kg)	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*
egg [1)]	**0.03**	*0.04*	**0.06**	*0.10*	**0.08**	*0.10*
yolk [2)]	**0.08**	*0.09*	**0.02**	*0.02*	**0.05**	*0.05*
white [2)]	**<LOQ**	*<LOQ*	**<LOQ**	*<LOQ*	**0.08**	*0.09*
muscle	**0.02**	*0.03*	**0.03**	*0.04*	**0.10**	*0.11*
liver	**0.10**	*0.20*	**0.15**	*0.20*	**0.10**	*0.12*
fat	**0.17**	*0.25*	**0.31**	*0.36*	**<LOQ**	*<LOQ*
skin with fat	**0.01**	*0.15*	**0.18**	*0.19*	**0.04**	*0.04*

[1)] plateau level [2)] taken during plateau phase [3)] Note, detailed data is given in section CA 6.4.1.4

Overall conclusion, BAS 750 F residues present in feed items are transferred to poultry commodities showing a linear dose-residue-dependency. Residues in eggs reach plateau level within 10 days indicating absence of accumulation for parent BAS 750 F, its metabolite M750F022 and 1,2,4-triazole. Residues in eggs and tissues decline rapidly within a few days after withdrawal of the dose.

CA 6.4.2 Ruminants

Report: CA 6.4.2/1
[redacted] 2015 a
Magnitude of residues in milk and tissues of dairy cows following multiple oral administration of BAS 750 F
2015/1107649

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), EPA 860.1340, EPA 860.1480, OECD 505 (Jan. 2007), OECD Guidance Document on Pesticide Analytical Methods (13 August 2007), OECD Guidance Document on Residue in Livestock (10 July 2013)

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

EXECUTIVE SUMMARY

A residue transfer study with BAS 750 F was conducted in lactating cows with nominal doses of 1.5 mg/kg, 7.5 mg/kg, 50 mg/kg and 150 mg/kg feed. Actual doses were 1.6 mg/kg, 7.5 mg/kg, 49.0 mg/kg and 141.3 mg/kg feed. This corresponds to doses of 0.04 mg/kg bw/d, 0.19 mg/kg bw/d, 1.04 mg/kg bw/d, and 3.74 mg/kg bw/d. Doses were applied for a period of 28 days followed by a depuration period of 3, 7 or 14 days. Animals of the control group received empty capsules only. Residues of parent BAS 750 F, M750F022 as well as the TDM group (triazole derivative metabolites), 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were analysed in samples of milk (including one representative example separated into cream and skim milk) as well as tissues (muscle, liver, kidney, different fat types).

Generally, the residue data obtained indicates a linear relationship between feed intake (BAS 750 F dose level) and the residue level in animal commodities for each of the analytes, parent BAS 750 F, and M750F022 as well as 1,2,4-triazole with absence of BAS 750 F-specific residues in untreated controls (BAS 750 F, M750F022). Concerning the TDMs, background levels above the LOQ were found, notably for 1,2,4-triazole and TA, occasionally for TAA, while for TLA no residues >LOQ were found.

Residues in milk did reach a plateau level within 3 days for both BAS 750 F and 1,2,4-triazole. The plateau levels found for the highest dose group (given as group mean, highest individual in parenthesis) were for BAS 750 F at 0.21 mg/kg (0.35 mg/kg) and for 1,2,4-triazole at 0.27 mg/kg (0.33 mg/kg). M750F022 measured at one sampling date representative of the plateau phase of the highest dose (group F, day 21) was quantified at 0.01 mg/kg. Upon withdrawal residues did decline rapidly, latest after 4 days of depuration to levels <LOQ (BAS 750 F) or to background levels (1,2,4-triazole) demonstrating that residues do not accumulate in milk.

In milk of the highest dose group, residues were localized mainly in the cream fraction for BAS 750 F (1.23 mg/kg) and M750F022 (0.06 mg/kg) while for 1,2,4-triazole residues were slightly higher in skim milk (0.24 mg/kg) than for cream (0.23 mg/kg).

Generally, much higher level were found for parent BAS 750 F than for its metabolite M750F022.

Residues in tissues were higher for BAS 750 F in liver compared to kidney and fat and much lower levels in muscle. For the highest dose group (150 mg/kg feed), group mean level (highest individual) in liver were 3.03 mg/kg (3.58 mg/kg), in kidney 1.29 mg/kg (1.88 mg/kg), in different fat types 0.60-1.71 mg/kg (1.20-2.29 mg/kg) while in muscle only 0.16 mg/kg (0.22 mg/kg).

For M750F022 residues were higher in fat compared to liver, kidney and muscle. For the highest dose group (150 mg/kg feed), group mean level (highest individual) in different fat types were 0.07 - 0.16 mg/kg (0.13 -0.21 mg/kg), in liver and kidney 0.04 mg/kg (0.04 mg/kg), while in muscle only 0.02 mg/kg (0.02 mg/kg). Generally, much higher level were found for parent BAS 750 F than for its metabolite M750F022.

For 1,2,4-triazole, residues were higher in muscle, liver, kidney compared to different fat types. For the highest dose group (150 mg/kg feed), group mean level (highest individual) in muscle were 0.28 mg/kg (0.33 mg/kg), in kidney 0.28 mg/kg (0.39 mg/kg), in liver 0.26 mg/kg (0.30 mg/kg) while in different fat types level were 0.11 - 0.17 mg/kg (0.19 -0.28 mg/kg).

Upon withdrawal residues did rapidly declined to levels below or near the LOQ demonstrating absence of accumulation for BAS 750 F (latest after 7 days for muscle, kidney, latest after 14 days for liver, perirenal/mesenterial fat, with 0.02 mg/kg after 14 days for subcutaneous fat) as well as for M750F022 (latest after 3 days for muscle, kidney, latest after 7 days for liver, latest after 14 days for perirenal/mesenterial fat). In subcutaneous fat residues also declined rapidly, with levels near the LOQ for both BAS 750 F and M750F022 (0.023 mg/kg) after 14 days of withdrawal.

To provide a general overview over the relative amounts of the three analytes parent BAS 750 F, metabolite M750F022 and 1,2,4-triazole, residue level determined at the highest dose (150 mg/kg feed) are summarized in the following table.

Table 6.4.2-1: Overview tissue distribution for BAS 750 F, M750F022 and 1,2,4-triazole in highly overdosed 100x dose group (150 mg/kg feed)[3)]

	BAS 750 F		M750F022		1,2,4-T	
dose group E (150 mg/kg feed)	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*
milk [1)]	**0.21**	*0.35*	**0.02**	*0.02*	**0.29**	*0.33*
cream [2)]	**1.23**	*1.95*	**0.06**	*0.07*	**0.23**	*0.29*
skim milk [2)]	**0.07**	*0.10*	**<LOQ**	*<LOQ*	**0.24**	*0.31*
muscle	**0.16**	*0.22*	**0.02**	*0.02*	**0.28**	*0.33*
liver	**3.03**	*3.58*	**0.04**	*0.04*	**0.26**	*0.30*
kidney	**1.29**	*1.88*	**0.04**	*0.04*	**0.28**	*0.39*
fat perirenal	**1.71**	*2.29*	**0.16**	*0.21*	**0.11**	*0.19*
fat mesenterial	**1.16**	*1.87*	**0.13**	*0.20*	**0.11**	*0.25*
fat subcutaneaous	**0.59**	*1.20*	**0.07**	*0.13*	**0.17**	*0.28*

[1)] plateau level [2)] taken during plateau phase [3)] Note, detailed data is given in section CA6.4.2.4.

In conclusion, BAS 750 F residues present in feed items are transferred to ruminant commodities showing a linear dose-residue-dependency. Residues in milk reach plateau level indicating absence of accumulation for parent BAS 750 F, its metabolite M750F022 and 1,2,4-triazole. Residues in milk and tissues decline rapidly after dose withdrawal.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:

Description:	BAS 750 F (TGAI)
Lot/Batch #:	COD-001800
CAS Number:	40487-42-1
Purity:	BAS 750 F: 99.61%
Development code:	BAS 750 F
Spiking levels:	BAS 750 F: 0.01 - 3.6 mg/kg M750F022, 1,2,4-T, TA, TAA, TLA: 0.01 - 0.1 mg/kg

2. Test Animals: lactating dairy cow

Species:	Holstein/Friesian/Ayrshire cross
Gender:	female
Age:	2 - 14 years
Weight at dosing:	508 - 758 kg (at begin of study, day-1)
Number of animals:	18
Acclimation period:	7 days
Diet:	2 x 4 kg non-medicated protein concentrate (Forage Link Reaction 18 Nuts, Carrs Billington), good quality hay, *ad libitum*
Water:	tap water, *ad libitum*
Housing:	group housed in 6 pens each containing 3 animals, straw bedding

Environmental conditions:

Temperature:	5-30°C (acclimation period: 6-26°C, dosing period: 5-30°C, depuration period: 9-27°C)
Humidity:	relative humidity was 22-98% (acclimation period: 33-95%, dosing period: 22-90%, depuration period: 26-98%)
Air change:	natural ventilation
Photoperiod:	natural light conditions

B. STUDY DESIGN

1. Dosing regime-

Oral: Amount of dose: BAS 750 F doses were
Group A (cows 1, 2, 3): 0 mg /kg feed
Group B (cows 4, 5, 6): 1.5 mg /kg feed
Group C (cows 7, 8, 9): 7.5 mg /kg feed
Group D (cows 10, 11, 12): 50 mg /kg feed
Group E (cows 13, 14, 15): 150 mg /kg feed
Group F (cows 16, 17, 18): 150 mg /kg feed
three subgroups for depuration with withdrawal of 3 days (F1), of 7 days (F2) and of 14 days (F3)

Food consumption: recorded daily
Vehicle: gelatine capsules (administered via balling gun)
Timing: once daily, after morning milking
Duration: 28 consecutive days
Withdrawal: 3, 7 or 14 days
Observations: twice daily (appearance and behavior)

2. Sample collection

Milk collection: twice daily; afternoon and morning milk pooled per cow
milk was taken on the following study days:
-1, 1, 3, 5, 7, 10, 14, 17, 21, 24, 28 as well as 29-41 (days of depuration: 1-13)
separation into skimmed milk and cream on study day 21

Interval from last dose to sacrifice:
< 24 hours: cows 2-15
3 days: cow 16
7 days: cow 18
14 days: cow 17, cow 1 (companion animal of cow 17)

Samples collected and analyzed:
muscle (composite of loin and hind-leg muscle),
fat (perirenal, mesenterial, subcutaneous)
liver (gall bladder removed, distal portions of each lobe),
kidney (both organs entirely)

3. Storage of samples: tissue samples were taken immediately after sacrifice, stored frozen at -20° C

4. Extraction and characterization-
Analytical method & type:

BASF Method L0272/01: BAS 750 F
L0309/01: M750F022
Method 01132: TDM

4.1 Principle of the methods

BASF method L0272/01
The limit of quantitation (LOQ) of the method is 0.01 mg/kg for milk, cream, skim milk, muscle, liver, kidney, and fat. BAS 750 F was extracted from protein-rich matrices with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract was centrifuged and partitioned at alkaline conditions against cyclohexane, prior to analysis by LC-MS/MS. BAS 750 F was extracted from fat-rich matrices with a mixture of acetonitrile and iso-hexane. An aliquot of the extract was centrifuged and partitioned against iso-hexane, prior to analysis by LC-MS/MS.

BASF method L0309/01
The limit of quantitation (LOQ) of the method is 0.01 mg/kg for milk, cream, skim milk, muscle, liver, kidney, and fat. M750F022 was extracted from protein-rich matrices with a mixture of methanol, water and hydrochloric acid. An aliquot of the extract is dried and dissolved in a methanol/water mixture, then subjected to clean-up step (SPE) prior to analysis by GC-MS. M750F022 was extracted from fat-rich matrices with a mixture of acetonitrile and iso-hexane. An aliquot of the extract was centrifuged and partitioned against iso-hexane, an aliquot of the acetonitrile phase is dried and dissolved in a methanol water mixture, subjected to clean-up step (SPE) prior to analysis by GC-MS.

BASF method 01132
The limit of quantitation (LOQ) of the method is 0.01 mg/kg for milk, cream, skim milk, muscle, liver, kidney, and fat. Triazole derivative metabolites (TDM) were extracted with a mixture of methanol/water, followed by filtration and clean-up (SPE) before analysis by LC-MS/MS. For determination of 1,2,4-triazole, an aliquot of the final extract was spiked with a stable isotopically labelled analyte as internal standard, then subjected to derivatization with dansyl chloride, passed through a cartridge, evaporated to dryness and re-dissolved in acetone and water. After filtration, extracts were analysed with LC-MS/MS for dansyl-1,2,4-triazole.

4.2 Recoveries

Concurrent recoveries obtained from control samples spiked with BAS 750 F and M750F022 were analyzed to demonstrate the validity of the method [see Table 6.4.2-2].

Table 6.4.2-2: L0272/01 and L0309/01: accuracy and precision (mean procedural recoveries)

matrix	fortification level *[mg/kg]*	BAS 750 F		M750F022	
		mean recovery *[%]*	**RSD** *[%]*	**mean recovery** *[%]*	**RSD** *[%]*
milk	0.01	86.9	11.3 (n=29)	105	8.5 (n=5)
	0.10	88.9	6.0 (n=29)	93.3	3.8 (n=5)
	overall	**88.0**	**8.7 (n=63)**	**99.1**	**8.9 (n=10)**
cream	0.01	86.3	7.5 (n=13)	102	4.9 (n=5)
	0.10	90.9	3.0 (n=13)	88.2	12.1 (n=8)
	0.50	89.5	n.a. (n=1)	n.a.	n.a.
	overall	**88.6**	**6.0 (n=27)**	**95.0**	**11.3 (n=16)**
skim milk	0.01	89.7	5.9 (n=10)	*n.a.*	*n.a.*
	0.10	91.5	2.3 (n=10)		
	overall	**90.6**	**4.4 (n=20)**		
muscle	0.01	80.1	9.9 (n=5)	75.4	7.3 (n=5)
	0.10	81.0	2.6 (n=5)	74.0	3.3 (n=5)
	overall	**80.5**	**6.8 (n=10)**	**74.7**	**5.5 (n=10)**
fat	0.01	79.8	8.4 (n=23)	101.0	17.3 (n=14)
	0.10	80.3	9.0 (n=23)	83.9	12.3 (n=14)
	0.30	73.9	n.a. (n=1)	n.a.	n.a.
	2.5	70.0	n.a. (n=1)	n.a.	n.a.
	overall	**80.2**	**8.2 (n=48)**	**92.2**	**18.1 (n=28)**
liver	0.01	82.4	10.8 (n=13)	96.1	12.5 (n=8)
	0.10	78.3	3.0 (n=13)	73.2	5.2 (n=8)
	0.60	78.3	n.a. (n=1)	n.a.	n.a.
	3.60	72.9	n.a. (n=1)	n.a.	n.a.
	overall	**80.0**	**8.3 (n=28)**	**84.7**	**17.6 (n=16)**
kidney	0.01	90.2	10.8 (n=13)	83.7	7.0 (n=6)
	0.10	82.6	7.0 (n=13)	76.6	4.5 (n=6)
	1.90	78.5	n.a. (n=1)	*n.a.*	*n.a.*
	overall	**86.1**	**10.1 (n=27)**	**80.1**	**7.4 (n=12)**

RSD = relative standard deviation, n.a. = not analysed

Average recoveries were in the required range of 70-100% with RSD being ≤ 20% for all analytes, BAS 750 F, M750F022.

Concurrent recoveries obtained from control samples spiked with TDMs were analyzed to demonstrate the validity of the method [see Table 6.4.2-3].

Table 6.4.2-3: Method L01132: accuracy and precision (mean procedural recoveries)

matrix	forti-fication level *[mg/kg]*	1,2,4-T		TA		TAA		TLA	
		mean rec *[%]*	**RSD** *[%]*	**mean rec** *[%]*	**RSD** *[%]*	**mean rec** *[%]*	**RSD** *[%]*	**mean rec** *[%]*	**RSD** *[%]*
milk	0.01	95.0	16.3 (n=46)	93.9	22.5 (n=37)	87.9	13.8 (n=38)	92.61	25.75 (n=37)
	0.10	82.9	15.3 (n=47)	83.6	13.6 (n=35)	90.1	11.3 (n=38)	97.7	10.2 (n=38)
	overall	**88.9**	**17.3 (n=93)**	**88.9**	**20.0 (n=72)**	**89.0**	**12.6 (n=76)**	**95.2**	**19.2 (n=75)**
cream	0.01	106	5.2 (n=5)	77.4	17.3 (n=5)	95.9	9.6(n=5)	98.2	7.3 (n=5)
	0.10	102	3.5 (n=5)	83.7	5.8 (n=5)	101	7.4 (n=5)	107	2.6 (n=5)
	overall	**104**	**4.7 (n=10)**	**80.6**	**12.5 (n=10)**	**98.4**	**8.5 (n=10)**	**103**	**6.8 (n=10)**
skim milk	0.01	85.3	19.0 (n=5)	94.5	25.9 (n=5)	99.4	14.2 (n=5)	99.7	14.8 (n=5)
	0.10	84.3	2.1 (n=4)	89.1	5.1 (n=5)	110	5.3(n=5)	110	3.9 (n=5)
	overall	**84.9)**	**13.6 (n=9)**	**91.8**	**18.3 (n=10)**	**105**	**11.0 (n=10)**	**105**	**11.0 (n=10)**
muscle	0.01	98.1	5.3 (n=5)	85.4	13.0 (n=5)	94.9	3.8 (n=5)	102	6.0 (n=5)
	0.10	90.5	2.8 (n=5)	73.4	10.9 (n=5)	97.1	2.7 (n=5)	98.3	2.8 (n=5)
	overall	**94.3**	**5.9 (n=10)**	**79.4**	**14.0 (n=10)**	**96.0**	**3.3 (n=10)**	**99.9**	**4.8 (n=10)**
fat	0.01	83.2	17.8 (n=14)	105	8.8 (n=11)	87.1	11.3(n=11)	96.6	14.4 (n=11)
	0.10	70.9	25.8 (n=13)	83.8	7.3 (n=11)	92.5	6.2 (n=11)	98.1	5.7 (n=11)
	overall	**77.3**	**22.5 (n=27)**	**94.6**	**14.2 (n=22)**	**89.8**	**9.3 (n=22)**	**97.3**	**10.7 (n=22)**
liver	0.01	93.2	9.5 (n=5)	105	9.5 (n=3)	110	5.6 (n=5)	103	6.4 (n=5)
	0.10	74.6	2.0 (n=4)	91.2	4.4 (n=3)	98.9	2.6 (n=5)	102	4.3 (n=5)
	overall	**84.9**	**13.7 (n=9)**	**98.1**	**10.4 (n=6)**	**104**	**6.9 (n=10)**	**103**	**5.2 (n=10)**
kidney	0.01	103	3.9 (n=4)	85.6	9.5 (n=5)	107	2.7 (n=5)	87.7	5.2 (n=5)
	0.10	75.4	2.8 (n=5)	89.1	8.7 (n=5)	89.7	2.7 (n=5)	90.7	3.8 (n=5)
	overall	**87.5**	**16.8 (n=9)**	**87.3**	**8.8 (n=10)**	**98.3**	**9.6 (n=10)**	**89.2**	**4.6 (n=10)**

mean Rec = mean recovery, RSD = relative standard deviation, n = number of single values, n.a. = not applicable

II. RESULTS AND DISCUSSION

1. Dose level

The dietary burden for ruminant (cow) was estimated based on preliminary information on residue levels available at the time of study start. Estimation were done according to current EFSA and OECD procedures (EFSA: Appendix G: Lundehn document 7031/VI/95 rev.4, July 1996, OECD: OECD Guidance document (ENV/JM/MONO(2013)8, vers Sept 04th, 2013) resulting in an estimated maximum residue intake for EU dairy (EFSA) of 2.9 mg/kg feed (0.11 mg/kg bw/d) and for beef of 20.1 mg/kg feed (0.48 mg/kg bw/d). In order to accommodate estimations obtained with both calculation procedures as well as increased residue intake as a result of future uses the study was conducted with four dose levels ranging from below the EFSA estimated feed intake (1.5 mg/kg feed), including dose levels 7.5 mg/kg, 50 mg/kg up to 150 mg/kg feed. The actual dose levels achieved in the study expressed as mg/kg feed (dry matter) and mg/kg bodyweight/day are shown in the table below.

Table 6.4.2-4: Dose levels (actual mean)

group	dose group	nominal dose *(mg/kg feed)*	actual concentration *(mg/kg feed)* [2)]	actual concentration *(mg/kg bw/d)* [1)]
1 (control)	A	0	0	0
2	B	1.5	1.57	0.035
3	C	7.5	7.49	0.193
4	D	50	49.02	1.042
5	E	150	141.25	3.740
6	F	150	148.70	3.418

1) based on the average mg BAS 750 F received per day and the body weight of each animal during the dosing period
2) based on the average feed intake during dosing period

2. Animal health

All animals remained healthy for the duration of the study as confirmed by regular veterinary inspections. Only minor observations were made (namely for animal 18 of group F on day 33 and for animal 3 of group A on day 22), no treatment was required, also feed intake, bodyweight, or milk yield were not affected (see below).

3. Feed intake, bodyweight, milk production

Administration of BAS 750 F does not affect feed intake, bodyweight and milk production. During acclimatization, the average individual hay consumption in each group was 7.7-10.2 kg (dry weight). The feed consumption remained constant over the dosing period and by week 4, average individual hay consumption was 7.97-11.4 kg. Similar hay consumption was noted during the depuration period. Over the course of the study, the bodyweight of each animal remained relatively constant. During the pre-trial period (day -14 to -1), bodyweight was 508 - 766 kg, during dosing, bodyweight was 502-780 kg. Milk production remained largely constant over pre-trial and dosing period. From day -7 to -1, the mean daily milk yield per animal was 6.64-20.70 kg. During the treatment period, average daily milk yield per animal ranged was 5.74-19.42 kg.

4. Residues

Residue levels of parent BAS 750 F, metabolite M750F022 as well as the TDM analytes 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA) were analyzed in milk (part 4.1) as well as tissues (part 4.2 namely muscle, liver, kidney, and fat types).

4.1 Residues in milk

The following tables provide a summary of the residues in milk of

4.1.1 BAS 750 F
4.1.2 metabolite M750F022
4.1.3 TDM metabolites.

Residue data is provided as the both the “group mean residue” as well as the “maximum individual residue” value (in brackets) in Table 6.4.2-5, Table 6.4.2-6 and Table 6.4.2-7.
The data includes residue levels occurring pre-treatment (day-1), during the treatment phase (days 1-28) as well as the subsequent depuration period (days 29-41). For one sampling timepoint representative of the plateau phase during treatment (milk of day 21) residue data is provided separately for cream and skim milk.

4.1.1 BAS 750 F in milk

The two highest dose groups (E: 150 mg/kg feed and D: 50 mg/kg feed) did show residues above LOQ. The group mean reached a plateau level around day 3 (E: 0.20 mg/kg, D: 0.06 mg/kg). The highest group mean value for group E was 0.253 mg/kg. The highest individual residue value was 0.354 mg/kg (day 21).
In the samples of the depuration group (F: dose of 150 mg/kg feed) residues declined rapidly from a level of 0.20 mg/kg to 0.10 mg/kg (3 days), 0.04 mg/kg (7 days) and 0.01 mg/kg (14 days of depuration) indicting absence of accumulation of BAS 750 F in milk.
Analysis of samples representative for the plateau phase (day 21) showed that residues are predominantly located in cream (group mean E: 1.23 mg/kg) with much lower levels in skim milk (0.07 mg/kg).
In the dose groups C (7.5 mg/kg feed), B (1.5 mg/kg feed) and A (control 0 mg/kg) residues were typically below the LOQ.

Table 6.4.2-5: Residues of BAS 750 F in milk (including skim milk and cream)

study day	BAS 750 F: group mean residue (highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
-1	ND	ND	ND	**<0.01**	ND	ND
1	ND	**<0.01**	**<0.01**	**0.025** *(0.029)*	**0.090** *(0.099)*	**0.096** *(0.112)*
3	**<0.01**	**<0.01**	**0.011** *(0.014)*	**0.058** *(0.064)*	**0.209** *(0.246)*	**0.200** *(0.282)*
5	ND	**<0.01**	**<0.01**	**0.047** *(0.048)*	**0.171** *(0.203)*	**0.257** *(0.357)*
7	**<0.01**	**<0.01**	**<0.01**	**0.053** *(0.058)*	**0.177** *(0.222)*	**0.240** *(0.337)*
10	**<0.01**	**<0.01**	**<0.01**	**0.061** *(0.067)*	**0.215** *(0.265)*	**0.240** *(0.337)*
14	**<0.01**	**<0.01**	**<0.01**	**0.080** *(0.110)*	**0.216** *(0.273)*	**0.273** *(0.344)*
17	**<0.01**	**<0.01**	**<0.01**	**0.064** *(0.078)*	**0.168** *(0.268)*	**0.162** *(0.210)*
21	**<0.01**	**<0.01**	**<0.01**	**0.064** *(0.083)*	**0.253** *(0.354)*	**0.248** *(0.368)*
24	**<0.01**	**<0.01**	**<0.01**	**0.056** *(0.063)*	**0.203** *(0.280)*	**0.242** *(0.283)*
28	**<0.01**	**<0.01**	**<0.01**	**0.053** *(0.059)*	**0.214** *(0.248)*	**0.201** *(0.258)*
29	**(0.077)**[4)]	-	-	-	-	**0.104** *(0.128)*
30	**<0.01**	-	-	-	-	**0.037** *(0.046)*
31	**<0.01**	-	-	-	-	**0.012** (0.015)
32	**<0.01**	-	-	-	-	**<0.01**
33	**<0.01**	-	-	-	-	**<0.01**
34	**<0.01**	-	-	-	-	**<0.01**
35	**<0.01**	-	-	-	-	**<0.01**
36	**<0.01**	-	-	-	-	**<0.01**
37	**<0.01**	-	-	-	-	**<0.01**
38	**<0.01**	-	-	-	-	**<0.01**
39	**<0.01**	-	-	-	-	**<0.01**
40	**<0.01**	-	-	-	-	**<0.01**
41	**<0.01**	-	-	-	-	**<0.01**
42	**<0.01**	-	-	-	-	-
mean (days 3-28)	**<0.01**	**<0.01**	**0.010** *(0.014)*	**0.060** *(0.110)*	**0.203** *(0.354)*	**0.229** *(0.368)*
cream (day21)	-	**<0.01**	**0.052** *(0.061)*	**0.424** *(0.459)*	**1.23** *(1.95)*	**1.46** *(2.16)*
skim milk (day21)	-	**<0.01**	**<0.01**	**0.012** *(0.016)*	**0.069** *(0.103)*	**0.044** *(0.073)*

‚ND denotes non-detection, "-" denotes non-analysis. Residues below the LOQ are denoted by <0.01 mg/kg.. For calculation of mean, the result of < 0.01 mg/kg was set to 0.01 mg/kg. Note, for groups A,B,C,D,E the group mean value of "<LOQ" is generally based on n=3 independent samples. [1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal [4)] This determination was confirmed in repeated analyses (2x in duplicates), yet appears unlikely to reflect the residue level.

4.1.2 M750F022 in milk

M750F022 levels were analysed in milk and cream samples (day 21) selected to represent the highest dose groups. In milk, detectable residues were determined at the highest dose group (group F, 150 mg/kg) with group mean residues of 0.021 mg/kg (highest individual 0.022 mg/kg). For group D (50 mg/kg), group mean and highest residue were both at the LOQ (0.01 mg/kg), therefore residues below the LOQ can be assumed for the lower dose groups (thus were not analysed). Residues in cream were about fivefold higher, namely in group F (0.099 mg/kg), group D (0.063 mg/kg), group C (0.011 mg/kg), and in group B <LOQ, taken together reflecting indicating a linear dose dependency. Residues in skim milk can be assumed to be lower than in whole milk, thus were not determined.

Table 6.4.2-6: Residues of M750F022 in milk (including cream and skim milk) collected at study day 21 (6.4/23)

study day 21	M750F022 : group mean residues (highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
milk	-	-	-	**0.010** *(0.010)*	-	**0.021** *(0.022)*
cream	-	**<0.01**	**0.011** *(0.014)*	**0.063** *(0.072)*	-	**0.099** *(0.108)*
skim milk	-	-	-	-	-	-

"-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples.

4.1.3 TDM in milk

In milk from treated animals, 1,2,4-triazole (1,2,4-T) was the only compound of the TDM group found at levels significantly different from the background (untreated control, Table 6.4/19). TAA and TLA were generally not detected (Table 6.4/25 and Table 6.4/26), while residues of TA if present in quantifiable amounts were comparable to levels of corresponding untreated controls (Table 6.4/24) and therefore considered largely treatment-unrelated (Note that TA was determined in skim milk at 0.022 mg/kg (highest individual 0.042 mg/kg) while residues in cream were below the LOQ. Importantly, this level was by far exceeded by the level of 1,2,4-triazole (0.242 mg/kg) in this sample).

Table 6.4.2-7: Residues of 1,2,4-T in milk (including cream and skim milk)

study day	1,2,4-T : group mean residue (highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
-1	**0.012** (0.014)	**0.012** (0.014)	**0.012** (0.014)	**0.011** (0.014)	**0.013** (0.014)	**<0.01**
1	**0.012** *(0.014)*	**0.011** *(0.013)*	**0.015** *(0.020)*	**0.029** *(0.033)*	**0.059** *(0.066)*	**0.052** *(0.062)*
3	**0.013** *(0.016)*	**0.014** *(0.017)*	**0.024** *(0.027)*	**0.069** *(0.071)*	**0.157** *(0.180)*	**0.161** *(0.175)*
5	**0.013** *(0.016)*	**0.015** *(0.015)*	**0.029** *(0.036)*	**0.096** *(0.099)*	**0.242** *(0.267)*	**0.200** *(0.216)*
7	**0.013** *(0.015)*	**0.016** *(0.018)*	**0.029** *(0.035)*	**0.106** *(0.112)*	**0.247** *(0.270)*	**0.181** *(0.213)*
10	**0.011** *(0.013)*	**0.015** *(0.016)*	**0.030** *(0.039)*	**0.104** *(0.110)*	**0.269** *(0.311)*	**0.224** *(0.250)*
14	**0.012** *(0.013)*	**0.018** *(0.019)*	**0.036** *(0.048)*	**0.121** *(0.137)*	**0.288** *(0.310)*	**0.276** *(0.318)*
17	**0.011** *(0.012)*	**0.017** *(0.017)*	**0.030** *(0.037)*	**0.129** *(0.136)*	**0.286** *(0.329)*	**0.254** *(0.263)*
21	**0.012** *(0.013)*	**0.015** *(0.017)*	**0.030** *(0.037)*	**0.127** *(0.130)*	**0.287** *(0.334)*	**0.244** *(0.259)*
24	**0.010** *(0.011)*	**0.012** *(0.014)*	**0.028** *(0.033)*	**0.118** *(0.121)*	**0.234** *(0.262)*	**0.221** *(0.228)*
28	**0.011** *(0.012)*	**0.012** *(0.013)*	**0.029** *(0.033)*	**0.108** *(0.118)*	**0.280** *(0.331)*	**0.189** *(0.229)*
29	**0.049**	-	-	-	-	**0.220** *(0.255)*
30	**0.015**	-	-	-	-	**0.173** *(0.220)*
31	**0.013**	-	-	-	-	**0.149** *(0.166)*
32	**0.012**	-	-	-	-	**0.098** *(0.102)*
33	**0.015**	-	-	-	-	**0.068** *(0.072)*
34	**0.016**	-	-	-	-	**0.043** *(0.051)*
35	**0.015**	-	-	-	-	**0.047**
36	**0.016**	-	-	-	-	**0.025**
37	**0.016**	-	-	-	-	**0.023**
38	**0.016**	-	-	-	-	**0.020**
39	**0.015**	-	-	-	-	**0.018**
40	**0.016**	-	-	-	-	**0.016**
41	**0.017**	-	-	-	-	**0.016**
42	**0.021**	-	-	-	-	
mean (days 3-28)	**0.012** *(0.016)*	**0.015** *(0.019)*	**0.030** *(0.048)*	**0.113** *(0.137)*	**0.267** *(0.334)*	**0.224** *(0.318)*
cream (day 21)	-	**0.011** *(0.012)*	**0.023** *(0.027)*	**0.092** *(0.094)*	**0.227** *(0.290)*	**0.179** *(0.205)*
skim milk (day 21)	-	**0.016** *(0.018)*	**0.029** *(0.035)*	**0.125** (0.129)	**0.242** *(0.305)*	**0.225** *(0.234)*

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples.
[1] 3 days withdrawal, [2] 7 days withdrawal, [3] 14 days withdrawal [4] representative of plateau phase (days 3-28)

Detectable residues of 1,2,4-triazole were seen in the highest dose group (150 mg/kg feed) while for TA, TAA and TLA residues were <LOQ or at levels considered treatment unrelated (maximal individual residue 0.018 mg/kg). The group mean reached a plateau level at day 10 at a level of around 0.27 mg/kg. On day 21, the highest group mean value was reached (0.287 mg/kg) as well as the highest individual residue 0.334 mg/kg. In the samples of the depuration group (dose of 150 mg/kg feed) residues declined rapidly from 0.22 mg/kg (day 29) to 0.15 mg/kg (day 31) and reached nearly background levels of 0.04 mg/kg on day 34 (note that this is close to background levels as the residue levels in untreated control varied around 0.01 - 0.02 mg/kg, with a maximal value at 0.049 mg/kg).

Analysis of samples representative for the plateau phase (day 21) showed 1,2,4-triazole residues levels are similar in milk (0.287 mg/kg) and fractions produced thereof, namely skim milk (0.242 mg/kg) and cream (0.227 mg/kg). In cream, TA was detected at quantifiable levels albeit much lower than 1,2,4-triazole (0.022 mg/kg) while TAA and TLA were not detected.

In the dose group D (50 mg/kg feed), 1,2,4-triazole residue levels were present at detectable amounts reaching a plateau around 0.120 mg/kg at day 14. The difference between the residues (group mean E: 0.274 mg/kg and D: 0.120 mg/kg) correspond well with the difference in dose level (E: 150 mg/kg feed and D: 50 mg/kg feed) indicating a linear dose dependency. In dose group C (7.5 mg/kg feed), 1,2,4-triazole levels reached a plateau at 0.030 mg/kg (starting day 5). In dose group B (1.5 mg/kg feed) levels were comparable to the untreated control (maximal 0.019 mg/kg).

Table 6.4.2-8: Residues of TA in milk (including cream and skim milk)

study day	TA : group mean residue (and highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
-1	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
1	**<0.01**	**ND**	**<0.01**	**<0.01**	**ND**	**<0.01**
3	**<0.01**	**ND**	**<0.01**	**ND**	**<0.01**	**<0.01**
5	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
7	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
10	**ND**	**ND**	**<0.01**	**ND**	**<0.01**	**<0.01**
14	**<0.01**	**<0.01**	**ND**	**<0.01**	**<0.01**	**<0.01**
17	**ND**	**ND**	**ND**	**<0.01**	**0.010** *(0.011)*	**<0.01**
21	**0.011** *(0.011)*	**0.013** *(0.016)*	**<0.01**	**0.014** *(0.014)*	**0.011** *(0.011)*	**0.014** *(0.016)*
24	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.013** *(0.018)*
28	**<0.01**	**<0.01**	**<0.01**	**0.011** *(0.012)*	**0.012** *(0.014)*	**<0.01**
mean (days 3-28)	**<0.01**	**0.011** *(0.016)*	**<0.01**	**0.010** *(0.014)*	**0.011** *(0.014)*	**0.011** *(0.018)*
cream (day 21)	-	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
skim milk (day 21)	-	**<0.01**	**<0.01**	**<0.01**	**0.022** *(0.042)*	**0.017** *(0.028)*

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples.

Table 6.4.2-9: Residues of TAA in milk (including cream and skim milk)

study day	TAA : group mean residue (highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
-1	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
1	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
3	**ND**	**ND**	**ND**	**ND**	**ND**	**<0.01**
5	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
7	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
10	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
14	**ND**	**ND**	**ND**	**ND**	**ND**	**<0.01**
17	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
21	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
24	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
28	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
cream (day 21)	-	**ND**	**ND**	**ND**	**ND**	**ND**
skim milk (day 21)	-	**ND**	**ND**	**ND**	**ND**	**ND**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis.

Table 6.4.2-10: Residues of TLA in milk (including cream and skim milk)

study day	TLA : group mean residue (highest individual) in mg/kg					
	Group A *(Control)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F** *(150 mg/kg)*
-1	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
1	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
3	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
5	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
7	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
10	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
14	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
17	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
21	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
24	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
28	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
cream (day 21)	-	**ND**	**ND**	**ND**	**ND**	**ND**
skim milk (day 21)	-	**ND**	**ND**	**ND**	**ND**	**ND**

ND denotes non-detection, "-" denotes non-analysis.

4.2 Residues in tissues (muscle, liver, kidney, fat)

The following tables provide a summary of the residues in tissues of
4.2.1 BAS 750 F
4.2.2 metabolite M750F022
4.2.3 TDM metabolites.

Residue data is provided as both the group mean as well as the maximum individual residue value (in brackets) in Table 6.4.2-11, Table 6.4.2-12, Table 6.4.2-13, Table 6.4.2-14, Table 6.4.2-15, and Table 6.4.2-16. The data includes residue levels occurring during the treatment phase as well as the subsequent depuration period.

4.2.1 BAS 750 F in tissue

For the highest dose group (150 mg/kg feed), the group mean residues in muscle were at 0.16 mg/kg (highest individual 0.22 mg/kg), in liver at 3.03 mg/kg (highest individual 3.58 mg/kg), in perirenal fat at 1.71 mg/kg (highest individual 2.29 mg/kg, note that level in other fat types were lower). In the samples of the depuration group (dose of 150 mg/kg feed) residues in tissues declined rapidly, with levels <LOQ latest after 7 days (muscle, kidney), after 14 days (liver, perirenal and mesenterial fat). For subcutaneous fat, levels at withdrawal day 14 day had declined to 0.023 mg/kg (from 1.47 mg/g at withdrawal day 3).
Residues in other dose groups were lower, if present above the LOQ, levels did indicate a linear dose dependency, namely for liver, kidney, fat (groups E-D-C-B) and for muscle (E-D).

Table 6.4.2-11: BAS 750 F residues in tissues

tissue	BAS 750 F : group mean (maximum individual) residues in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**0.073** (0.105)	**0.163** *(0.221)*	**0.063**	**<0.01**	**ND**
liver	**<0.01**	**0.031** *(0.034)*	**0.150** *(0.182)*	**0.993** *(1.40)*	**3.03** *(3.58)*	**0.885**	**0.021**	**<0.01**
kidney	**<0.01**	**0.012** *(0.014)*	**0.048** *(0.074)*	**0.291** *(0.505)*	**1.29** *(1.88)*	**0.275**	**<0.01**	**ND**
fat (perir.)	**<0.01**	**0.017** *(0.018)*	**0.049** *(0.059)*	**0.649** *(0.900)*	**1.71** *(2.29)*	**0.536**	**0.017**	**<0.01**
fat (mesen.)	**<0.01**	**0.018** *(0.018)*	**0.053** *(0.077)*	**0.528** *(0.566)*	**1.16** *(1.87)*	**2.25**	**0.023**	**<0.01**
fat (subcut.)	**<0.01**	**0.015** *(0.017)*	**0.023** *(0.041)*	**0.483** *(0.78)*	**0.594** *(1.20)*	**1.47**	**0.322**	**0.023**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

4.2.2 M750F022 in tissue

For the highest dose group (150 mg/kg feed), the group mean residues in muscle were at 0.016 mg/kg (highest individual 0.018 mg/kg), in liver at 0.038 mg/kg (highest individual 0.044 mg/kg), in perirenal fat at 0.16 mg/kg (highest individual 0.21 mg/kg, note that residues in mesenterial fat and subcutaneous fat generally was lower).
In the samples of the depuration group (dose of 150 mg/kg feed) residues declined rapidly, with residues <LOQ in muscle latest after 3 days of depuration, residues at or below the LOQ in liver latest after 3 days of depuration, in kidney after 7 days of depuration, in perirenal and mesenterial fat latest 14 days of depuration. In subcutaneous fat, decline was also rapid from 0.11 mg/kg (3d-withdrawal-animal) to 0.023 mg/kg (14d-withdrawal-animal).
Residues in other dose groups were lower. In muscle, residues were below the LOQ for group D. Levels in liver, kidney and fat indicate a linear dose dependency (groups E-D-C).

Table 6.4.2-12: M750F022 residues in tissues

tissue	M750F022 : group mean (maximum individual) residues in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	-	-	-	**<0.01**	**0.016** *(0.018)*	**<0.01**	**<0.01**	**<0.01**
liver	-	-	**<0.01**	**0.021** *(0.022)*	**0.038** *(0.044)*	**0.016**	**ND**	**0.010**
kidney	-	-	**<0.01**	**0.019** *(0.020)*	**0.041** *(0.043)*	**0.012**	**<0.01**	**<0.01**
fat (perir.)	-	-	**<0.01**	**0.082** *(0.089)*	**0.163** *(0.212)*	**0.096**	**0.051**	**<0.01**
fat (mesen.)	-	-	**0.01** *(0.011)*	**0.083** *(0.090)*	**0.125** *(0.203)*	**0.149**	**0.054**	**<0.01**
fat (subcut.)	-	-	**<0.01**	**0.052** *(0.077)*	**0.073** *(0.130)*	**0.109**	**0.068**	**0.023**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

4.2.3 TDM in tissue

In tissues, TDM residues were present above the LOQ, with 1,2,4-triazole as the predominant component. Residues of TLA were > LOQ. Residues of TAA also were below the LOQ except for residues in largely treatment unrelated residues in kidney (levels similar to untreated control).
For TA, quantifiable residue levels also appear to be to a large extend treatment unrelated (since groups B, C, and D are comparable to untreated control group A). Dose group E shows a treatment related increase of TA level (in muscle E: 0.178 mg/kg compared to A: 0.03 mg/kg, in liver E: 0.65 mg/kg compared to A: 0.14 mg/kg, in kidney E: 0.19 mg/kg compared to A: 0.04 mg/kg), in perirenal fat E: 0.06 mg/kg compared to A: 0.01 mg/kg), in mesenterial fat E: 0.02 mg/kg compared to A: 0.01 mg/kg), in subcutaneous fat E: 0.06 mg/kg compared to A: 0.02 mg/kg). Upon withdrawal, TA levels rapidly declined within 3 days, yet remaining at levels >LOQ for the rest of the depuration phase.

For the highest dose group (150 mg/kg feed), the group mean residues of 1,2,4-triazole in muscle were at 0.28 mg/kg (highest individual 0.33 mg/kg), in liver at 0.26 mg/kg (highest individual 0.30 mg/kg), in kidney at 0.28 mg/kg (highest individual 0.39 mg/kg). In different fat types, the group mean were at 0.11-0.17 mg/kg (highest individual residues was in subcutaneous fat at 0.28 mg/kg).
In the samples of the depuration group (dose of 150 mg/kg feed) residues declined rapidly to levels near the LOQ after a depuration of 14 days (muscle, liver 0.02 mg/kg, kidney 0.01 mg/kg, fat <0.01 mg/kg).
Residues in other dose groups were lower. Group mean residue level for groups D-C-B are in accordance with a linear dose dependency taking into account residue levels around the LOQ (up to 0.024 mg/kg for mesenterial fat).

Table 6.4.2-13: Residues of 1,2,4-T in tissues

tissue	**1,2,4-T : group mean (maximum individual) residues in mg/kg**							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	**0.013** *(0.015)*	**0.011** *(0.012)*	**0.028** *(0.030)*	**0.105** *(0.108)*	**0.283** *(0.328)*	**0.141**	**0.047**	**0.016**
liver	**0.011** *(0.013)*	**0.014** *(0.016)*	**0.031** *(0.034)*	**0.121** *(0.124)*	**0.257** *(0.301)*	**0.166**	**0.048**	**0.016**
kidney	**0.012** *(0.015)*	**0.013** *(0.017)*	**0.028** *(0.032)*	**0.086** *(0.117)*	**0.279** *(0.386)*	**0.139**	**0.044**	**0.014**
fat (perir.)	**0.010** *(0.011)*	**<0.01**	**0.014** *(0.017)*	**0.016** *(0.028)*	**0.114** *(0.191)*	**<0.01**	**<0.01**	**<0.01**
fat (mesen.)	**0.024** *(0.052)*	**<0.01**	**0.011** *(0.011)*	**0.018** *(0.035)*	**0.105** *(0.254)*	**0.085**	**<0.01**	**<0.01**
fat (subcut.)	**<0.01**	**<0.01**	**0.017** *(0.021)*	**0.031** *(0.065)*	**0.171** *(0.277)*	**0.040**	**0.010**	**<0.01**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

Table 6.4.2-14: Residues of TA in tissues

tissue	TA : group mean (maximum individual) residues in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	**0.026** *(0.034)*	**0.038** *(0.058)*	**0.050** *(0.063)*	**0.056** *(0.066)*	**0.178** *(0.255)*	**0.051**	**0.037**	**0.080**
liver	**0.141** (0.179)	**0.143** (0.168)	**0.167** (0.216)	**0.216** (0.229)	**0.652** (0.777)	**0.231**	**0.221**	**0.404**
kidney	**0.038** *(0.052)*	**0.043** *(0.050)*	**0.048** *(0.065)*	**0.047** *(0.068)*	**0.188** *(0.231)*	**0.064**	**0.083**	**0.120**
fat (perir.)	**0.013** *(0.018)*	**<0.01**	**0.015** *(0.023)*	**0.011** *(0.013)*	**0.055** *(0.083)*	**<0.01**	**<0.01**	**<0.01**
fat (mesen.)	**0.013** *(0.019)*	**<0.01**	**0.015** *(0.020)*	**0.012** *(0.015)*	**0.020** *(0.034)*	**0.016**	**<0.01**	**<0.01**
fat (subcut.)	**0.017** *(0.030)*	**0.012** *(0.015)*	**0.022** *(0.037)*	**0.015** *(0.023)*	**0.064** *(0.085)*	**<0.01**	**<0.01**	**0.012**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

Table 6.4.2-15: Residues of TAA in tissues

tissue	TAA : group mean (maximum individual) residues in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**ND**	**<0.01**
liver	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
kidney	**0.011** *(0.014)*	**0.013** *(0.016)*	**0.011** *(0.013)*	**0.014** *(0.019)*	**0.027** *(0.036)*	**<0.01**	**<0.01**	**0.016**
fat (perir.)	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**ND**	**ND**	**ND**
fat (mesen.)	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**ND**	**ND**
fat (subcut.)	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**ND**	**ND**	**ND**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

Table 6.4.2-16: Residues of TLA in tissues

tissue	TLA : group mean (maximum individual) residues in mg/kg							
	Group A *(0 mg/kg)*	**Group B** *(1.5 mg/kg)*	**Group C** *(7.5 mg/kg)*	**Group D** *(50 mg/kg)*	**Group E** *(150 mg/kg)*	**Group F1[1)]** *(150 mg/kg)*	**Group F1[2)]** *(150 mg/kg)*	**Group F1[3)]** *(150 mg/kg)*
muscle	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
liver	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
kidney	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
fat (perir.)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
fat (mesen.)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
fat (subcut.)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

Note, <0.01 denotes residues below LOQ, ND denotes non-detection, "-" denotes non-analysis. Note, for groups A, B, C, D, E the group mean value of "<LOQ" is generally based on n=3 independent samples. For groups F1, F2, F3 n=1 independent sample.
[1)] 3 days withdrawal, [2)] 7 days withdrawal, [3)] 14 days withdrawal

III. CONCLUSION

Generally, the residue data obtained indicates a linear relationship between feed intake (BAS 750 F dose level) and the residue level in animal commodities for each of the analytes, parent BAS 750 F, and M750F022 as well as 1,2,4-triazole with absence of BAS 750 F-specific residues in untreated controls (BAS 750 F, M750F022). Concerning the TDMs, background levels above the LOQ were found, notably for 1,2,4-triazole and TA, occasionally for TAA, while for TLA no residues >LOQ were found.

Residues in milk did reach a plateau level within 3 days for both BAS 750 F and 1,2,4-triazole. The plateau levels found for the highest dose group (given as group mean, highest individual results in parenthesis) were for BAS 750 F at 0.21 mg/kg (0.35 mg/kg) and 1,2,4-triazole at 0.27 mg/kg (0.33 mg/kg). M750F022 measured at one sampling date representative of the plateau phase of the highest dose (group F, day 21) was quantified at 0.01 mg/kg. Upon withdrawal residues did decline rapidly, latest after 4 days of depuration to levels <LOQ (BAS 750 F) or to background levels (1,2,4-triazole) demonstrating that residues do not accumulate in milk.

In milk of the highest dose group, residues were localized mainly in the cream fraction for BAS 750 F (1.23 mg/kg) and M750F022 (0.06 mg/kg) while for 1,2,4-triazole residues were slightly higher in skim milk (0.24 mg/kg) than for cream (0.23 mg/kg).

Generally, much higher level were found for parent BAS 750 F than for its metabolite M750F022.

Residues in tissues were higher for BAS 750 F in liver compared to kidney and fat and much lower levels in muscle. For the highest dose group (150 mg/kg feed), group mean level (highest individual) in liver were 3.03 mg/kg (3.58 mg/kg), in kidney 1.29 mg/kg (1.88 mg/kg), in different fat types 0.60-1.71 mg/kg (1.20-2.29 mg/kg) while in muscle only 0.16 mg/kg (0.22 mg/kg).

For M750F022 residues were higher in fat compared to liver, kidney and muscle. For the highest dose group (150 mg/kg feed), group mean levels (highest individual) in different fat types were 0.07 - 0.16 mg/kg (0.13 -0.21 mg/kg), in liver and kidney 0.04 mg/kg (0.04 mg/kg), while in muscle only 0.02 mg/kg (0.02 mg/kg). Generally, much higher levels were found for parent BAS 750 F than for its metabolite M750F022.

For 1,2,4-triazole, residues were higher in muscle, liver, kidney compared to different fat types. For the highest dose group (150 mg/kg feed), group mean levels (highest individual) in muscle were 0.28 mg/kg (0.33 mg/kg), in kidney 0.28 mg/kg (0.39 mg/kg), in liver 0.26 mg/kg (0.30 mg/kg) while in different fat types level were 0.11 - 0.17 mg/kg (0.19 - 0.28 mg/kg).

Upon withdrawal, residues did rapidly decline to levels below or near the LOQ demonstrating absence of accumulation for BAS 750 F (latest after 7 days for muscle, kidney, latest after 14 days for liver, perirenal/mesenterial fat, with 0.02 mg/kg after 14 days for subcutaneous fat) as well as for M750F022 (latest after 3 days for muscle, kidney, latest after 7 days for liver, latest after 14 days for perirenal/mesenterial fat). In subcutaneous fat residues also declined rapidly, with levels near the LOQ for both BAS 750 F and M750F022 (0.023 mg/kg) after 14 days of withdrawal.

To provide a general overview over the relative amounts of the three analytes parent BAS 750 F, metabolite M750F022 and 1,2,4-triazole, residue levels determined at the highest dose (150 mg/kg feed) are summarized in the following table.

Table 6.4.2-17: Overview tissue distribution for BAS 750 F, M750F022 and 1,2,4-triazole in highly overdosed 100x dose group (150 mg/kg feed)

	BAS 750 F		**M750F022**		**1,2,4-T**	
dose group E (150 mg/kg feed)	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*	*group mean (mg/kg)*	*highest individual (mg/kg)*
milk [1)]	**0.21**	*0.35*	**0.02**	*0.02*	**0.29**	*0.33*
cream [2)]	**1.23**	*1.95*	**0.06**	*0.07*	**0.23**	*0.29*
skim milk [2)]	**0.07**	*0.10*	**<LOQ**	*<LOQ*	**0.24**	*0.31*
muscle	**0.16**	*0.22*	**0.02**	*0.02*	**0.28**	*0.33*
liver	**3.03**	*3.58*	**0.04**	*0.04*	**0.26**	*0.30*
kidney	**1.29**	*1.88*	**0.04**	*0.04*	**0.28**	*0.39*
fat perirenal	**1.71**	*2.29*	**0.16**	*0.21*	**0.11**	*0.19*
fat mesenterial	**1.16**	*1.87*	**0.13**	*0.20*	**0.11**	*0.25*
fat subcutaneaous	**0.59**	*1.20*	**0.07**	*0.13*	**0.17**	*0.28*

[1)] plateau level [2)] taken during plateau phase [3)]Note, detailed data is given in section CA6.4.4.

In conclusion, BAS 750 F residues present in feed items are transferred to ruminant commodities showing a linear dose-residue-dependency. Residues in milk reach plateau level indicating absence of accumulation for parent BAS 750 F, its metabolite M750F022 and 1,2,4-triazole. Residues in milk and tissues decline rapidly after dose withdrawal.

Report: CA 6.4.2/2
Guedez Orozco A.A.,Heger N., 2016 a
Determination of the fatty conjugates metabolites of M750F022 (Reg. No. 6011210) in animal matrices
2016/1001326
Guidelines: SANCO/3029/99 rev. 4 (11 July 2000)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

Based on residue levels of first, free M750F022 and second, of the sum of M750F022 releasing compounds (expressed as M750F022) in several samples representing important hen matrices, the factors between total M750F022 residue and free M750F022 residue were calculated. For the purpose of deriving estimates of maximal total content of M750F022 residue (including conjugates) based on residue data for free M750F022, matrix-specific correction factors are proposed. For kidney, it proposed to apply the correction factor of 1.0 for liver. For skin with fat, it is proposed to apply the correction factor of 4.0 for fat.

matrix	correction factor
liver	1.0
muscle	1.5
egg	1.5
fat, skin with fat	4.0

I. MATERIAL AND METHODS

A. MATERIALS

1.Test Material: *not relevant*

2.Test Commodity: *not relevant*

B. STUDY DESIGN AND METHODS

1. Test procedure *not relevant*

2. Description of analytical procedures

BASF method L0309/02

This analytical method allows the quantitation of fatty acid conjugates of M750F022 in animal matrices. M750F022 is released from the fatty acid conjugates by alkaline treatment (10 M NaOH) and subsequently detected by GC/MS. The conditions for the alkaline cleavage were tested and reported in the metabolism study (DocID: 2015/1001001). Cleavage efficiency was confirmed representatively with fatty acid conjugate M750F025. Validation of the method was performed within this study for M750F025 in muscle, liver, egg and fat (see section CA4.3).

Fatty acid conjugates of M750F022

The limit of quantitation (LOQ) of M750F022 is 0.01 mg/kg for all matrices.
Fat, skin: Fatty acid conjugates of M750F022 are extracted from fat-rich matrices with iso-hexane followed by liquid-liquid partitions with acetonitrile (2x). The acetonitrile phase is treated with NaOH (10 M), subjected to SPE clean-up, followed by the determination of M750F022 by GC/MS.
Muscle, liver: Fatty acid conjugates of M750F022 are extracted from protein-rich matrices with MeOH. The extract is treated with NaOH (10 M), subjected to SPE clean-up followed by determination of M570F022 by GC/MS.

M750F022

The limit of quantitation (LOQ) of M750F022 is 0.01 mg/kg for all matrices.
Fat, skin: M750F022 is extracted from fat-rich matrices with acetonitrile/iso-hexane followed by liquid/liquid partition with iso-hexane, subjected to SPE clean-up, followed by the determination of M750F022 by GC/MS.
Muscle, liver: M750F022 is extracted from protein-rich matrices with MeOH. The extract is treated with NaOH (10 M) and cyclohexane or dichloromethane. An aliquot of the cyclohexane or dichloromethane phase is dried and dissolve in MeOH/H_20 (50/50), subjected to SPE clean-up followed by determination of M570F022 by GC/MS.

3. Recoveries

Average recoveries were in the range of 70-110% with RSDs ≤20% for analyte M750F022. Concurrent recoveries obtained from control samples spiked with M750F022 were analysed to demonstrate the validity of the method.

Table 6.4.2-18: Procedural Recoveries M750F022 (fragment m/z 295)

Matrix	Fortification Level [mg/kg]	M750F022 m/z 295			
		Mean [%]	SD [±]	RSD [%]	n
Egg	0.01; 0.1	91.1	17	19	6
Fat	0.01; 0.1	88.6	5.4	6.1	6
Liver	0.01; 0.1; 1	69.7	18	25	7
Muscle	0.01; 0.1	89.9	12	13	6
Overall:		84.0	15	17	25

Table 6.4.2-18: Procedural Recoveries M750F025 (measured as M750F022 at m/z 295 fragment)

Matrix	Fortification Level [mg/kg]	M750F025 m/z 295			
		Mean [%]	SD [±]	RSD [%]	n
Egg	0.01; 0.1	85.5	5.0	5.8	10
Fat	0.01; 0.1	68.0	4.8	7.1	10
Liver	0.01; 0.1; 1	82.7	11	13	10
Muscle	0.01; 0.1	93.8	7.0	7.4	6
Overall:		82.5	11.8	12.6	40

II. RESULTS AND DISCUSSION

Conjugates of M750F022 carrying various acyl moieties (fatty acids) were quantitated in selected samples which had been obtained as part of the hen feeding study (see section CA6.4/1). Using BASF Method No. L0309/02 (LOQ of 0.01 mg/kg), free M750F022 was determined (see column (a) in table below) as well as the sum of M750F022 and conjugates, expressed as M750F022 (see column (b) in table below). (Note, during the study, a validation of the analytical method L0309/02 was conducted in the matrix corresponding to the samples analyzed. M750F025, a hexadecanoate conjugate of M750F022, was used to represent the fraction of fatty acid conjugates, details are provided in section CA4.3).

Correction factors calculated for each sample were used as basis to propose matrix-specific correction factors for the purpose of calculating the total content of M750F022 residue (including conjugates) based on residue data for free M750F022.

Table 6.4.2-20: Residues of M750F022 and fatty acid conjugates in hen matrices

samples [1]			residues [2] [expressed as M750F022 equivalents]		correction factor [3]	
matrix	sample [4]	M750F022 *[mg/kg]*	(a) M750F022 *[mg/kg]*	(b) M750F022&conjugates *[mg/kg]*	factor calculated (b/a)	factor proposed
egg	F	0.053	0.060	0.071	1.18	1.5
	C	< 0.01	0.010	0.015	1.54	
	D	0.015	0.019	0.023	1.22	
	F	0.052	0.060	0.092	1.54	
	C	< 0.01	0.010	0.014	1.38	
	D	0.016	0.021	0.022	1.07	
fat	C	0.36	0.040	0.094	2.35	4.0
	D	0.071	0.069	0.30	4.28	
	E	0.36	0.36	1.3	3.53	
skin with fat	D	0.041	0.043	0.067	1.55	4.0
	E	0.19	0.20	0.73	3.71	
liver	D	0.2	0.23	0.25	1.10	1.0
	E	0.033	0.046	0.045	1.00	
muscle	D	< 0.01 *(0.0083)*[5]	0.014	0.017	1.27	1.5
	E	0.037	0.047	0.070	1.48	

[1] samples from hen feeding study (CA 6.4.1/1, DocID 2015/1106667, analysed by method L0309/1
[2] re-analysis using BASF method L0309/2 to determine first, content of free M750F022 and second, content of M750F022&fatty acid conjugates.
[3] the correction factor is calculated as sum of M750F022&fatty acid conjugates divided by free M750F022.
[4] details on samples selected are provided in the study report DocID2016/1001326)
[5] Residue level below LOQ is provided in parenthesis.

III. CONCLUSION

Based on residue levels of first, free M750F022 and second, of the sum of M750F022 releasing compounds (expressed as M750F022) in several samples representing important hen matrices, the factors between total M750F022 residue and free M750F022 residue were calculated. For the purpose of deriving estimates of maximal total content of M750F022 residue (including conjugates) based on residue data for free M750F022, matrix-specific correction factors are proposed. For kidney, it proposed to apply the correction factor of 1.0 for liver. For skin with fat, it is proposed to apply the correction factor of 4.0 for fat.

matrix	correction factor
liver	1.0
muscle	1.5
egg	1.5
fat, skin with fat	4.0

CA 6.4.3 Pigs

No study is needed for pig (see CA 6.2) and therefore is not provided in the present dossier. Estimations on magnitude of residues in commodities of pig can be based on the feeding study in ruminants (cow study, see CA 6.4.2). Notably, it can be assumed that BAS 750 F residues present in feed items are transferred to pig commodities showing a linear dose-residue-dependency. It also can be assumed that residues in pig tissues decline rapidly after dose withdrawal.

CA 6.4.4 Fish

A fish feeding study is not required. Currently no test method or guidance document is available. As a consequence waiving of this particular data requirement is considered acceptable according to the “Guidance document for applicants on preparing dossiers for the approval of a chemical new active substance and the renewal of the chemical active substance according to regulation (EU) No. 283/2013 and regulation (EU) No. 284/2013” (SANCO/10181/2013-rev.2 of 2-May-2013).

CA 6.5 Effects of Processing

CA 6.5.1 Nature of the residue

Report: CA 6.5.1/1
Hassink J.,Bartmann S., 2014 a
BAS 750 F: Hydrolysis at 90°C, 100°C and 120°C
2014/1170665
Guidelines: EEC 7035/VI/95 rev. 5, OECD 507 - Nature of the residues in processed commodities - High temperature hydrolysis
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

A standard hydrolysis study, performed with 14C-BAS 750 F labeled at the chlorophenyl ring (C-label) as well as with 14C-BAS 750 F labeled at the triazole ring (T-label) showed hydrolytic stability under conditions representative of the following processing procedures: pasteurization (pH 4, 20 min, 90 °C), baking, brewing, boiling (pH 5, 60 min, 100 °C), and sterilization (pH 6, 20 min, 120 °C). HPLC analysis showed that no major loss of radioactivity occurred upon treatment as well as absence of any degradation product exceeding 2% of total radioactivity. Chiral HPLC analysis confirmed absence of any significant change of the enantiomer ratio.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description: **C-label:**
[chlorophenyl-U-C14]-BAS 750 F, spec. activity 7.878 MBq/mg
T-label:
[triazole-3(5)-C14]-BAS 750 F, spec. activity 5.46 MBq/mg

Lot/Batch #: **C-label:** CFQ41561
T-label: 1062-2001

Purity: **C-label:** 98.9% (radiochemical purity)
T -label: 98.8% (radiochemical purity)

CAS#: 40487-42-1
Development code: BAS 750 F

B. STUDY DESIGN AND METHODS

1. Test procedure

Different processes (pasteurisation, baking, brewing, boiling, and sterilisation) were simulated in order to investigate any potential degradation of BAS 750 F during industrial processing or household preparation, The study was performed with ^{14}C-BAS 750 F labelled either at the chlorophenyl ring (C-label) or at the triazole ring (T-label).

Figure 6.5.1-1: molecular structure of [chlorophenyl-U-C14]-BAS 750 F (C-label)

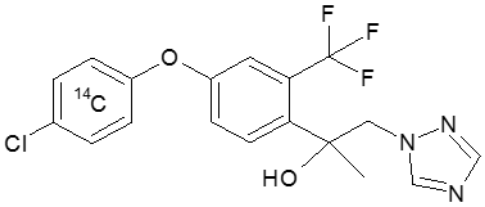
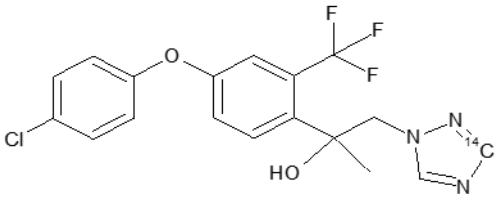
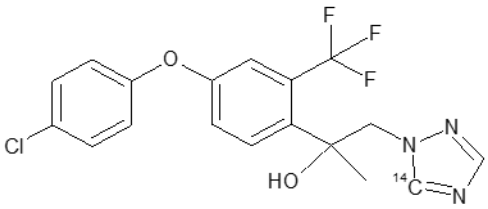


Figure 6.5.1-2: molecular structure of [triazole-3(5)-C14]-BAS 750 F (T-label)





The ^{14}C-labeled test substance was dissolved in aqueous buffer solutions of different pH values and heated according to the parameters given in Table 6.5.1-1

Table 6.5.1-1: Conditions tested in the standard hydrolysis study

temperature (°C)	pH	time (min)	simulated processing procedure
90	4	20	pasteurisation
100	5	60	baking, brewing, boiling
120	6	20	sterilisation (in the dark)

2. Description of analytical procedures

Total radioactivity was determined by liquid scintillation counting (LSC), composition of the radioactive residue was analysed directly by HPLC (radiodetection) without any prior work-up. If necessary, samples were stored frozen prior to analysis.

Two HPLC systems were used:
System 1 was used for chromatographic confirmation of radiochemical purity prior to incubation as well as for analysis of chromatographic profile subsequent to incubation (investigation of potential degradation products).
System 2 was used for the chiral separation of the two enantiomers of BAS 750 F. Samples were cleaned-up by solid phase extraction. The C18 SPE cartridges were loaded with 5 ml of each sample, washed with 1 ml water and eluted with 5 mL ethanol. 1 ml of the eluate was evaporated under nitrogen to dryness and dissolved in 1 ml n-Heptane/ethanol/2-propanol (500 + 25 + 25) for HPLC injection.

II. RESULTS AND DISCUSSION

1. Components of residue at hydrolytic conditions

Total radioactivity (TAR) before and after incubation were similar, indicating absence of major loss of radioactivity (see Table 6.5.1-2). Before and after incubation, BAS 750 F accounted for almost all of the radioactivity, indicating absence of any degradation product at levels of 2% TAR or higher.

Table 6.5.1-2: BAS 750 F levels before and after incubation

test material	T-label			C-label		
	pH 4, 90°C (20 min)	pH 5, 100°C (60 min)	pH 6, 120°C (20 min)	pH 4, 90°C (20 min)	pH 5, 100°C (60 min)	pH 6, 120°C (20 min)
	Total Radioactivity (TAR)					
	% TAR	% TAR	% TAR	% TAR	% TAR	% TAR
total (prior to treatment) [1)]	100.0	100.0	100.0	100.0	100.0	100.0
total (post treatment)	110.3	110.2	105.2	110.2	108.3	110.1
BAS 750 F	110.3	110.2	103.8	107.9	107.1	107.3
unknown [2)]	n.d.	n.d.	1.4	2.3	1.2	2.8

[1)] actual concentration of each sample was determined by LSC before each incubation to use as value for 100% TAR.
[2)] sum of unknown peaks, each < 2% TAR

2. Enantiomer ratio during hydrolytic conditions

The relative amounts of S-enantiomer and R-enantiomer before and after incubation were similar, indicating absence of any significant change of the enantiomer ratio upon incubation at hydrolytic conditions (see Table 6.5.1-3).

Table 6.5.1-3: Enantiomer ratio of BAS 750 F before and after treatment

test material	T-label					C-label				
	BAS 750 F *%TAR*			others *%TAR*	sum [1] *%TAR*	BAS 750 F *%TAR*			others *%TAR*	sum [1] *%TAR*
	R [2]	S [2]	sum	-	-	R [2]	S [2]	sum	-	-
before treatment										
pH 4, 90°C	51.2	48.8	100.0	-	100.0	49.1	50.1	99.2	0.8	100.0
pH 5, 100°C	49.6	50.4	100.0	-	100.0	50.1	48.7	98.8	1.2	100.0
pH 6, 120°C	50.1	49.9	100.0	-	100.0	48.4	50.2	98.5	1.5	100.0
after treatment										
pH 4, 90°C	54.4	55.1	109.5	0.7	110.2	55.0	54.5	109.5	0.7	110.2
pH 5, 100°C	55.7	54.5	110.2	-	110.2	52.9	55.4	108.3	-	108.3
pH 6, 120°C	53.3	51.9	105.2	-	105.2	55.4	54.0	109.4	0.7	110.1

[1] The actual concentration of each sample was determined by LSC before each test and set to 100 % TAR.
[2] R denotes R enantiomer (no. 5934591), S denotes S-enantiomer (no. 5434588)

III. CONCLUSION

Under conditions representative of industrial and household processing procedures, BAS 750 F is stable, namely representing pasteurisation (pH 4, 90 °C, 20 min), baking, boiling, brewing (pH 5, 100 °C, 60 min) and sterilisation (pH 6, 120 °C, 20 min). Notably, no degradation product exceeding 2% of total radioactivity was detected. Also, no change in the isomer ratio was observed. In conclusion, as BAS 750 F can be regarded as stable to hydrolysis, the nature of the residue is not affected by processing operations.

CA 6.5.2 Distribution of the residue in inedible peel and pulp

The representative uses to be evaluated in this dossier (cereals) are crops with no peel or edible peel only. Therefore, studies on the distribution between peel and pulp are not required.

CA 6.5.3 Magnitude of residues in processed commodities

Report:	CA 6.5.3/1 Plier S.,Elze M., 2015 a Determination of residues of BAS 750 F (Reg.No. 5834378) in wheat and its processed products after two applications of BAS 750 01 F in Germany, 2014 2014/1315283
Guidelines:	OECD 508 Magnitude of the Pesticide Residues in Processed Commodities (2008), OECD 509 Crop Field Trial (2009), OECD Series on Testing and Assessment No. 96 (2008) - Magnitude of Pesticide Residues in Processed Commodities, EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7035/VI/95 rev. 5, BBA IV 3-3, BBA IV 3-4, IVA Guideline IA-III (1992)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

During the 2014 growing season, three field trials were conducted with the product BAS 750 01 F in order to investigate the residue behavior of BAS 750 F in wheat and its processed products. The field trials were located in representative wheat growing areas in Germany. Each field trial included one treated plot. In addition, an application-free control plot was also included in one of the three field trials. The test item BAS 750 01 F, an EC formulation of BAS 750 F, was twice foliar applied at an exaggerated target rate of 4.5 L product/ha (nominal rate of 0.45 kg/ha BAS 750 F). The applications were made at crop growth stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha. Samples were taken at the following time points:

- DALA 0 (plants at BBCH 69, application control)
- DALA 7-9 (plants at BBCH 71, sub-samples processed into silage)
- DALA 45-60 (BBCH 89 (grain at BBCH 89)

The processing of wheat was conducted using sub-samples taken from grain (BBCH 89, DALA 45-60) as well as plants (BBCH 71, DALA 7-9). The following fractions of wheat were generated following industrial processing procedures at a laboratory scale: wet silage, wilted silage, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction.

Determination of residue levels of BAS 750 F and TDM (1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) in wheat grain (at commercial harvest, three separate trials) and processed fractions thereof allow the calculation of (average) transfer factors. Note, for 1,2,4-triazole and triazole lactic acid, transfer factors could not be calculated due to residue level <LOQ.

For BAS 750 F the average transfer factors for germ (TF=1.26), middlings (TF=2.69), shorts (TF=3.56), bran (TF=3.01), and aspirated grain fractions (TF=34.78) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, gluten, gluten feed meal, starch, whole meal flour, whole grain bread and milled byproducts. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.24 for wet silage and TF= 1.69 for wilted silage).

For TA the average transfer factors for germ (TF=1.33), middlings (TF=2.66), bran (TF=2.82), whole meal flour (TF=1.15) and shorts (TF=4.20) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, gluten, gluten feed meal as well as starch, whole grain bread, milled by-products and aspirated grain fractions. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.28 for wet silage and TF= 1.26 for wilted silage).

For TAA the average transfer factors for middlings (TF=1.34), gluten (TF=1.34), bran (TF=1.71), and shorts (TF=1.99) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, germ, gluten feed meal as well as starch, whole meal flour, whole grain bread, milled by-products and aspirated grain fractions. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.96 for wet silage and TF= 1.79 for wilted silage).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 01 F
Description: BAS 750 01 F
Lot/Batch #: FD-140113-0006
Purity: BAS 750 01 F (BAS /50 F 100 g/L, nominal)
CAS#: 40487-42-1
Development code: BAS 750 F
Spiking levels: 0.01, 1.0, 5.0 and 50.0 mg/kg (BAS 750 F)
0.01, 1.0, 2.0, 4.0 mg/kg (TDMs: 1,2,4-T, TA, TAA, TLA)

2. Test Commodity:
Crop: wheat
Type: *not applicable*
Variety: Cubus, Akteur, Ritmo
Botanical name: *Triticum aestivum* L.

Crop parts(s) or processed commodity:
whole plant without roots,
wet silage, wilted silage
grain,
bran,
flour,
germ,
middlings,
shorts,
gluten,
gluten feed meal,
starch,
whole meal flour,
whole grain bread,
milled byproducts,
aspirated grain fraction

Sample size: 0.5-107 kg (depending on the sample)

B. STUDY DESIGN

1. Test procedure

During the 2014 growing season, three field trials were conducted with the product BAS 750 01 F in order to investigate the residue behavior of BAS 750 F in wheat and its processed products. The field trials were located in representative wheat growing areas in Germany. Each field trial included one treated plot. In addition, an application-free control plot was also included in one of the three field trial (L140181). The test item BAS 750 01 F, an EC formulation of BAS 750 F, was twice foliar applied at an exaggerated target rate of 4.5 L product/ha (nominal rate of 0.45 kg/ha BAS 750 F). The applications were made at crop growth stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha. Samples were taken at the following time points:

- DALA 0 (plants at BBCH 69, application control)
- DALA 7-9 (plants at BBCH 71, sub-samples processed into silage)
- DALA 45-60 (BBCH 89 (grain at BBCH 89, sub-samples were processed into flour etc.)

The processing of wheat was conducted using sub-samples taken from grain (BBCH 89, DALA 45-60) as well as plants (BBCH 71, DALA 7-9). The following fractions of wheat were generated following industrial processing procedures at a laboratory scale:
wet silage, wilted silage, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction.

2. Description of analytical procedures

The level of residues was determined for BAS 750 F (BASF method L0076/09, LOQ of 0.01 mg/kg) and for the triazole derivative metabolites (TDM) 1,2,4-T, TA, TAA, TLA (BASF method L0170/02, LOQ of 0.01 mg/kg for each of the four analytes).

Principle of the method L0076/09: The analyte is extracted with methanol/water/HCl (70/25/5, v/v/v). After extraction with cyclohexane, an aliquot is concentrated, dissolved in methanol/water (50/50, v/v) and transferred into an autosampler vial for LC-MS/MS analysis (method validated for BAS 750 F analysis in plant matrices).

Principle of the method L0170/02: The analytes are extracted with methanol/water (4/1, v/v), an aliquot is filtered, concentrated and cleaned-up by a simple dispersive C18-SPE-step. The analytes are determined by LC-DMS/MS/MS (method validated for TDM analysis in plant matrices, i.e. 1,2,4-T, TA, TAA, TLA).

Recovery results are summarized below in Table 6.5.3-1

Table 6.5.3-1: Summary of fortification experiments

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0076/09		**BAS 750 F**			
whole plant (no root), wet silage, wilted silage, grain, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction	0.01, 1.0, 5.0, 50	44	86.2	6.9	8.0
method L0170/02		**1,2,4-triazole**			
whole plant (no root), wet silage, wilted silage, grain, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction	0.01, 1.0, 2.0, 4.0	68	97.3	12	13
method L0170/02		**TA**			
whole plant (no root), wet silage, wilted silage, grain, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction	0.01, 1.0, 2.0, 4.0	61	89.4	16	18

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	RSD (%)
method L0170/02		**TAA**			
whole plant (no root), wet silage, wilted silage, grain, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction	0.01, 1.0, 2.0, 4.0	64	89.5	14	16
method L0170/02		**TLA**			
whole plant (no root), wet silage, wilted silage, grain, bran, flour, germ, middlings, shorts, gluten, gluten feed meal, starch, whole meal flour, whole grain bread, milled byproducts and aspirated grain fraction	0.01, 1.0, 2.0, 4.0	66	90.6	15	16

II. RESULTS AND DISCUSSION

The transfer of BAS 750 F and triazole derivative metabolites (TDM) from wheat grain and plant to processed fractions was determined following two applications of BAS 750 01 F at exaggerated rates of a (nominal) rate of 0.45 kg BAS 750 F/ha at crop growth stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha.

To assess the comparability among the three trials (i.e. amount of formulated product applied and residues in the unprocessed sample material), indicative samples were taken prior to harvest, plants at DALA 0 and DALA 7-9 as well as grain at DALA 45-60. For all three trials similar residue levels of BAS 750 F were determined in plant directly after last application (5.9 mg/kg, 7.0 mg/kg and 7.9 mg/kg decreasing to 3.0-4.4 mg/kg within 7-9 days). Residues in grain showed some variation with one trial having higher residues in grain (0.013 mg/kg) than the other two trials (0.017 and 0.034 mg/kg). No residues of BAS 750 F were detected in the untreated control.

Residues of TDMs in plants directly after the last application (whole plant, no roots) were lower (TA up to 0.27 mg/kg, TAA up to 0.15 mg/kg, TLA up to 0.11 mg/kg with TAA and TLA decreasing by approximately 40% until DALA 7-9). Samples taken from the untreated control plot show the treatment unrelated background levels, with detectable TDM amounts in plants of TA (0.1 mg/kg), of TLA and TAA (0.08 mg/kg) and of grain (TA 0.4 mg/kg, TAA 0.17 mg/kg). The residue levels of TDM determined in samples of the untreated control plot and processed fractions thereof are summarized in Table 6.5.3-6.

BAS 750 F

BAS 750 F residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.13 or 0.017 or 0.034 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculate an average transfer factor (TF). Results are summarized in Table 6.5.3-2.

Transfer factors indicating reduction of residues during processing (TF < 1.00), were obtained for flour, gluten, gluten feed meal as well as starch, whole meal flour, whole grain bread and milled by-products. Increase of residue levels are determined for germ (TF=1.26), middlings (TF=2.69), shorts (TF=3.56), bran (TF=3.01), and aspirated grain fractions (TF=34.78).

BAS 750 F residue levels in silage compared with residue levels in plant (prior to processing 6.9 or 2.5 or 3.2 mg/kg) were used to calculate transfer factors for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculate an average transfer factor (TF). Transfer factor indicates a slight increase of residues in silage (1.24 for wet silage and 1.69 for wilted silage).

Table 6.5.3-2: Transfer factors for BAS 750 F in wheat processed fractions

matrix	BAS 750 F [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF 1	TF 2	TF 3	TF mean
RAC GRAIN [1)]	***0.13***	***0.017***	***0.034***	**1.00**	**1.00**	**1.00**	**1.00**
bran	*0.31*	*0.063*	*0.10*	2.38	3.71	2.94	**3.01**
flour	*< 0.01*	*< 0.01*	*< 0.01*	< 0.08	< 0.59	< 0.29	**< 0.32**
germ	*0.11*	*0.031*	*0.038*	0.85	1.82	1.12	**1.26**
middlings	*0.25*	*0.066*	*0.077*	1.92	3.88	2.26	**2.69**
shorts	*0.34*	*0.077*	*0.12*	2.62	4.53	3.53	**3.56**
gluten	*0.072*	*< 0.01*	*0.015*	0.55	< 0.59	0.44	**< 0.53**
gluten feed meal	*0.038*	*< 0.01*	*< 0.01*	0.29	< 0.59	< 0.29	**< 0.39**
starch	*< 0.01*	*< 0.01*	*< 0.01*	< 0.08	< 0.59	< 0.29	**< 0.32**
whole meal flour	*0.10*	*0.017*	*0.027*	0.77	1.00	0.79	**0.85**
whole grain bread	*0.070*	*< 0.01*	*0.019*	0.54	< 0.59	0.56	**< 0.56**
milled byproducts	*0.081*	*0.019*	*0.014*	0.62	1.12	0.41	**0.72**
aspirated grain fraction	*5.0*	*0.37*	*1.5*	38.46	21.76	44.12	**34.78**
RAC PLANT [2)]	***6.9***	***2.5***	***3.2***	**1.00**	**1.00**	**1.00**	**1.00**
silage, wet	*7.6*	*3.6*	*3.8*	1.10	1.44	1.19	**1.24**
silage, wilted	*8.0*	*4.7*	*6.5*	1.16	1.88	2.03	**1.69**
plant (DALA 0) [3)]	*7.0*	*7.6*	*5.9*	-	-	-	-
plant (DALA 7-9) [3)]	*3.1*	*3.0*	*4.4*	-	-	-	-
grain (DALA 45-60) [3)]	*0.14*	*0.024*	*0.028*	-	-	-	-

[1)] RAC, sub-sample of grain used for processing
[2)] RAC, whole plant without roots, sub-sample of plant used for processing
[3)] samples taken on field (purpose: application control)
[4)] transfer factor is equivalent to the US "concentration factor"
[5)] trial numbers are L140181 (trial 1), L140182 (trial 2), L140183 (trial 3)
[6)] for calculation purposes residue level of "< 0.01" are set to a value of "0.01"

1,2,4-triazole

No residues above the limit of quantitation were found in any field sample or processed fraction (both for untreated and treated samples). Transfer factors were therefore not determined.

TA

TA residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.27 – 0.48 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculated an average transfer factor (TF). Results for TA are summarized in Table 6.5.3-3

Transfer factors below or around 1.00 were obtained for flour, gluten, gluten feed meal as well as starch, whole meal flour, whole grain bread, milled by-products and aspirated grain fractions. Increase of residue levels are indicated by the transfer factors for germ (TF=1.33), for middlings (TF=2.66), for bran (TF=2.82) and for shorts (TF=4.20).

For silage, average transfer factors indicate a slight increase of residues (wet silage TF=1.28, wilted silage TF=1.26). TAA levels in plant samples prior to processing were 0.13 - 0.31 mg/kg.

Table 6.5.3-3: Transfer factors for TA in wheat processed fractions

matrix	TA [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	mean TF
RAC GRAIN [1)]	***0.48***	***0.27***	***0.35***	**1.00**	**1.00**	**1.00**	**1.00**
bran	*1.0*	*0.95*	*1.0*	2.08	3.52	2.86	**2.82**
flour	*0.20*	*0.19*	*0.18*	0.42	0.70	0.51	**0.54**
germ	*0.18*	*0.71*	*0.34*	0.38	2.63	0.97	**1.33**
middlings	*1.2*	*0.74*	*0.96*	2.50	2.74	2.74	**2.66**
shorts	*1.7*	*1.6*	*1.1*	3.54	5.93	3.14	**4.20**
gluten	*0.067*	*0.14*	*0.18*	0.14	0.52	0.51	**0.39**
gluten Feed Meal	*0.090*	*0.052*	*0.094*	0.19	0.19	0.27	**0.22**
starch	*< 0.01*	*< 0.01*	*< 0.01*	< 0.02	< 0.04	< 0.03	**< 0.03**
whole meal flour	*0.44*	*0.41*	*0.35*	0.92	1.52	1.00	**1.15**
whole grain bread	*0.35*	*0.37*	*0.30*	0.73	1.37	0.86	**0.99**
milled byproducts	*0.28*	*0.36*	*0.039*	0.58	1.33	0.11	**0.67**
aspirated grain fraction	*0.33*	*0.26*	*0.22*	0.69	0.96	0.63	**0.76**
RAC PLANT [2)]	***0.31***	***0.13***	***0.19***	**1.00**	**1.00**	**1.00**	**1.00**
wet silage	*0.28*	*0.17*	*0.31*	0.90	1.31	1.63	**1.28**
wilted silage	*0.18*	*0.23*	*0.27*	0.58	1.77	1.42	**1.26**
plant (DALA 0) [3)]	*0.26*	*0.090*	*0.12*	-	-	-	-
plant (DALA 7-9) [3)]	*<0.01*	*0.14*	*0.29*	-	-	-	-
grain (DALA 45-60) [3)]	*0.44*	*0.43*	*0.36*	-	-	-	-

1) RAC, sub-sample of grain used for processing
2) RAC, whole plant without roots, sub-sample of plant used for processing
3) samples taken on field (purpose: application control)
4) transfer factor is equivalent to the US "concentration factor"
5) trial numbers are L140181 (trial 1), L140182 (trial 2), L140183 (trial 3)
6) for calculation purposes residue level of "< 0.01" are set to a value of "0.01"

TAA

TAA residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.12 – 0.32 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculated an average transfer factor (TF). Results for TAA are summarized in Table 6.5.3-4
Transfer factors below or around 1.00 were obtained for flour, germ, gluten feed meal as well as starch, whole meal flour, whole grain bread, milled by-products and aspirated grain fractions. Increase of residue levels are indicated by the transfer factors for middlings (TF=1.34), gluten (TF=1.34), bran (TF=1.71) and shorts (TF=1.99).

For silage, average transfer factors indicate increase of residues (wet silage TF=1.96, wilted silage TF=1.79). TAA levels in plant samples prior to processing were 0.06 - 0.1 mg/kg.

Table 6.5.3-4: Transfer factors for TAA in wheat processed fractions

matrix	TAA [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	mean TF
RAC GRAIN[1)]	***0.32***	***0.20***	***0.12***	**1.00**	**1.00**	**1.00**	**1.00**
bran	*0.41*	*0.27*	*0.30*	1.28	1.35	2.50	**1.71**
flour	*0.26*	*0.16*	*0.18*	0.81	0.80	1.50	**1.04**
germ	*0.15*	*0.14*	*0.14*	0.47	0.70	1.17	**0.78**
middlings	*0.48*	*0.22*	*0.17*	1.50	1.10	1.42	**1.34**
shorts	*0.73*	*0.34*	*0.24*	2.28	1.70	2.00	**1.99**
gluten	*0.28*	*0.23*	*0.24*	0.88	1.15	2.00	**1.34**
gluten feed meal	*0.19*	*0.19*	*0.14*	0.59	0.95	1.17	**0.90**
starch	*0.012*	*< 0.01*	*< 0.01*	0.04	< 0.05	< 0.08	**< 0.06**
whole meal flour	*0.25*	*0.18*	*0.17*	0.78	0.90	1.42	**1.03**
whole grain bread	*0.38*	*0.15*	*0.15*	1.19	0.75	1.25	**1.06**
milled byproducts	*0.20*	*0.13*	*0.19*	0.63	0.65	1.58	**0.95**
aspirated grain fraction	*0.20*	*0.083*	*0.14*	0.63	0.42	1.17	**0.74**
RAC PLANT [2)]	***0.099***	***0.056***	***0.10***	**1.00**	**1.00**	**1.00**	**1.00**
wet silage	*0.25*	*0.082*	*0.19*	2.53	1.46	1.90	**1.96**
wilted silage	*0.13*	*0.11*	*0.21*	1.31	1.96	2.10	**1.79**
plant (DALA 0) [3)]	*0.14*	*0.049*	*0.15*	-	-	-	-
plant (DALA 7-9) [3)]	*0.090*	*0.058*	*0.10*	-	-	-	-
grain (DALA 45-60) [3)]	*0.16*	*0.093*	*0.14*	-	-	-	-

1) RAC, sub-sample of grain used for processing
2) RAC, whole plant without roots, sub-sample of plant used for processing
3) samples taken on field (purpose: application control)
4) transfer factor is equivalent to the US "concentration factor"
5) trial numbers are L140181 (trial 1), L140182 (trial 2), L140183 (trial 3)
6) for calculation purposes residue level of "< 0.01" are set to a value of "0.01"

TLA

1,2,4-triazole

TLA residue levels in grain prior to processing were below the LOQ of 0.01 mg/kg. Transfer factors were therefore not determined. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=2.96 for wet silage and TF= 3.19 for wilted silage). Results for TAA are summarized in Table 6.5.3-5.

Table 6.5.3-5: Transfer factors for TLA in wheat processed fractions

matrix	TLA [mg/kg]			Transfer factor [6]			
	trial 1[5]	trial 2[5]	trial 3[5]	TF1	TF2	TF3	mean TF
RAC GRAIN [1]	***< 0.01***	***< 0.01***	***< 0.01***	-	-	-	-
bran	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
flour	*0.023*	*< 0.01*	*< 0.01*	-	-	-	-
germ	*0.022*	*< 0.01*	*0.084*	-	-	-	-
middlings	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
shorts	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
gluten	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
gluten feed meal	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
starch	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
whole meal flour	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
whole grain bread	*< 0.01*	*< 0.01*	*0.011*	-	-	-	-
milled byproducts	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-
aspirated grain fraction	*0.018*	*0.017*	*0.019*	-	-	-	-
RAC PLANT [2]	***0.10***	***0.042***	***0.040***	**1.00**	**1.00**	**1.00**	**1.00**
wet silage	*0.20*	*0.11*	*0.17*	2.00	2.62	4.25	**2.96**
wilted silage	*0.22*	*0.12*	*0.18*	2.20	2.86	4.50	**3.19**
plant (DALA 0) [3]	*0.11*	*0.050*	*0.083*	-	-	-	-
plant (DALA 7-9) [3]	*0.047*	*0.046*	*0.052*	-	-	-	-
grain (DALA 45-60) [3]	*< 0.01*	*< 0.01*	*< 0.01*	-	-	-	-

[1] *RAC, sub-sample of grain used for processing*
[2] *RAC, whole plant without roots, sub-sample of plant used for processing*
[3] *samples taken on field (purpose: application control)*
[4] *transfer factor is equivalent to the US "concentration factor"*
[5] *trial numbers are L140181 (trial 1), L140182 (trial 2), L140183 (trial 3)*

Table 6.5.3-6: Summary of residues in untreated samples

matrix	residues [mg/kg]		
	TA	**TAA**	**TLA**
wet silage	*0.14*	*0.11*	*0.15*
wilted silage	*0.11*	*0.11*	*0.14*
bran	*0.83*	*0.29*	*< 0.01*
flour	*0.072*	*0.16*	*< 0.01*
germ	*0.28*	*0.12*	*< 0.01*
middlings	*0.37*	*0.19*	*< 0.01*
shorts	*0.37*	*0.28*	*< 0.01*
gluten	*0.067*	*0.21*	*< 0.01*
gluten feed meal	*0.046*	*0.10*	*< 0.01*
starch	*< 0.01*	*0.014*	*< 0.01*
whole meal flour	*0.20*	*0.19*	*< 0.01*
whole grain bread	*0.24*	*0.12*	*< 0.01*
milled byproducts	*0.18*	*0.12*	*< 0.01*
aspirated grain fraction	*0.20*	*0.11*	*0.021*
plant (DALA 0) [1)]	*0.10*	*0.081*	*0.087*
plant (DALA 7-9) [1)]	*0.13*	*0.081*	*0.074*
grain (DALA 45-60) [1)]	*0.42*	*0.17*	*< 0.01*

[1)] *samples were taken on the field*

III. CONCLUSION

BAS 750 F and TDM residue data in wheat grain (at commercial harvest, three separate trials) and processed fractions thereof allow the calculation of (average) transfer factors. Note, for 1,2,4-triazole and triazole lactic acid, transfer factors could not be calculated due to residue level <LOQ.

For BAS 750 F the average transfer factors for germ (TF=1.26), middlings (TF=2.69), shorts (TF=3.56), bran (TF=3.01), and aspirated grain fractions (TF=34.78) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, gluten, gluten feed meal, starch, whole meal flour, whole grain bread and milled byproducts. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.24 for wet silage and TF= 1.69 for wilted silage).

For TAA the average transfer factors for middlings (TF=1.34), gluten (TF=1.34), bran (TF=1.71), and shorts (TF=1.99) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, germ, gluten feed meal as well as starch, whole meal flour, whole grain bread, milled by-products and aspirated grain fractions. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.96 for wet silage and TF= 1.79 for wilted silage).

For TA the average transfer factors for germ (TF=1.33), middlings (TF=2.66), bran (TF=2.82), and shorts (TF=4.20) indicate an increase of residues upon processing, while TF below 1.00 were obtained for flour, gluten, gluten feed meal as well as starch, whole meal flour, whole grain bread, milled by-products and aspirated grain fractions. Residue data from wheat plant and silage produced thereof indicates an increase of residues upon silaging (TF=1.28 for wet silage and TF= 1.26 for wilted silage).

Report:	CA 6.5.3/2 Plier S.,Elze M., 2015 b Determination of residues of BAS 750 F (Reg.No. 5834378) in barley and its processed products after two applications of BAS 750 01 F in Germany, 2014 2014/1315282
Guidelines:	IVA Guideline IA-III (1992), BBA IV 3-3, BBA IV 3-4, OECD 508 Magnitude of the Pesticide Residues in Processed Commodities (2008), OECD 509 Crop Field Trial (2009), OECD-ENV/JM/MONO(2008)23, EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7035/VI/95 rev. 5
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landesentwicklung, Dresden, Germany)

EXECUTIVE SUMMARY

During the 2014 growing season, three field trials were conducted with the formulated product BAS 750 01 F in order to investigate the residue behavior of BAS 750 F in barley and its processed products. The field trials were located in representative barley growing areas in Germany. Each field trial included one treated plot. In addition, an application-free control plot was also included in one of the three field trial. The test item BAS 750 01 F, an EC formulation of BAS 750 F was twice foliar applied at an exaggerated target rate of 4.5 L product/ha (nominal rate: 0.45 kg/ha BAS 750 F). The applications were made at crop stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha. Samples were taken at the following timepoints,

- DALA 0 (plants at BBCH 69, purpose application control)
- DALA 43-56 (grain at BBCH 89)

The processing of barley was conducted using sub-samples taken from grain (BBCH 89, DALA 43-56). The following fractions of barley were generated following industrial processing procedures at a laboratory scale: pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried) and brewers yeast.

Determination of residue levels of BAS 750 F and TDM (1,2,4-triazole, triazole alanine, triazole acetic acid, triazole lactic acid) in barley grain (at commercial harvest, three separate trials) and processed fractions thereof allow the calculation of (average) transfer factors. Note, only for 1,2,4-triazole transfer factors could not be calculated due to residue level <LOQ.

For BAS 750 F the average transfer factors for flour (TF=3.78), bran (TF=4.90), malt sprouts (TF=1.24), and dried brewers grain (TF=2.31) while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and brewers yeast.

For TA the average transfer factors flour (TF=1.21), bran (TF=1.64), malt sprouts (TF=2.84), and brewers yeast (TF=1.18) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and dried brewers grain.

For TAA the average transfer factors for flour (TF=2.08), bran (TF=1.50), and malt sprouts (TF=2.66) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and brewers yeast.

For TLA the average transfer factors for flour (TF=3.57), and beer (TF=2.44) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, bran, brewing malt, malt sprouts, dried brewers grain and brewers yeast.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 01 F
Description: BAS 750 01 F
Lot/Batch #: FD-140113-0006
Purity: BAS 750 01 F (BAS /50 F 100 g/L, nominal)
CAS#: 40487-42-1
Development code: BAS 750 F

Spiking levels: 0.01, 1.0, 5.0 and 50.0 mg/kg (BAS 750 F)
0.01, 1.0 mg/kg (TDM: 1,2,4-T, TA, TAA, TLA)

2. Test Commodity:
Crop: barley
Type: *not applicable*
Variety: Quench, Grace
Botanical name: *Hordeum vulgare* L.
Crop parts(s) or processed commodity: pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast
Sample size: 0.1-53 kg (depending on sample)

B. STUDY DESIGN

1. Test procedure

During the 2014 growing season, three field trials were conducted with the formulated product BAS 750 01 F in order to investigate the residue behavior of BAS 750 F in barley and its processed products. The field trials were located in representative barley growing areas in Germany. Each field trial included one treated plot. In addition, an application-free control plot was also included in one of the three field trial (L140178). The test item BAS 750 01 F, an EC formulation of BAS 750 F was twice foliar applied at an exaggerated target rate of 4.5 L product/ha (nominal rate: 0.45 kg/ha BAS 750 F). The applications were made at crop stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha. Samples were taken at the following timepoints,

- DALA 0 (plants at BBCH 69, purpose application control)
- DALA 43-56 (grain at BBCH 89, sub-samples processed into flour etc.)

The processing of barley was conducted using sub-samples taken from grain (BBCH 89, DALA 43-56). The following fractions of barley were generated following industrial processing procedures at a laboratory scale: pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried) and brewers yeast.

2. Description of analytical procedures

The level of residues was determined for BAS 750 F (BASF method L0076/09, LOQ of 0.01 mg/kg) and for the triazole derivative metabolites (TDM) 1,2,4-T, TA, TAA, TLA (BASF method L0170/02, LOQ of 0.01 mg/kg for each of the four analytes).

Principle of the method L0076/09: The analyte is extracted with methanol/water/HCl (70/25/5, v/v/v). After extraction with cyclohexane, an aliquot is concentrated, dissolved in methanol/water (50/50, v/v) and transferred into an autosampler vial for LC-MS/MS analysis (method validated for BAS 750 F analysis in plant matrices).

Principle of the method L0170/02: The analytes are extracted with methanol/water (4/1, v/v), an aliquot is filtered, concentrated and cleaned-up by a simple dispersive C18-SPE-step. The analytes are determined by LC-DMS/MS/MS (method validated for TDM analysis in plant matrices, i.e. 1,2,4-T, TA, TAA, TLA).

A summary on the results of the recovery experiments is given in Table 6.5.3-7

Table 6.5.3-7: Summary of the fortification experiments

matrix	fortification level (mg/kg)	summary recoveries			
		n	mean (%)	SD (+/-)	n
method L0076/09		**BAS 750 F**			
whole plant (no root), grain, pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast	0.01, 1.0, 5.0, 50	31	88.3	5.9	6.7
method L0170/02		**1,2,4-triazole**			
whole plant (no root), grain, pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast	0.01, 1.0	46	94.9	9.6	10
method L0170/02		**TA**			
whole plant (no root), grain, pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast	0.01, 1.0	42	90.3	13	14
method L0170/02		**TAA**			
whole plant (no root), grain, pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast	0.01, 1.0	46	89.2	13	15
method L0170/02		**TLA**			
whole plant (no root), grain, pearled barley (pot barley), flour, bran, brewing malt, malt sprouts, beer, brewers grain (dried), brewers yeast	0.01, 1.0	46	90.8	12	14

II. RESULTS AND DISCUSSION

The transfer of BAS 750 F and triazole derived metabolites (TDM) from barley grain to processed fractions was determined following two applications of BAS 750 01 F at exaggerated rates of a (nominal) rate of 0.45 kg BAS 750 F/ha at crop growth stages BBCH 49 and BBCH 69 using a spray volume of 200 L/ha. To assess the comparability among the three trials (i.e. amount of formulated product applied and residues in the unprocessed sample material), indicative samples were taken prior to harvest, plants at DALA 0 and grain at DALA 43-56. For all three trials similar residue levels of BAS 750 F were determined in plant directly after last application (12.0 mg/kg, 9.3 mg/kg and 15.0 mg/kg) as well as in grain at DALA 43-56 (0.35mg/kg, 0.21 mg/kg, 0.23 mg/kg). No residues of BAS 750 F were detected in the untreated control.

Residues of TDMs in plants directly after the last application (whole plant, no roots) were lower (TA up to 0.076 mg/kg, TAA up to 0.076 mg/kg, TLA up to 0.12 mg/kg). Samples taken from the untreated control plot show the treatment-unrelated background levels, with detectable TDM amounts in plants of TA (0.023 mg/kg), TAA (0.073 mg/kg) of TLA (0.067 mg/kg) and in grain of TA (0.096 mg/kg), TAA (0.11 mg/kg) of TLA (0.033 mg/kg). The residue levels of TDM determined in samples of the untreated control plot and processed fractions thereof are summarized in Table 6.5.3-12

BAS 750 F

BAS 750 F residue levels in processed grain fractions compared with residue levels in grain (in sub-sample taken prior to processing 0.40 or 0.24 or 0.22 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and thus used to calculate the average transfer factor (TF). Results are summarized in

Table 6.5.3-8. Transfer factors indicating reduction of residues during processing (TF < 1.00), were obtained for pot barley, brewing malt, beer, and brewers yeast. Increase of residue levels are determined for flour (TF=3.78), bran (TF=4.90), malt sprouts (TF=1.24), and dried brewers grain (TF=2.31).

Table 6.5.3-8: Transfer factors for BAS 750 F in barley processed fractions

matrix	BAS 750 F [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	**mean TF**
RAC GRAIN [1)]	***0.40***	***0.24***	***0.22***	**1.00**	**1.00**	**1.00**	**1.00**
pearled barley (pot b.)	*0.065*	*0.029*	*0.018*	0.16	0.12	0.08	**0.12**
flour	*1.8*	*0.88*	*0.70*	4.50	3.67	3.18	**3.78**
bran	*1.7*	*1.2*	*1.2*	4.25	5.00	5.45	**4.90**
brewing malt	*0.20*	*0.12*	*0.067*	0.50	0.50	0.30	**0.43**
malt sprouts	*0.67*	*0.23*	*0.24*	1.68	0.96	1.09	**1.24**
beer	*< 0.01*	*< 0.01*	*< 0.01*	< 0.03	< 0.04	< 0.05	**< 0.04**
brewers grain (dried)	*0.95*	*0.58*	*0.47*	2.38	2.42	2.14	**2.31**
brewers yeast	*0.076*	*0.064*	*0.042*	0.19	0.27	0.19	**0.22**
plant (DALA 0)	*12*	*9.3*	*15*	-	-	-	-
grain (DALA 43-56) [2)]	*0.35*	*0.21*	*0.23*	-	-	-	-

1) RAC, sub-sample of grain used for processing
2) RAC, whole plant without roots, sub-sample of plant used for processing
3) samples taken on field (purpose: application control)
4) transfer factor is equivalent to the US "concentration factor"
5) trial numbers are L140178 (trial 1), L140179 (trial 2), L140180 (trial 3).
6) for calculation purposes residue level of "< 0.01" are set to a value of "0.01".

1,2,4-triazole

No residues above the limit of quantitation were found in any field sample or processed fraction (both for untreated and treated samples). Transfer factors were therefore not determined.

TA

TA residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.20 – 0.53 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculate an average transfer factor (TF). Results for TA are summarized in Table 6.5.3-9. Transfer factors below or around 1.00 were obtained for pot barley, brewing malt, beer, and dried brewers grain. Increase of residue levels are indicated by the average transfer factors for flour (TF=1.21), bran (TF=1.64), malt sprouts (TF=2.84), and brewers yeast (TF=1.18).

Table 6.5.3-9: Transfer factors for TA in barley processed fractions

matrix	TA [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	mean TF
RAC GRAIN [1)]	***0.25***	***0.20***	***0.53***	**1.00**	**1.00**	**1.00**	**1.00**
pearled barley (pot b.)	*0.21*	*0.27*	*0.15*	0.84	1.35	0.28	**0.82**
flour	*0.27*	*0.24*	*0.71*	1.08	1.20	1.34	**1.21**
bran	*0.52*	*0.47*	*0.26*	2.08	2.35	0.49	**1.64**
brewing malt	*0.045*	*0.29*	*0.27*	0.18	1.45	0.51	**0.71**
malt sprouts	*0.33*	*1.1*	*0.91*	1.32	5.50	1.72	**2.85**
beer	*< 0.01*	*< 0.01*	*< 0.01*	< 0.04	< 0.05	< 0.02	**< 0.04**
brewers grain (dried)	*< 0.01*	*< 0.01*	*< 0.01*	< 0.04	< 0.05	< 0.02	**< 0.04**
brewers yeast	*0.15*	*0.56*	*0.069*	0.60	2.80	0.13	**1.18**
plant (DALA 0)	*0.076*	*0.055*	*0.049*	-	-	-	-
grain (DALA 43-56) [2)]	*0.16*	*0.55*	*0.54*	-	-	-	-

1) RAC, sub-sample of grain used for processing
2) RAC, whole plant without roots, sub-sample of plant used for processing
3) samples taken on field (purpose: application control)
4) transfer factor is equivalent to the US "concentration factor"
5) trial numbers are L140178 (trial 1), L140179 (trial 2), L140180 (trial 3).
6) for calculation purposes residue level of "< 0.01" are set to a value of "0.01".

TAA

TAA residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.17 – 0.19 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculated an average transfer factor (TF). Results for TA are summarized in

Table 6.5.3-10. Transfer factors below or around 1.00 were obtained for pot barley, brewing malt, beer, dried brewers grain and brewers yeast. Increase of residue levels are indicated by the average transfer factors for flour (TF=2.08), bran (TF=1.50), and malt sprouts (TF=2.66).

Table 6.5.3-10: Transfer factors for TAA in barley processed fractions

matrix	**TAA [mg/kg]**			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	mean TF
RAC GRAIN [1)]	***0.19***	***0.18***	***0.17***	**1.00**	**1.00**	**1.00**	**1.00**
pearled barley (pot b.)	*0.16*	*0.11*	*0.12*	0.84	0.61	0.71	**0.72**
flour	*0.36*	*0.38*	*0.38*	1.89	2.11	2.24	**2.08**
bran	*0.22*	*0.24*	*0.34*	1.16	1.33	2.00	**1.50**
brewing malt	*0.17*	*0.20*	*0.14*	0.89	1.11	0.82	**0.94**
malt sprouts	*0.40*	*0.57*	*0.46*	2.11	3.17	2.71	**2.66**
beer	*0.028*	*< 0.01*	*0.025*	0.15	< 0.06	0.15	**< 0.12**
brewers grain (dried)	*0.022*	*0.011*	*0.014*	0.12	0.06	0.08	**0.09**
brewers yeast	*0.042*	*0.044*	*0.038*	0.22	0.24	0.22	**0.23**
plant (DALA 0)	*0.074*	*0.059*	*0.076*	-	-	-	-
grain (DALA 43-56) [2)]	*0.17*	*0.18*	*0.19*	-	-	-	-

1) *RAC, sub-sample of grain used for processing*
2) *RAC, whole plant without roots, sub-sample of plant used for processing*
3) *samples taken on field (purpose: application control)*
4) *transfer factor is equivalent to the US "concentration factor"*
5) *trial numbers are L140178 (trial 1), L140179 (trial 2), L140180 (trial 3)*
6) *for calculation purposes residue level of "< 0.01" are set to a value of "0.01"*

TLA

TA residue levels in processed grain fractions compared with residue levels in grain (prior to processing 0.055 – 0.14 mg/kg) were used to calculate transfer factor for each trial (TF1, TF2, TF3). Overall, comparable transfer factors were obtained and used to calculate an average transfer factor (TF). Results for TLA are summarized in

Table 6.5.3-11. Transfer factors below or around 1.00 were obtained for pot barley, bran, brewing malt, malt sprouts, dried brewers grain and brewers yeast. Increase of residue levels are indicated by the average transfer factors for flour (TF=3.57), and beer (TF=2.44).

Table 6.5.3-11: Transfer factors for TLA in barley processed fractions

matrix	TLA [mg/kg]			Transfer factor [4) 6)]			
	trial 1[5)]	trial 2[5)]	trial 3[5)]	TF1	TF2	TF3	mean TF
RAC GRAIN [1)]	***0.055***	***0.14***	***0.14***	**1.00**	**1.00**	**1.00**	**1.00**
pearled barley (pot b.)	*0.028*	*0.077*	*0.073*	0.51	0.55	0.52	**0.53**
flour	*0.36*	*0.54*	*0.045*	6.55	3.86	0.32	**3.57**
bran	*0.035*	*0.11*	*0.055*	0.64	0.79	0.39	**0.61**
brewing malt	*0.021*	*0.032*	*0.011*	0.38	0.23	0.08	**0.23**
malt sprouts	*< 0.01*	*< 0.01*	*< 0.01*	< 0.18	< 0.07	< 0.07	**< 0.11**
beer	*0.25*	*0.15*	*0.24*	4.55	1.07	1.71	**2.44**
brewers grain (dried)	*< 0.01*	*< 0.01*	*< 0.01*	< 0.18	< 0.07	< 0.07	**< 0.11**
brewers yeast	*0.040*	*0.042*	*0.021*	0.73	0.30	0.15	**0.39**
plant (DALA 0)	*0.087*	*0.12*	*0.062*	-	-	-	-
grain (DALA 43-56) [3)]	*0.049*	*0.17*	*0.13*	-	-	-	-

1) RAC, sub-sample of grain used for processing
2) RAC, whole plant without roots, sub-sample of plant used for processing
3) samples taken on field (purpose: application control)
4) transfer factor is equivalent to the US "concentration factor"
5) trial numbers are L140178 (trial 1), L140179 (trial 2), L140180 (trial 3)
6) for calculation purposes residue level of "< 0.01" are set to a value of "0.01"

Table 6.5.3-12: Summary of residues in the untreated samples

matrix	**residues [mg/kg]**		
	TA	**TLA**	**TAA**
pearled barley (pot barley)	*0.026*	*0.087*	*0.010*
flour	*0.14*	*0.18*	*0.033*
bran	*0.079*	*0.13*	*0.046*
brewing malt	*0.075*	*0.14*	*< 0.01*
malt sprouts	*0.31*	*0.32*	*< 0.01*
beer	*< 0.01*	*0.023*	*0.14*
brewers grain (dried)	*0.029*	*0.011*	*< 0.01*
brewers yeast	*0.038*	*0.036*	*< 0.01*
plant (DALA 0)	*0.023*	*0.073*	*0.067*
grain (DALA 52) [1)]	*0.096*	*0.11*	*0.033*

1) samples were taken on the field

III. CONCLUSION

BAS 750 F and TDM residue data in barley grain (at commercial harvest, three separate trials) and processed fractions thereof allow the calculation of (average) transfer factors. Note, only for 1,2,4-triazole transfer factors could not be calculated due to residue level <LOQ.

For BAS 750 F the average transfer factors for flour (TF=3.78), bran (TF=4.90), malt sprouts (TF=1.24), and dried brewers grain (TF=2.31) while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and brewers yeast.

For TA the average transfer factors flour (TF=1.21), bran (TF=1.64), malt sprouts (TF=2.84), and brewers yeast (TF=1.18) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and dried brewers grain.

For (TAA) the average transfer factors for flour (TF=2.08), bran (TF=1.50), and malt sprouts (TF=2.66) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, brewing malt, beer, and brewers yeast.

For (TLA) the average transfer factors for flour (TF=3.57), and beer (TF=2.44) indicate an increase of residues upon processing, while a TF below 1.00 was obtained for pot barley, bran, brewing malt, malt sprouts, dried brewers grain and brewers yeast.

CA 6.6 Residues in Rotational Crops

To investigate residues in rotational crops resulting from the use of BAS 750 F both a nature of the residue study (CA 6.6.1) as well as a magnitude of the residue study (CA 6.6.2) was conducted. These studies provide detailed information, therefore a theoretical consideration of the nature and level of BAS 750 F residue would not provide additional knowledge and therefore is not included in the present dossier.

The metabolism and distribution of BAS 750 F in succeeding crops was investigated using the active substance, radiolabelled (^{14}C) either in the chlorophenyl ring (C-label) or in the triazole ring (T-label).
In the absence of any metabolite indicating cleavage of the ether bridge (e.g. no occurrence of C-labeled single-ring structures or T-labeled double-ring structures), the trifluorophenylmethyl ring (TFMP ring) in the middle of the parent backbone can be considered covered by the C-labeled samples. An additional study with a TFMP-label can be expected to provide the same results as the study with the C-label, thus would not provide additional information, and therefore was not conducted. For both, C-label and T-label, the test item was a mixture of ^{14}C-BAS 750 F and ^{13}C-BAS 750 F. The molecular structures and the position of isotopes are shown below:

BAS 750 F
= Reg. No. 5834378
= CAS No. 1417782-03-6

Figure 6.6/1 Chlorophenyl ring-U-C14-BAS 750 F (C-label)

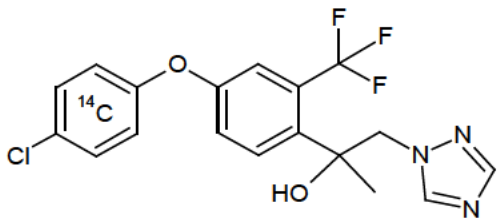


Figure 6.6/2 Chlorophenyl-1-C13-BAS 750 F (C-label)

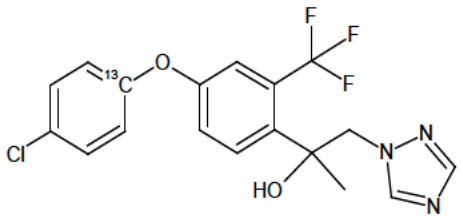


Figure 6.6/3 Triazole-3(5)-C14-BAS 750 F (T-label)

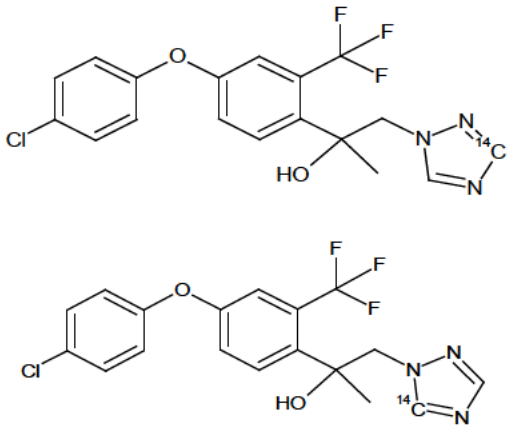
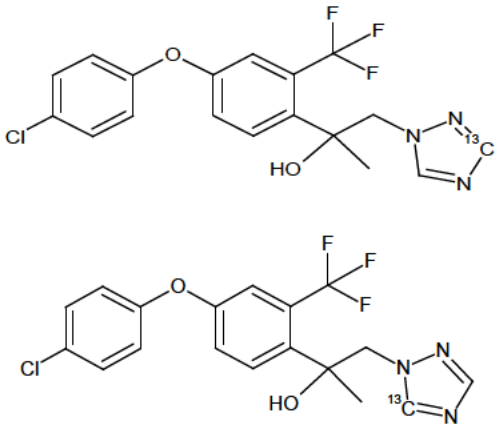


Figure 6.6/4 Triazole-3(5)-C13-BAS 750 F (T-label)



CA 6.6.1 Metabolism in rotational crops

Report:	CA 6.6.1/1 Rabe U.,Glaessgen W., 2015 a Confined rotational crop study with 14C LS 5834378 2015/1001871
Guidelines:	OECD 502 Metabolism in Rotational Crops (January 2007), EPA 860.1850: Confined Accumulation in Rotational Crops, EPA 860.1000, PMRA Residue Chemistry Guidelines Section 97.13 Confined Accumulation in Rotational Crops (Canada)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

A confined rotational crop study was conducted with ^{14}C-BAS 750 F either labeled in the chlorophenyl-ring (C-label) or labeled in the triazole ring (T-label). The active substance was applied as EC formulation (EXP 5834378F-AW) to bare loamy sand soil at an application rate of 1 x 300 g as/ha, corresponding an initial BAS 750 F concentration in soil of 0.1 mg/kg. The nature and the concentration of radioactive residues were investigated in representative rotational crops, namely spinach leaf, white radish root and top as well as in spring wheat forage, hay, straw/chaff and grain after plant back intervals of 30, 120 and 365 days. Plant samples were harvested at maturity. Additional immature stages of spinach and wheat forage (used for hay production) were taken from spinach (cultivation interval 25 to 28 days), and wheat forage (used for hay production, cultivation interval 49 to 53 days). Soil samples were taken after ploughing and after harvest of the mature crops for each plant back interval. Homogenized samples were analysed including combustion (total radioactive residue, TRR), solvent extraction, solubilization treatment and HPLC chromatography (radiodetection).

TRR data indicates uptake of radioactive residue from the soil for all representative crops. In most crop parts, residues remained similar or decreased at longer replant intervals. For all crop parts, several fold higher residues were seen with the T-label compared with the C-label indicating the presence (and plant uptake) of T-label-specific cleavage products (as defined by absence of C-label). The largest difference was determined for grain (DAT 30) with TRR of 2.311 mg/kg (T-label) and 0.014 mg/kg (C-label).

Extractability by solvents of parent BAS 750 F and TDMs generally was high. With the T-label solvent extracted >90% TRR except for straw/hay (82% or higherTRR). With the C-label extractability was moderate to high, with lower extractability correlating with lower TRR (spinach/radish/forage), high solubilization by enzymes (grain) and matrix type (straw/hay). Further radioactivity was released by solubilization (by treatment with ammonia and enzymes additional amounts of the residue were characterized as being associated with plant constituents, notably starch and cellulose/hemicellulose). The amounts of final residue indicate that to a low extent, radiocarbon was incorporated into insoluble cell constituents. For spinach, radish, forage and grain the final residue was < 0.01 mg/kg (in samples with low total radioactive residue representing higher proportions of the TRR). For hay (corresponding to dried forage) and straw the final residue was 0.05 mg/kg or below. Taken together the residues either identified or characterized, the residue resulting from BAS 750 F soil treatment was quantitatively investigated.

The amount of parent BAS 750 F in rotational crops is generally lower than the amount of TDMs as seen in the metabolic profile of T-labeled samples. Major amounts of BAS 750 F are only seen in spinach leaf (up to 25% TRR in immature leaf, with TDMs amounting to 65% TRR). In other T-labeled samples, BAS 750 F was present at < 6% TRR. Notably, BAS 750 F was not detected in grain. This was confirmed in C-labeled samples, where BAS 750 F was determined at similar absolute amounts (samples of spinach and radish up to 0.01 mg/kg, samples of forage at 0.015 mg/kg, samples of straw up to 0.10 mg/kg). The parent BAS 750 F is present in the plant as a racemic mixture of the S-enantiomer and the R-enantiomer, thus the 1:1 ratio of the test item remained unchanged indicating absence of preferential metabolisation and/or uptake of one of the two enantiomers.

The TDM amounts, since generally exceeding the BAS 750 F amounts, explain the different TRRs obtained with both labels. Particular high levels were determined in T-labeled grain (up to 3.2 mg/kg), while levels in C-labeled grain were < 0.04 mg/kg. Note, that plants were grown in plastic boxes with limited drainage. It is assumed that this has enhanced the accessibility of TDM for plant uptake. Consequently, quantitative data obtained in this study is to be considered indicative only. Data on the magnitude of the residue under field conditions, is provided in section 6.6.2 (BAS 750 F field rotational crop study with representative rotational crops).

The metabolic pathway thus consists of cleavage of the parent backbone structure at the triazole bridge, releasing 1,2,4-triazole, which is further transformed to the derivative metabolites TA, TAA, and TLA. This cleavage is assumed to occur mainly in the soil (see below) but might occur also within the plant. The data obtained shows that parent and cleavage products are taken up by rotational crops from the soil followed by translocation within the plant.

In summary, the metabolism of BAS 750 F in rotational crops has been comprehensively studied and is thus well understood. Parent BAS 750 F and the TDMs were identified as the major components of the residue. No components specific to rotational crops were detected. Based on the residue level found a field rotational crop study is required.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 750 F

Description:
C-label:
Chlorophenyl-U-C14-BAS 750 F,
Chlorophenyl-1-C13-BAS 750 F
T-label:
Triazole-3(5)-C14-BAS 750 F,
Triazole-3(5)-C13-BAS 750 F

Lot/Batch No.:
CFQ41561
(Chlorophenyl-U-C14-BAS 750 F, 7.878 MBq/mg)
RS4-2012-173A2
(Chlorophenyl-1-C13-BAS 750 F)
1063-1101
(Triazole-3(5)-C14-BAS 750 F, 5.4 MBq/mg)
1077-1001
(Triazole-3(5)-C13-BAS 750 F)

Purity:

Chlorophenyl-U-C14-BAS 750 F:	99.1%
(radiochemical purity 98.9%)	
Chlorophenyl-1-C13-BAS 750 F:	97.7%
Triazole-3(5)-C14-BAS 750 F:	98.9%
(radiochemical 98.2%)	
Triazole-3(5)-C13-BAS 750 F:	97.1%

CAS No.: 1417782-03-6
Development code: BAS 750 F
Stability test compound: The test item was stable over the test period.

2. Test Commodity:

Crop:	spinach	radish	wheat
Type:	*(not relevant)*	white	spring
Variety:	*Corvette*	*April Cross*	*Thasos*
Botanical name:	*Spinacia oleracea*	*Raphanus sativus*	*Triticum aestivum*
Crop part / processed commodity:	immature leaf, mature leaf	top, root,	forage, hay, straw, grain

3. Soil: sandy loam (properties given in Table 6.6.1-1).

Table 6.6.1-1: Soil Physicochemical Properties

soil series	soil type	pH	OM %	sand %	silt%	slay %	maximal water holding capacity	CEC cmol/kg
Bruch West	sandy loam [1]	7.4 [2]	2.54 [3]	72.1	16.6	11.2	26.6 g / 100 g dry soil	11.2

[1] USDA scheme, [2] ($CaCl_2$) , [3] organic matter, corresponds to the total organic carbon (TOC)

B. STUDY DESIGN

The study was conducted during the period from March 2013 to April 2015 by BASF Agricultural Center, Limburgerhof, Germany.

1. Test procedure

The representative crops spinach, white radish and spring wheat were grown in plastic containers (0.365 m x 0.56 m = 0.20 m^2) filled with a sandy loam soil. Aging of the soil and subsequent cultivation of crops was done under natural climatic conditions except for absence of rain, i.e. depending on the outdoor climatic conditions, boxes were located either in a phytotron or a glass house or a vegetation hall (glass roof).

Application of the test item was performed in an automatic spray track system. The test item (a mixture of ^{14}C-/^{13}C-BAS 750 F combined with the blank EC formulation EXP 5834378F-AW and water) was applied to bare soil at a nominal application rate of 300 g as/ha.
The crops spinach, white radish, and spring wheat were cultivated after soil aging intervals of 30d (31d), 120d (122d), and 365d (364d) with the C-label (T-label) including a simulated "ploughing" step prior to start of a cultivation period (i.e. mixing 20 cm top soil layer).

2. Sampling

For rotational crops both mature and immature growth stages were sampled. For spinach both as immature leaf (BBCH 15-19) and mature leaf (BBCH 49) were sampled with the root remaining in the soil. Radish was sampled when mature (BBCH49), and separated into edible part (radish root) and the remaining green part (radish top). Wheat was harvested both as immature green plant (wheat forage BBCH37-39) and mature straw and ears (BBCH 89). A subsample of wheat forage was dried to obtain wheat hay (BBCH37-39). Mature ears were separated by threshing into wheat grain and chaff. Chaff and straw were combined to obtain the sample wheat straw. For soil samples were taken immediately after "ploughing" (i.e. at the end of soil aging intervals) as well as after harvest (i.e. crop maturation). Plant and soil samples were stored frozen at -20°C or below during the course of the study.

3. Description of analytical procedures

Aliquots of homogenised plant or soil samples were subjected to oxidative combustion (liquid scintillation measurement) to determine total radioactive residue (TRR combusted). In addition, aliquots were subjected to repetitive solvent extraction (methanol three times, water two times). ERR was calculated as the sum of radioactivity in the extracts. Residual radioactive residues (RRR) was determined by combustion of the residue after solvent extraction. TRR calculated was obtained as the sum of ERR and RRR.

The nature of the residue in extracts was investigated using two different HPLC methods (radiodetection). Assignment of chromatographic peaks and identification of components of the residue was based on co-chromatography with ^{14}C-labelled reference items as well as on comparison of retention times and elution pattern (metabolic profile). Further characterization of the RRR was done by sequential solubilization including treatments with aqueous ammonia, amylases/amyloglucosidase, macerozyme/cellulase, and tyrosinase/laccase.

II. RESULTS AND DISCUSSION

1. TOTAL RADIOACTIVE RESIDUE (TRR)

TRR in soil

TRR in soil was determined directly after "ploughing" as well as after harvest of a mature crop (see Table 6.6.1-2). For both labels (C-label/T-label), residue levels were similar and only slightly decreased from the start of crop cultivation until the date of harvesting (30 DAT: 0.094/0.094 mg/kg, 120 DAT: 0.080/0.095 mg/kg, 365 DAT: 0.064/0.072 mg/kg). No significant change of the TRR was observed during crop cultivation.

Table 6.6.1-2: TRR of soil treated with ^{14}C-BAS 750 F

soil samples	C-label		T-label	
	DAT [1)]	TRR [2)] *[mg/kg]*	DAT [1)]	TRR [2)] *[mg/kg]*
plant back interval:	**30**		**31**	
soil after ploughing	30	0.094	31	0.094
after harvest of mature crops				
soil post cultivation of spinach	71	0.083	75	0.109
soil post cultivation of radish	98	0.108	101	0.072
soil post cultivation of wheat	135	0.087	136	0.081
plant back interval:	**120**		**122**	
soil after ploughing	120	0.080	122	0.095
after harvest of mature crops				
soil post cultivation of spinach	161	0.086	165	0.094
soil post cultivation of radish	177	0.065	181	0.075
soil post cultivation of wheat	264	0.085	270	0.072
plant back interval:	**365**		**364**	
soil after ploughing	365	0.064	364	0.072
after harvest of mature crops				
soil post cultivation of spinach	405	0.070	410	0.075
soil post cultivation of radish	426	0.063	425	0.067
soil post cultivation of wheat	502	0.054	502	0.068

[1)] days after treatment (bare soil application of ^{14}C-BAS 750 F), [2)] TRR=total radioactive residue determined by combustion

TRR in plants

TRR for rotational crops of different replant intervals was determined by combustion ("TRR combusted"). For samples extracted, comparable values were obtained for the "TRR calculated" (sum of ERR and RRR) and were therefore used as "100% TRR" for all further calculations (see Table 6.6.1-3, for non-extracted samples "TRR combusted" was used). In contrast to soil samples, significantly different results were obtained with the two labels.

Table 6.6.1-3: TRR in rotational crops cultivated on ^{14}C-BAS 750 F- treated soil

crop parts	C-label			T-label		
	DAP [1]	**TRR** [2] *combusted [mg/kg]*	**TRR** [2] *calculated [mg/kg]*	**DAP**	**TRR** *combusted [mg/kg]*	**TRR** *calculated [mg/kg]*
plant back interval 30/31 DAT [1]						
spinach (immature)	28	*0.016*	*0.013*	25	*0.055*	*0.052*
spinach (mature)	41	*0.014*	*0.009*	44	*0.063*	*0.057*
radish (top)	68	*0.013*	*0.011*	70	*0.194*	*0.186*
radish (root)	68	*0.010*	*0.009*	70	*0.281*	*0.267*
wheat (forage)	49	*0.027*	*0.021*	53	*0.318*	*0.288*
wheat (hay)	49	*0.085*	*0.076*	53	*0.761*	*0.681*
wheat (straw) [4]	105	*0.240*	*0.239*	105	*1.058*	*1.039*
wheat (grain)	105	*0.015*	*0.014*	105	*2.400*	*2.311*
plant back interval 120/122 DAT [1]						
spinach (immature)	33	*0.011*	*0.009*	32	*0.114*	*0.116*
spinach (mature)	41	*0.016*	*0.014*	43	*0.171*	*0.150*
radish (top)	57	*0.006*	*0.006*	59	*0.209*	*0.197*
radish (root)	57	*0.009*	*0.008*	59	*0.206*	*0.198*
wheat (forage)	50	*0.030*	*0.024*	52	*0.417*	*0.387*
wheat (hay)	50	*0.181*	*0.155*	52	*2.561*	*2.260*
wheat (straw) [4]	144	*0.105*	*0.094*	148	*1.102*	*1.008*
wheat (grain)	144	*0.039*	*0.039*	148	*3.389*	*3.252*
plant back interval 365/364 DAT [1]						
spinach (immature)	27	*0.007*	- [3]	33	*0.096*	*0.094*
spinach (mature)	40	*0.007*	- [3]	46	*0.108*	*0.097*
radish (top)	61	*0.005*	- [3]	61	*0.100*	*0.100*
radish (root)	61	*0.005*	- [3]	61	*0.093*	*0.098*
wheat (forage)	55	*0.012*	*0.010*	54	*0.189*	*0.193*
wheat (hay)	55	*0.035*	*0.033*	54	*0.873*	*0.860*
wheat (straw) [4]	137	*0.078*	*0.076*	138	*0.947*	*0.916*
wheat (grain)	137	*0.032*	*0.033*	138	*2.258*	*2.221*

[1] *DAT=days after soil treatment (soil aging interval), DAP=days after planting/sowing (cultivation interval),*
[2] *TRR=sum of ERR and RRR (ERR: methanol extract and water extract, RRR: residues after solvent extraction),*
[3] *no extraction performed,*
[4] *straw samples including chaff fraction (see Study design)*

With the C-label, (calculated) TRR was at low levels (maximal 0.02 mg/kg, decreasing further at longer replant intervals) was seen for spinach (similar results for mature and immature leaf), for radish (similar results for root and top) and for wheat forage. Higher residues in wheat hay essentially reflect the loss of water during its production from forage (0.08, 0.16, 0.03 mg/kg for the three replant intervals). In straw (including chaff), levels were higher at short replant interval (30 DAT: 0.24 mg/kg) and decreased to 0.10 mg/kg (120 DAT) and 0.08 mg/kg (365 DAT). The TRR in grain was low at 30 DAT (0.014 mg/kg) increasing towards longer replant intervals (120 DAT: 0.039 mg/kg, 365 DAT: 0.033 mg/kg).

With the T-label, throughout replant intervals DAT31/122/364, significantly higher TRR level were found in spinach (mature leaf: 0.06/0.15/0.10 mg/kg, similar in immature leaf), in radish (root: 0.27/0.20/0.10 mg/kg, similar in top), in forage (0.29/0.39/0.19 mg/kg) and straw (1.0/1.0/0.9 mg/kg). Note that the residues in hay (0.68/2.26/0.86 mg/kg) essentially reflect the loss of water during its production from forage. In grain the largest TRR difference between T-label (2.3/3.3/2.2 mg/kg) and C-label (<0.04 mg/kg) was seen.

Taken together, the TRR values obtained indicate uptake of radioactive residue from the soil for all representative crops. In most crop parts, residues remained similar or decreased at longer replant intervals (excepted for C-labeled grain). For all crop parts, several fold higher residues were seen with the T-label compared with the C-label indicating the presence (and plant uptake) of T-label-specific cleavage products (as defined by absence of C-ring). Note that in crop metabolism studies similar label-specific differences were seen (see section CA 6.2).
The largest difference was determined for grain (DAT30/31) with TRR of 2.3 mg/kg (T-label) and only 0.014 mg/kg (C-label). Note that plants were cultivated in plastic boxes (see section Material and Methods) with limited drainage. This is assumed to enhance the accessibility of mobile compounds to uptake by plant roots.

2. EXTRACTABILITY

Data on extractability of radioactive residue is summarized in Table 6.6.1-4 (C-label) and Table 6.6.1-5 (T-label). With the two labels, significantly different results were obtained.

With the C-label, solvent extraction of spinach and radish resulted in low RRR (maximum 0.005 mg/kg). Given the low TRR in these samples, the RRR represented up to 17-60% TRR while the corresponding ERR was 40-82% TRR (ERR was extracted mainly by methanol, while water extracted only an additional 2-6% TRR with the exception of radish top where water extraction amounted to 8-12% TRR). Similar extractability was seen for wheat forage. RRR was low (max 0.014 mg/kg, representing up to 61% of the low TRR). Hay containing several fold higher residues than forage (water loss during production from forage) showed similar extractabilities resulting in RRR up to 58% TRR (representing 0.09 mg/kg). Both for forage and hay, methanol extraction (35-70% TRR) was more effective than water (only additional 2-7% TRR).
In straw/chaff, the proportion of the residue not extracted by solvent was high (35%, 53% and 66% TRR for DAT 30, 120, 365). With longer replant intervals, the methanol extractable residue decreased from 54% to 26% TRR, while the water extractable residue decreased from 12 to 8% TRR.

In grain, the predominant proportion of the residue was not extracted by solvent resulting in RRR of 79 to 92% TRR. Extraction with methanol and water did retrieve similar amounts of residue (7-11% TRR), with the exception of one grain sample (30 DAT, where methanol did not extract any detectable residue).

Table 6.6.1-4: Extractability of radioactive residues in rotational crops (C-Label)

crop part	distribution of radioactive residues								
	TRR [1]	methanol extract		water extract		ERR [1]		RRR [1]	
	[mg/kg]	**%TRR**	*[mg/kg]*	**%TRR**	*[mg/kg]*	**%TRR**	*[mg/kg]*	**%TRR**	*[mg/kg]*
plant back interval 30 DAT [1]									
spinach, immature	*0.013*	**80.5**	*0.010*	**2.2**	*0.0003*	**82.7**	*0.011*	**17.3**	*0.002*
spinach, mature	*0.009*	**78.0**	*0.007*	**1.8**	*0.0002*	**79.8**	*0.007*	**20.2**	*0.002*
radish, top	*0.011*	**67.3**	*0.007*	**12.0**	*0.0013*	**79.3**	*0.008*	**20.7**	*0.002*
radish, root	*0.009*	**53.2**	*0.005*	**2.4**	*0.0002*	**55.6**	*0.005*	**44.4**	*0.004*
wheat, forage	*0.021*	**68.7**	*0.015*	**2.1**	*0.0005*	**70.8**	*0.015*	**29.2**	*0.006*
wheat, hay	*0.076*	**70.0**	*0.053*	**5.0**	*0.0038*	**74.9**	*0.057*	**25.1**	*0.019*
wheat, straw [4]	*0.239*	**53.7**	*0.128*	**11.6**	*0.0278*	**65.3**	*0.156*	**34.7**	*0.083*
wheat, grain	*0.014*	**0.0**	*0.000*	**7.8**	*0.0011*	**7.8**	*0.001*	**92.2**	*0.013*
plant back interval 120 DAT [1]									
spinach, immature	*0.009*	**63.9**	*0.006*	**5.0**	*0.0005*	**68.9**	*0.006*	**31.1**	*0.003*
spinach, mature	*0.014*	**53.8**	*0.007*	**6.0**	*0.0008*	**59.8**	*0.008*	**40.2**	*0.005*
radish, top	*0.006*	**31.7**	*0.002*	**8.3**	*0.0005*	**39.9**	*0.002*	**60.1**	*0.004*
radish, root	*0.008*	**54.2**	*0.005*	**4.1**	*0.0003*	**58.2**	*0.005*	**41.8**	*0.004*
wheat, forage	*0.024*	**40.0**	*0.010*	**3.5**	*0.0009*	**43.5**	*0.011*	**56.5**	*0.014*
wheat, hay	*0.155*	**35.0**	*0.054*	**6.8**	*0.0106*	**41.8**	*0.065*	**58.2**	*0.090*
wheat, straw [4]	*0.094*	**38.7**	*0.036*	**8.7**	*0.0082*	**47.4**	*0.045*	**52.6**	*0.050*
wheat, grain	*0.039*	**11.4**	*0.004*	**9.9**	*0.0039*	**21.2**	*0.008*	**78.8**	*0.031*
plant back interval 365 DAT [1]									
wheat, forage	*0.010*	**34.8**	*0.004*	**4.6**	*0.0005*	**39.4**	*0.004*	**60.6**	*0.006*
wheat, hay	*0.033*	**44.5**	*0.014*	**6.1**	*0.0020*	**50.6**	*0.016*	**49.4**	*0.016*
wheat, straw [4]	*0.076*	**25.5**	*0.019*	**8.3**	*0.0063*	**33.8**	*0.026*	**66.2**	*0.050*
wheat, grain	*0.033*	**7.3**	*0.002*	**7.8**	*0.0025*	**15.1**	*0.005*	**84.9**	*0.028*

[1] DAT=days after soil treatment, TRR=total radioactive residue calculated as sum of ERR and RRR, ERR=extractable radioactive residue calculated as sum of the methanol extract and water extract, RRR=radioactive residue after solvent extraction, for precise values see tables 6.6.1-8 and 6.6.1-9 (further characterization of RRR), [4] straw samples including chaff fraction

With the T-label, extractabilities were higher, thus correlating with the higher TRR when compared to the C-label (see section 1 on TRR). In addition, no replant-interval-dependency was observed.
Solvent extraction of spinach and radish samples resulted in RRR <5% TRR (maximum 0.009 mg/kg, thus similar to RRR with the C-label). Extraction was most efficient with methanol (81% TRR or higher) while water extracted <5% TRR (except for radish tops 8-14% TRR).
For forage and hay similar extractability pattern were observed with RRR at 6-13% TRR, the ERR was mostly extracted with methanol (forage 85% TRR, hay 57-83% TRR), to lesser extent with water (forage 6% TRR, hay 11-30%).

Table 6.6.1-5: Extractability of radioactive residues in rotational crops (T-Label)

crop part	distribution of radioactive residues								
	TRR [1)]	**methanol extract**		**TRR** [1)]		**ERR** [1)]		**TRR** [1)]	
	[mg/kg]	**% TRR**	*[mg/kg]*	**% TRR**	*[mg/kg]*	**% TRR**	*[mg/kg]*	**% TRR**	*[mg/kg]*
plant back interval 31 DAT [1)]									
spinach, immature	*0.052*	**93.4**	*0.049*	**2.4**	*0.0012*	**95.7**	*0.050*	**4.3**	*0.002*
spinach, mature	*0.057*	**95.2**	*0.054*	**1.3**	*0.0008*	**96.6**	*0.055*	**3.4**	*0.002*
radish, top	*0.186*	**87.3**	*0.162*	**7.7**	*0.0144*	**95.1**	*0.177*	**4.9**	*0.009*
radish, root	*0.267*	**92.7**	*0.248*	**4.2**	*0.0112*	**96.9**	*0.259*	**3.1**	*0.008*
wheat, forage	*0.288*	**87.0**	*0.251*	**5.8**	*0.0167*	**92.8**	*0.267*	**7.2**	*0.021*
wheat, hay	*0.681*	**78.9**	*0.537*	**13.5**	*0.0921*	**92.4**	*0.630*	**7.6**	*0.052*
wheat, straw [4)]	*1.039*	**57.6**	*0.598*	**24.6**	*0.2559*	**82.2**	*0.854*	**17.8**	*0.185*
wheat, grain	*2.311*	**47.5**	*1.097*	**46.0**	*1.0634*	**93.5**	*2.161*	**6.5**	*0.150*
plant back interval 122 DAT [1)]									
spinach, immature	*0.116*	**93.1**	*0.108*	**2.3**	*0.0026*	**95.4**	*0.110*	**4.6**	*0.005*
spinach, mature	*0.150*	**92.2**	*0.139*	**2.7**	*0.0040*	**94.9**	*0.143*	**5.1**	*0.008*
radish, top	*0.197*	**81.4**	*0.160*	**14.1**	*0.0278*	**95.5**	*0.188*	**4.5**	*0.009*
radish, root	*0.198*	**91.8**	*0.182*	**4.3**	*0.0086*	**96.2**	*0.190*	**3.8**	*0.008*
wheat, forage	*0.387*	**84.9**	*0.328*	**6.4**	*0.0247*	**91.3**	*0.353*	**8.7**	*0.034*
wheat, hay	*2.260*	**56.7**	*1.282*	**30.0**	*0.6772*	**86.7**	*1.959*	**13.3**	*0.302*
wheat, straw [4)]	*1.008*	**68.2**	*0.687*	**20.0**	*0.2018*	**88.2**	*0.889*	**11.8**	*0.119*
wheat, grain	*3.252*	**49.3**	*1.603*	**46.4**	*1.5087*	**95.7**	*3.111*	**4.3**	*0.141*

Table 6.6.1-5: Extractability of radioactive residues in rotational crops (T-Label)

crop part	distribution of radioactive residues								
	TRR [1)]	methanol extract		TRR [1)]		ERR [1)]		TRR [1)]	
	[mg/kg]	% TRR	*[mg/kg]*	% TRR	*[mg/kg]*	% TRR	*[mg/kg]*	% TRR	*[mg/kg]*
plant back interval 364 DAT [1)]									
spinach, immature	*0.094*	**94.6**	*0.089*	**2.1**	*0.0020*	**96.7**	*0.091*	**3.3**	*0.003*
spinach, mature	*0.097*	**94.2**	*0.091*	**2.3**	*0.0022*	**96.4**	*0.093*	**3.6**	*0.003*
radish, top	*0.100*	**83.6**	*0.083*	**12.9**	*0.0128*	**96.5**	*0.096*	**3.5**	*0.004*
radish, root	*0.098*	**96.4**	*0.094*	**2.3**	*0.0023*	**98.7**	*0.097*	**1.3**	*0.001*
wheat, forage	*0.193*	**85.6**	*0.165*	**6.2**	*0.0120*	**91.8**	*0.177*	**8.2**	*0.016*
wheat, hay	*0.860*	**83.0**	*0.715*	**10.8**	*0.0928*	**93.8**	*0.807*	**6.2**	*0.053*
wheat, straw [4)]	*0.916*	**48.9**	*0.448*	**33.8**	*0.3095*	**82.7**	*0.757*	**17.3**	*0.159*
wheat, grain	*2.221*	**40.6**	*0.901*	**52.9**	*1.1742*	**93.4**	*2.075*	**6.6**	*0.146*

[1)] *TRR=total radioactive residue calculated as sum of ERR and RRR, ERR=extractable radioactive residue calculated as sum of the methanol extract and water extract, RRR=radioactive residue after solvent extraction, for precise values see* Table 6.6.1-11 (further characterization of RRR), [4)] straw samples including the chaff fraction

In straw/chaff, extractability was >82% TRR (methanol extraction with 49-68% TRR exceeding water extraction with 20-34% TRR). Given TRR at 1.0 mg/kg, the RRR of <18% represented residue levels of up to 0.19 mg/kg.
In grain, extractability was very high (93% TRR or higher) with similar amounts obtained by methanol and water extraction steps (41-53% TRR). The RRR (< 7% TRR) represented up to 0.15 mg/kg.

Taken together, the extractability data shows that the higher TRR with the T-label correlate with higher extractability. This indicates that the T-label specific components of the residue (see section 1 on TRR) are not only present at high levels but also to a large extent solvent-extractable (and thus accessible to further analysis). Only smaller amounts of T-label specific compounds remain associated with the RRR (reflected by the T-label RRR exceeding the C-label RRR when expressed in mg/kg).

3. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

Identified residue components

Data on identification of radioactive components of the residue (ERR and RRR) is summarized in Table 6.6.1-6 (C-label) and Table 6.6.1-7 (T-label). For the C-label, representative data on identity of residue components could be obtained for all crop parts (except grain), notably for spinach (30 DAT, 120 DAT), radish (30 DAT), wheat forage, hay, straw (DAT30, DAT120, DAT365). For the T-label, data on identity of residue components could be obtained for all crop parts/replant intervals including grain. Correlating with TRR and extractability data, significantly different results were obtained with the two labels.

With the C-label, the identification rate appears to reflect the amounts of residue retrieved by extraction and solubilization, thus is high for spinach at DAT30 (>85% TRR, lower at DAT120) and moderate for radish at DAT30 (55 and 62% TRR for root and top). Importantly, the identification rate is fully attributed to unchanged parent BAS 750 F as the only identified component of the radioactive residue. Notably, no residues of parent BAS 750 F were found in grain. Thus, in spinach (mature leaf), parent BAS 750 F amounted to 85% TRR (0.008 mg/kg, DAT30) and 52% of TRR (0.007 mg/kg, DAT120). Similar values were obtained for immature leaf (91% and 61% TRR). In radish, parent BAS 750 F amounted to 55% TRR (top, DAT30) and 62% (root, DAT30). In forage, parent BAS 750 F amounted to 70%, 44%, 18% TRR (DAT30, 120, 365), in hay 62%, 36% and 41% TRR and in straw 43%, 36%, 24% indicating a decrease of BAS 750 F from DAT30 to DAT365 (forage: from 0.015 to 0.002 mg/kg, hay: from 0.046 to 0.013 mg/kg, for straw: from 0.101 to 0.018 mg/kg).

With the T-label, identification rate was generally high with >88% TRR in spinach, >78% TRR in radish, > 89% TRR in grain, >83% TRR in forage/hay, and >73% TRR in straw.
Unchanged parent BAS 750 F was detected at levels of 0.01 mg/kg in spinach (mature, immature leaf) and radish top, at level of <0.015 mg/kg in forage and <0.043 mg/kg in straw, thus similar absolute amounts as seen in C-labeled samples representing however much smaller proportions of the residue (spinach maximum 25% TRR, radish top 6 % TRR, forage/hay/straw at maximum 5 % TRR). Parent was not present in detectable amounts in spinach leaf, radish top and wheat forage of DAT122/365, or generally in radish roots and wheat grain.

The predominant components of the residue were the triazole derived metabolites 1,2,4-triazole, triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA). Different to parent, similar levels for all aging interval were seen.
In spinach of all replant intervals, TA was the predominant component with 53-56% of TRR (corresponding to 0.03 – 0.08 mg/kg). The second most abundant component was TLA with 18-33% TRR (0.01 – 0.05 mg/kg). Lower amounts were seen for 1,2,4-triazole (8-12% TRR and TAA (4-5% TRR). Similar results were found for immature spinach with TA amounting up to 71% TRR (0.07 mg/kg). In these samples, BAS 750 F was present in major amounts (14% and 25% in mature and immature spinach).
TA was also the predominant component in radish root (62-79% TRR, 0.08-0.17 mg/kg) and radish top (45-94% TRR), with TLA as the second most abundant component (0-11% TRR), and lower levels of TAA (0-4.5% TRR) and 1,2,4-triazole (0-2.6% TRR). BAS 750 F was present only in minor amounts (<6% TRR).
In forage and hay, TA and TLA were present in similar amounts (forage: TA 38-44% TRR, TLA 34-38% TRR, hay: TA 32-46% TRR, TLA 24-38% TRR, lower amounts of TAA (8-10% TRR) and 1,2,4-triazole (0-4% TRR) whereas BAS 750 F was present only in minor amounts (at maximum at 5% TRR).
In straw, TLA was the component present in highest amounts (34-39% TRR), followed by TA (13-33%) and TAA (15-19% TRR), BAS 750 F was present only in minor amounts (<5% TRR)
In grain, a different metabolite pattern was seen: the predominant component was TA (43-73 % TRR), the second most abundant was TAA (20-24% TRR), together amounting to 63-94% TRR. 1,2,4-triazole was only seen in lower amounts (15% TRR at 30 DAT, 1% at 365 DAT), as was TLA (14% TRR at 30 DAT). BAS 750 F was not detected in grain.

Characterized residue components

Characterization of radioactive residue addressed soluble component (characterization of ERR by HPLC, refer to study report for detailed data) as well as non-soluble components (characterization of the RRR by enzyme treatment). Data on solubilization by sequential solubilization of RRR is summarized in Table 6.6.1-8 (C-label) and Table 6.6.1-9 (T-label). Label specific differences were seen upon sequential treatment with ammonia (AM), starch-degrading enzymes amylase/amyloglucosidase (A/G), cell wall degrading enzymes macerozyme/cellulase (M/C) and polyphenol-oxidases tyrosinase/laccase (T/L).

With the C-label, RRR of spinach (mature leaf, DAT30, 0.002 mg/kg), radish root (30 DAT, 0.004 mg/kg) and radish top (30 DAT, 0.002 mg/kg) were subjected to AM followed by enzyme digestion (M/C) obtaining similar results. For spinach, treatment of the RRR (20% TRR) resulted in characterization of 10% TRR (3% AM, 7% M/C, leaving a final residue of 6% TRR (0.0005 mg/kg). For radish top, treatment of the RRR (21% TRR) resulted in characterization of 10% TRR (4% AM, 6% M/C, leaving a final residue of 10% TRR (0.0010 mg/kg). For radish root, treatment of the RRR (44% TRR) resulted in characterization of 14% TRR (4% AM, 10% M/C, leaving a final residue of 31% TRR (0.0029 mg/kg).

RRR of forage from replant intervals DAT30 (0.006 mg/kg), DAT120 (0.014 mg/kg), and DAT365 (0.006 mg/kg) was subjected to AM followed by enzyme digestion (M/C, the DAT365 sample in addition to T/L). Treatment of RRRs (representing 29%, 57% and 61% TRR) resulted in characterization of 12%, 38% and 20% TRR leaving final residues of <0.004 mg/kg (<13-32% TRR). Similar characterization was obtained for RRR of hay (0.019 mg/kg, 0.091 mg/kg, 0.016 mg/kg). Treatments resulted in characterization of 12%, 34% and 15% TRR leaving final residues of <0.04 mg/kg.

RRR of straw from replant intervals DAT30 (0.083 mg/kg), DAT120 (0.050 mg/kg), and 365 DAT (0.050 mg/kg) was subjected to AM followed by enzyme digestion (M/C, T/L). Treatment of RRRs (representing 35%, 53% and 66% TRR) resulted in characterization of 11%, 20% and 14% TRR leaving final residues of up to 0.05 mg/kg (21-51%% TRR). Most solubilization was obtained with AM and M/C while T/L added only 1.9% TRR or less.

RRR of grain from replant intervals DAT30 (0.01 mg/kg), DAT120 (0.03 mg/kg), and 365 DAT (0.03 mg/kg) was subjected to AM followed by two enzyme digestion steps (M/C, A/G). Treatment of RRRs (representing 92%, 79% and 85% TRR) resulted in characterization of the major proportion of the RRR (representing 72%, 62% and 50% TRR) leaving final residues of <0.008 mg/kg. The three treatments released similar amounts of the residue (15-30% TRR).

With the T-label, RRR of forage from replant intervals DAT30 (0.021 mg/kg), DAT120 (0.034 mg/kg), and 365 DAT (0.016 mg/kg) was subjected to solubilization including treatment with AM, with M/C, and with T/L). Treatment of RRRs (representing 7-8% TRR) resulted in characterization of 3.5-5.5% TRR leaving final residues of <0.01 mg/kg (<3% TRR).
Similar characterization was obtained for RRR of hay (0.05 mg/kg, 0.30 mg/kg, 0.05 mg/kg representing 6-13% TRR). Treatments resulted in characterization of 4-10% TRR leaving final residues of <3% TRR (up to 0.06 mg/kg).

RRR of straw from replant intervals DAT30 (0.19 mg/kg), DAT120 (0.12 mg/kg), and DAT365 (0.16 mg/kg) was subjected to AM followed by enzyme digestion (M/C, and T/L). Treatment of RRRs (representing 12-18% TRR) resulted in characterization of 6-12% TRR leaving final residues of up to 0.05 mg/kg (<6% TRR). Most solubilization was obtained with AM (6-10%), less with M/C (<2% TRR) and T/L (<1% TRR).

RRR of grain from replant intervals DAT30 (0.15 mg/kg), DAT120 (0.14 mg/kg), and DAT365 (0.15 mg/kg) was subjected to AM followed by two enzyme digestion steps (A/AG, M/C). Treatment of RRRs (representing 4-7% TRR) resulted in characterization of the major proportion of the RRR (representing 3.6-5.4% TRR) leaving final residues of <0.7 % TRR (maximum 0.017 mg/kg). AM and A/G together released higher amounts than M/C indicating preferential association with starch compounds.

Taking the identification and characterization data together the TDM group appears to be the predominant component of the treatment-related residue (note, metabolites in common with a range of other azole fungicides). While TDMs were the most abundant components in all matrices, in the grain matrix, TDMs were the single component identified as well as present at particular high levels. Unchanged parent was also identified, albeit at much lower amounts (major amounts were only seen in spinach samples of the shortest replant interval, while occurrence in other samples was only minor non-detectable). In contrast, in C-labeled samples only unchanged parent BAS 750 F was detected (TDMs are excluded from radiodetection), explaining largely the differences of TRR and extractability obtained with both labels. Characterization by solubilization indicates that both TDMs and parent BAS 750 F associate with plant constituents, namely starch and cell wall polymers. Note, radioactivity represented in the final residue indicates that to a certain extent radiolabel is incorporated into non-solubilizable cell constituents.

Table 6.6.1-6: C-label: summary of identified components (ERR and RRR of rotational crop matrices [1])

component	matrices															
	spinach (imm)		spinach (mature)		radish top		radish root		wheat forage		wheat hay		wheat straw[3]		wheat grain	
	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]
plant back interval 30 DAT																
BAS 750 F	**91.2**	*0.012*	**85.2**	*0.008*	**54.8**	*0.006*	**61.5**	*0.006*	**70.4**	*0.015*	**61.6**	*0.046*	**42.5**	*0.101*	- [3]	- [3]
SUM [4]	**91.2**	*0.012*	**85.2**	*0.008*	**54.8**	*0.006*	**61.5**	*0.006*	**70.4**	*0.015*	**61.6**	*0.046*	**42.5**	*0.101*	-	-
plant back interval 120 DAT																
BAS 750 F	**60.8**	*0.006*	**51.7**	*0.007*	- [3]	- [3]	- [3]	- [3]	**43.7**	*0.011*	**35.7**	*0.055*	**35.7**	*0.034*	- [3]	- [3]
SUM [4]	**60.8**	*0.006*	**51.7**	*0.007*	-	-	-	-	**43.7**	*0.011*	**35.7**	*0.055*	**35.7**	*0.034*	-	-
plant back interval 365 DAT																
BAS 750 F	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	**17.7**	*0.002*	**41.0**	*0.013*	**23.9**	*0.018*	- [3]	- [3]
SUM [4]	-	-	-	-	-	-	-	-	**17.7**	*0.002*	**41.0**	*0.013*	**23.9**	*0.018*	-	-

[1] ERR=extractable radioactive residue, RRR=radioactive residue after solvent extraction, SUM includes all identified compounds, [2] straw including chaff, [3] not detected

Table 6.6.1-7: T-label: summary of identified components (ERR and RRR of rotational crop matrices[1])

component	matrices															
	spinach (imm)		spinach (mature)		radish top		radish root		wheat forage		wheat hay		wheat straw [2]		wheat grain	
	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]	%TRR	[mg/kg]
plant back interval 31 DAT																
1,2,4-triazole	**10.5**	*0.005*	**7.7**	*0.004*	- [3]	- [3]	- [3]	- [3]	**4.0**	*0.012*	**5.0**	*0.034*	**6.3**	*0.065*	**14.7**	*0.339*
TA	**42.9**	*0.022*	**56.0**	*0.032*	**45.4**	*0.084*	**61.8**	*0.165*	**43.1**	*0.124*	**45.6**	*0.311*	**13.2**	*0.137*	**42.5**	*0.982*
TAA	**2.5**	*0.001*	**5.5**	*0.003*	**4.2**	*0.008*	- [3]	- [3]	**10.9**	*0.031*	**20.8**	*0.142*	**15.0**	*0.156*	**20.0**	*0.462*
TLA	**8.8**	*0.005*	**18.3**	*0.010*	**22.4**	*0.042*	**30.9**	*0.083*	**33.7**	*0.097*	**23.7**	*0.162*	**35.3**	*0.366*	**13.8**	*0.319*
BAS 750 F	**25.2**	*0.013*	**13.9**	*0.008*	**5.6**	*0.010*	- [3]	- [3]	**5.0**	*0.015*	**4.5**	*0.031*	**4.1**	*0.043*	- [5]	- [5]
SUM [4]	**89.9**	*0.047*	**101.5**	*0.058*	**77.6**	*0.144*	**92.8**	*0.248*	**96.7**	*0.279*	**99.7**	*0.679*	**73.8**	*0.767*	**91.0**	*2.103*
plant back interval 122 DAT																
1,2,4-triazole	- [3]	- [3]	**12.4**	*0.019*	- [3]	- [3]	- [3]	- [3]	**3.9**	*0.015*	**4.5**	*0.101*	**2.6**	*0.026*	- [3]	- [3]
TA	**60.1**	*0.070*	**52.9**	*0.080*	**93.5**	*0.184*	**62.6**	*0.124*	**44.3**	*0.171*	**31.7**	*0.717*	**33.1**	*0.333*	**72.6**	*2.361*
TAA	**3.2**	*0.004*	- [5]	- [5]	- [5]	- [5]	- [5]	- [5]	**7.9**	*0.030*	**10.0**	*0.226*	**18.0**	*0.182*	**21.2**	*0.689*
TLA	**25.0**	*0.029*	**34.2**	*0.051*	- [5]	- [5]	**23.0**	*0.046*	**36.4**	*0.141*	**36.6**	*0.827*	**34.2**	*0.344*	- [3]	- [3]
BAS 750 F	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [5]	- [5]	**1.3**	*0.030*	**1.3**	*0.014*	- [3]	- [3]
SUM [4]	**88.4**	*0.102*	**99.5**	*0.150*	**93.5**	*0.184*	**85.6**	*0.169*	**92.4**	*0.357*	**84.1**	*1.901*	**89.2**	*0.899*	**93.8**	*3.050*
plant back interval 365 DAT																
1,2,4-triazole	- [5]	- [5]	**7.9**	*0.008*	**2.6**	*0.003*	- [3]	- [3]	- [3]	- [3]	**4.2**	*0.036*	**4.0**	*0.036*	**1.1**	*0.023*
TA	**71.1**	*0.067*	**56.2**	*0.054*	**77.5**	*0.077*	**79.1**	*0.077*	**37.7**	*0.073*	**38.3**	*0.330*	**16.6**	*0.152*	**64.2**	*1.425*
TAA	- [5]	- [5]	**3.7**	*0.004*	**4.5**	*0.004*	- [5]	- [5]	**8.7**	*0.017*	**11.7**	*0.101*	**18.9**	*0.173*	**24.3**	*0.539*
TLA	**25.0**	*0.023*	**33.6**	*0.032*	**10.7**	*0.011*	**17.5**	*0.017*	**37.5**	*0.072*	**37.5**	*0.323*	**38.6**	*0.354*	- [3]	- [3]
BAS 750 F	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	- [3]	**0.8**	*0.008*	- [3]	- [3]
SUM [4]	**96.1**	*0.090*	**101.4**	*0.098*	**95.2**	*0.095*	**96.6**	*0.095*	**83.8**	*0.162*	**91.7**	*0.789*	**78.9**	*0.722*	**89.5**	*1.987*

[1] ERR=extractable radioactive residue, RRR=radioactive residue after solvent extraction, SUM includes all identified compounds, [2] straw including chaff, [3] not detected

Table 6.6.1-8: C-label: characterization of RRR of spinach and radish matrices

solubilization fraction[1)]	matrices							
	spinach (imm)		spinach (mature)		radish top		radish root	
	% TRR	[mg/kg]	% TRR	[mg/kg]	% TRR	[mg/kg]	% TRR	[mg/kg]
plant back interval 30 DAT								
RRR	**17.3**	*0.0022*	**20.2**	*0.0018*	**20.7**	*0.0022*	**44.4**	*0.0041*
AM solubilizate [2)]	- [3)]	- [3)]	**2.9**	*0.0003*	**4.0**	*0.0004*	**4.2**	*0.0004*
AM residue	- [3)]	- [3)]	**14.3**	*0.0013*	**15.5**	*0.0016*	**40.7**	*0.0038*
A/G solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
A/G residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
M/C solubilizate	- [3)]	- [3)]	**7.3**	*0.0007*	**5.7**	*0.0006*	**10.1**	*0.0009*
M/C residue	- [3)]	- [3)]	**6.1**	*0.0005*	**10.3**	*0.0011*	**31.0**	*0.0029*
T/L solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
T/L residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
SUM SOLUBILIZED	- [3)]	- [3)]	**10.2**	*0.0009*	**9.7**	*0.0010*	**14.2**	*0.0013*
FINAL RESIDUE	- [3)]	- [3)]	**6.1**	*0.0005*	**10.3**	*0.0011*	**31.0**	*0.0029*
plant back interval 120 DAT								
RRR	**31.1**	*0.0029*-[3]	**40.2**	*0.0055*	**60.1**	*0.0037*	**41.8**	*0.0035*
AM solubilizate [2)]	- [3)]	- [3)]	**5.2**	*0.0007*	- [3)]	- [3)]	- [3)]	- [3)]
AM residue	- [3)]	- [3)]	**31.7**	*0.0043*	- [3)]	- [3)]	- [3)]	- [3)]
A/G solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
A/G residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
M/C solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
M/C residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
T/L solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
T/L residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
SUM SOLUBILIZED	-[3]	-[3]	**5.2**	*0.0007*	-[3]	-[3]	-[3]	-[3]
FINAL RESIDUE	-[3]	-[3]	**31.7**	*0.0043*	-[3]	-[3]	-[3]	-[3]
plant back interval 365 DAT								
RRR	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
AM solubilizate [2)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
AM residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
A/G solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
A/G residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
M/C solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
M/C residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
T/L solubilizate	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
T/L residue	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
SUM SOLUBILIZED	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]
FINAL RESIDUE	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]	- [3)]

[1)] AM, ammonia, A/G amylase/amyluglucosidase, M/C macerozyme/cellulase, T/L tyrosinase/laccase, [2)] combined ammonia solubilizate from two solubilisation steps in the cases of spring wheat hay, straw and grain; only one solubilisation step was performed in the cases of mature spinach, white radish top and root and spring wheat forage, 3) not analysed

Table 6.6.1-9: C-label: characterization of RRR of in wheat matrices

solubilization fraction[1)]	wheat matrices							
	forage		hay		straw [4)]		grain	
	% TRR	[mg/kg]	% TRR	[mg/kg]	% TRR	[mg/kg]	% TRR	[mg/kg]
plant back interval 30 DAT								
RRR	**29.2**	*0.0062*	**25.1**	*0.0189*	**34.7**	*0.0828*	**92.2**	*0.0132*
AM solubilizate [2)]	**6.1**	*0.0013*	**8.5**	*0.0064*	**7.7**	*0.0183*	**23.2**	*0.0033*
AM residue	**21.6**	*0.0046*	**17.1**	*0.0129*	**25.9**	*0.0618*	**63.5**	*0.0091*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**28.4**	*0.0041*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**31.3**	*0.0045*
M/C solubilizate	**5.4**	*0.0012*	**3.9**	*0.0030*	**2.2**	*0.0053*	**20.4**	*0.0029*
M/C residue	**13.3**	*0.0029*	**9.6**	*0.0072*	**22.8**	*0.0544*	**10.5**	*0.0015*
T/L solubilizate	-[3]	-[3]	-[3]	-[3]	**0.9**	*0.0020*	-[3]	-[3]
T/L residue	-[3]	-[3]	-[3]	-[3]	**21.6**	*0.0516*	-[3]	-[3]
SUM SOLUBILIZED	**11.5**	*0.0025*	**12.4**	*0.0094*	**10.8**	*0.0257*	**71.9 [2)]**	*0.0103*
FINAL RESIDUE	**13.3**	*0.0029*	**9.6**	*0.0072*	**21.6**	*0.0516*	**10.5**	*0.0015*
plant back interval 122 DAT								
RRR	**56.5**	*0.0138*	**58.2**	*0.0901*	**52.6**	*0.0495*	**78.8**	*0.0309*
AM solubilizate [2)]	**20.0**	*0.0049*	**5.4**	*0.0084*	**8.5**	*0.0081*	**17.5**	*0.0069*
AM residue	**35.1**	*0.0086*	**48.8**	*0.0756*	**43.2**	*0.0407*	**59.5**	*0.0234*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**29.6**	*0.0116*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**26.5**	*0.0104*
M/C solubilizate	**18.0**	*0.0044*	**28.9**	*0.0447*	**9.1**	*0.0085*	**14.9**	*0.0059*
M/C residue	**14.9**	*0.0036*	**21.9**	*0.0339*	**33.8**	*0.0318*	**8.5**	*0.0034*
T/L solubilizate	-[3]	-[3]	-[3]	-[3]	**1.9**	*0.0018*	-[3]	-[3]
T/L residue	-[3]	-[3]	-[3]	-[3]	**29.3**	*0.0275*	-[3]	-[3]
SUM SOLUBILIZED	**38.0**	*0.0093*	**34.3**	*0.0530*	**19.5**	*0.0184*	**62.0**	*0.0244*
FINAL RESIDUE	**14.9**	*0.0036*	**21.9**	*0.0339*	**29.3**	*0.0275*	**8.5**	*0.0034*
plant back interval 365 DAT								
RRR	**60.6**	*0.0063*	**49.4**	*0.0161*	**66.2**	*0.0500*	**84.9**	*0.0276*
AM solubilizate [2)]	**8.7**	*0.0009*	**8.0**	*0.0026*	**8.6**	*0.0065*	**14.7**	*0.0048*
AM residue	**44.0**	*0.0046*	**34.8**	*0.0113*	**54.9**	*0.0415*	**65.6**	*0.0213*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**18.3**	*0.0060*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]
M/C solubilizate	**8.6**	*0.0009*	**5.8**	*0.0019*	**3.9**	*0.0029*	**17.2**	*0.0056*
M/C residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**25.2**	*0.0082*
T/L solubilizate	**3.0**	*0.0003*	**1.6**	*0.0005*	**1.7**	*0.0013*	-[3]	-[3]
T/L residue	**32.2**	*0.0033*	**32.2**	*0.0105*	**50.8**	*0.0384*	-[3]	-[3]
SUM SOLUBILIZED	**20.4**	*0.0021*	**15.4**	*0.0050*	**14.3**	*0.0108*	**50.2**	*0.0163*
FINAL RESIDUE	**32.2**	*0.0033*	**32.2**	*0.0105*	**50.8**	*0.0384*	**25.2**	*0.0082*

[1)] AM, ammonia, A/G amylase/amyluglucosidase, M/C macerozyme/cellulase, T/L tyrosinase/laccase, [2)] combined ammonia solubilizate from two solubilisation steps in the cases of spring wheat hay, straw and grain; only one solubilisation step was performed in the cases of mature spinach, white radish top and root and spring wheat forage, 3) not analysed, [4)] straw samples include chaff

Table 6.6.1-10: T-label: characterization of RRR of in wheat matrices

solubilization fraction[1)]	wheat forage		wheat hay		wheat straw [4]		wheat grain	
	% TRR	*[mg/kg]*	*% TRR*	*[mg/kg]*	*% TRR*	*[mg/kg]*	*% TRR*	*[mg/kg]*
plant back interval 31 DAT								
RRR	**7.2**	*0.0208*	**7.6**	*0.0519*	**17.8**	*0.1851*	**6.5**	*0.1499*
AM solubilizate [2)]	**3.5**	*0.0100*	**3.7**	*0.0249*	**9.5**	*0.0990*	**1.9**	*0.0436*
AM residue	**2.7**	*0.0079*	**3.0**	*0.0208*	**6.6**	*0.0687*	**4.4**	*0.1020*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**3.1**	*0.0712*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**0.9**	*0.0216*
M/C solubilizate	-[3]	-[3]	**0.7**	*0.0044*	**1.5**	*0.0155*	**0.5**	*0.0109*
M/C residue	-[3]	-[3]	**2.1**	*0.0144*	**4.9**	*0.0504*	**0.4**	*0.0097*
T/L solubilizate	-[3]	-[3]	-[3]	-[3]	**0.5**	*0.0055*	-[3]	-[3]
T/L residue	-[3]	-[3]	-[3]	-[3]	**4.3**	*0.0450*	-[3]	-[3]
SUM SOLUBILIZED	**3.5**	*0.0100*	**4.3**	*0.0294*	**11.6**	*0.1201*	**5.4**	*0.1257*
FINAL RESIDUE	**2.7**	*0.0079*	**2.1**	*0.0144*	**4.3**	*0.0450*	**0.4**	*0.0097*
plant back interval 365 DAT								
RRR	**8.7**	*0.0338*	**13.3**	*0.3017*	**11.8**	*0.1188*	**4.3**	*0.1410*
AM solubilizate [2)]	**3.0**	*0.0116*	**7.3**	*0.1652*	**4.3**	*0.0430*	**1.6**	*0.0505*
AM residue	**5.0**	*0.0193*	**5.4**	*0.1219*	**6.3**	*0.0637*	**2.6**	*0.0830*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**1.7**	*0.0558*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**0.7**	*0.0215*
M/C solubilizate	**2.4**	*0.0094*	**2.6**	*0.0586*	**1.7**	*0.0167*	**0.3**	*0.0107*
M/C residue	**2.4**	*0.0091*	**2.6**	*0.0597*	**4.6**	*0.0465*	**0.2**	*0.0078*
T/L solubilizate	-[3]	-[3]	-[3]	-[3]	**0.5**	*0.0049*	-[3]	-[3]
T/L residue	-[3]	-[3]	-[3]	-[3]	**3.9**	*0.0395*	-[3]	-[3]
SUM SOLUBILIZED	**5.4**	*0.0210*	**9.9**	*0.2238*	**6.4**	*0.0646*	**3.6**	*0.1170*
FINAL RESIDUE	**2.4**	*0.0091*	**2.6**	*0.0597*	**3.9**	*0.0395*	**0.2**	*0.0078*
plant back interval 365 DAT								
RRR	**8.2**	*0.0159*	**6.2**	*0.0532*	**17.3**	*0.1587*	**6.6**	*0.1460*
AM solubilizate [2)]	**4.5**	*0.0088*	**3.1**	*0.0266*	**7.2**	*0.0657*	**4.1**	*0.0902*
AM residue	**3.4**	*0.0065*	**2.6**	*0.0227*	**8.6**	*0.0788*	**2.8**	*0.0616*
A/G solubilizate	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**1.3**	*0.0285*
A/G residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]
M/C solubilizate	**0.8**	*0.0015*	**0.5**	*0.0041*	**1.6**	*0.0145*	**0.3**	*0.0068*
M/C residue	-[3]	-[3]	-[3]	-[3]	-[3]	-[3]	**0.7**	*0.0165*
T/L solubilizate	**0.2**	*0.0004*	**0.2**	*0.0014*	**0.7**	*0.0061*	-[3]	-[3]
T/L residue	**2.3**	*0.0045*	**1.9**	*0.0165*	**5.9**	*0.0540*	-[3]	-[3]
SUM SOLUBILIZED	**5.5**	*0.0107*	**3.7**	*0.0320*	**9.4**	*0.0863*	**5.7**	*0.1256*
FINAL RESIDUE	**2.3**	*0.0045*	**1.9**	*0.0165*	**5.9**	*0.0540*	**0.7**	*0.0165*

[1)] AM, ammonia, A/G amylase/amyluglucosidase, M/C macerozyme/cellulase, T/L tyrosinase/laccase, [2)] combined ammonia solubilizate from two solubilisation steps in the cases of spring wheat hay, straw and grain; only one solubilisation step was performed in the cases of mature spinach, white radish top and root and spring wheat forage, 3) not analysed, [4)] straw samples include chaff

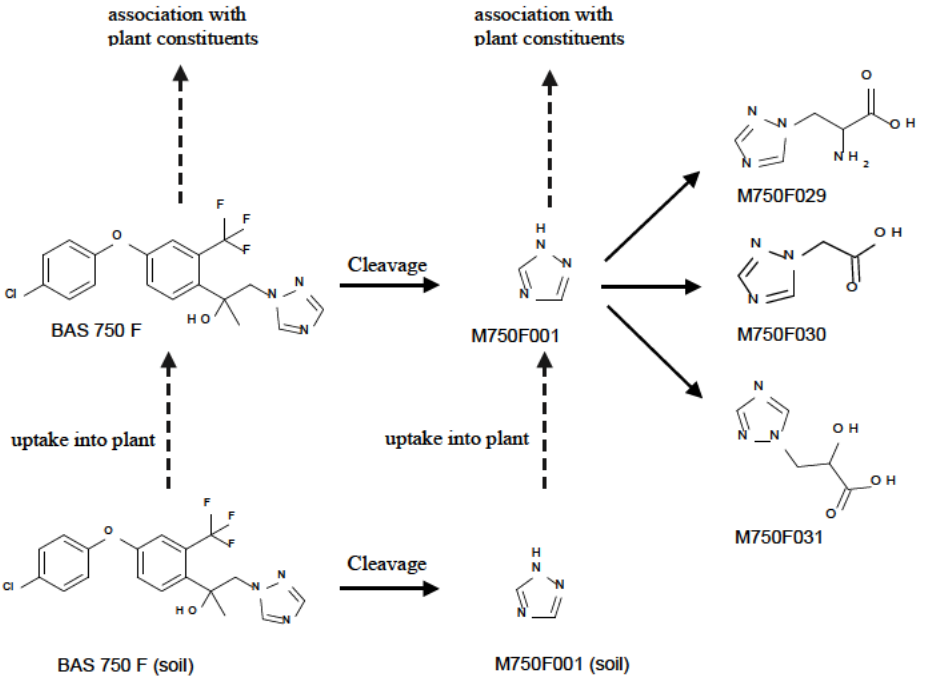
4. proposed metabolic pathway

In rotational crops, the metabolic profile is, in principle, the result of active substance degradation in soil, uptake of unchanged parent and/or soil metabolites into the plant, their translocation and/or further transformation within in the plant. In the present study, the metabolic profile of BAS 750 F in rotational crops cultivated on BAS 750 F treated soil was investigated in the representative crops wheat, radish and spinach using two labels, C-label and T-label. Different results are obtained with the two labels, however taken together a consistent picture of the metabolic pathway in the matrices of wheat (forage, hay, straw/chaff, grain), radish (root, top), and spinach (immature leaf, mature leaf) is seen, as shown in Figure 6.6.1-1.

In rotational crops cultivated on BAS 750 F treated soil, the residue includes mainly two components, parent BAS 750 F and triazole derivative metabolites (TDM). The TDM metabolites are generated by cleavage of the parent molecule at the triazole bridge. This cleavage step can, in principle, occur both in the plant or in the soil, followed by uptake of the cleavage products into plant (see section 5). Note that both components, unchanged parent and TDM, were also detected in crop metabolism studies (foliar spray application, see section CA 6.2). In addition, a small proportion of the radioactive residue appears to be associated with insoluble plant cell constituents being released only after solubilization treatments. In rotational crops, no further compounds were identified. In conclusion, no components specific to rotational crops were detected.

In conclusion, the metabolism of BAS 750 F in rotational crops can be considered well elucidated.

Figure 6.6.1-1: proposed metabolic pathway of ^{14}C-BAS 750 F in rotational crops



5. Uptake and translocation in the plant

In rotational crops, the metabolic profile of an active substance is potentially influenced by soil metabolites and their plant uptake and translocation. Determination of total radioactive residue (TRR) does indicate label-specific uptake and translocation. The C-label enables radiodetection specifically of parent BAS 750 F (due to non-detectability of TDM). In contrast, the T-label captures both the parent molecule as well as the TDM. Due to the fact that TDM are the predominant component of the T-labeled radioactive residue, the T-label largely represents the TDM. Taking results obtained with both labels together, different uptake and translocation properties can be deduced for parent and TDMs.

This data shows, the unchanged parent BAS 750 F is to a certain extent, taken up into plant followed by association with plant constituents. Possibly also within the plant, parent BAS 750 F is cleaved at the triazole bridge generating 1,2,4-triazole and the other triazole derivative metabolites TA, TAA, and TLA. The main proportion of TDMs in rotational crops however appear to result rather from degradation of BAS 750 F in the soil followed by preferential uptake of T-labeled cleavage products, namely 1,2,4-triazole into the plant where further transformation (TA, TAA, TLA) and association with plant constituents can occur.

Comparison of metabolic profiles in different rotational crops representing leafy vegetables, root vegetable, and cereals provides a consistent picture. Subsequent to uptake from soil, parent BAS 750 F and TDMs are translocated within the plant, namely the leaf (spinach), top (radish), and plant, (cereal forage/hay/straw). While BAS 750 F is not translocated into the grain, TDMs show high concentration into grain. In conclusion, uptake from soil and translocation within plant is high for TDM, and significantly lower for parent BAS 750 F.

6. Enantiomer ratio

Enantiomer-specific analysis of parent BAS 750 F in samples representing all representative crops and both labels (see Table 6.6.1-11) shows that both S-enantiomer and R-enantiomer are present as racemic mixture confirming that the 1:1 ratio of the test substance applied to bare soil remains unchanged. The unchanged enantiomer ratio indicates absence of preferential metabolisation and /or translocation of one of the two enantiomers in rotational crops.

Table 6.6.1-11: Determination of isomer ratio of BAS 750 F in rotational crop matrices

matrix	S-enantiomer [%]	R-enantiomer [%]
C-label		
application formulation	51.65	48.35
application formulation	48.97	51.03
spinach, mature	56.44	43.56
radish, root	49.57	50.43
wheat, forage	50.94	49.06
wheat, hay	57.84	42.16
T-label		
application formulation	50.51	48.87
spinach, immature	52.96	47.04

Assignment of the two HPLC peaks to the R- and the S-enantiomer was done based on comparison of elution profiles using HPLC system LC08 (see CA 5.1/1, DocID 2015/1107610, Fig. 358)

7. Storage stability

Directly after harvesting, samples were transferred in a freezer and stored -20 °C or below until extraction. Extracts were stored in a refrigerator. Specific samples, namely grain and straw (T-label, 364 DAT) analysed within 6 months after harvest, show only presence of components identified in this study, thus confirming absence of qualitative differences to the samples with longer storage intervals. This observation is consistent with storage stability investigations in crop metabolism studies (wheat, soybean, grape). Storage stability in extracts was confirmed in representative methanol extracts (see Table 6.6.1-12). The metabolite profile after extended storage was comparable to the profile obtained in the initial analysis of the extract, thus confirming stability of the radioactive residue in extracts of at least 8 months.

Table 6.6.1-12: Storage intervals extract samples storage of extract

matrix	storage interval[1)] (analysis 1) *[days]*	storage interval[1)] (analysis 2) *[days]*
C-label		
spinach, immature (30 DAT)	72	350
spinach, mature (30 DAT)	127	380
spinach, mature (120 DAT)	59	378
radish, top (30 DAT)	115	349
radish, root (30 DAT)	115	512
wheat, forage (30 DAT)	75	351
wheat, hay (30 DAT)	71	379
wheat, straw (30 DAT)	60	459
T-label		
spinach, immature (31 DAT)	43	512
spinach, mature (31 DAT)	44	365
radish, top (31 DAT)	16	286
radish, root (31 DAT)	16	450
wheat, forage (31 DAT)	47	286
wheat, hay (31 DAT)	46	452
wheat, straw (31 DAT)	14	284
wheat, grain (31 DAT)	14	448

1) interval from extraction to analysis

III. CONCLUSION

Metabolism of BAS 750 F was investigated in three representative succeeding crops spinach (leafy vegetable), radish (root and tuber) and wheat (cereal) using test substance either labeled in the chlorophenyl ring (C-label) or labeled in the triazole ring (T-label). The study included soil aging intervals of 30, 120 and 365 days following bare soil application of the maximal annual use rate of 300 g as/ha, corresponding to a soil concentration of 0.1 mg/kg (depth 20 cm). Note that regarding the use of BAS 750 F supported in the present dossier, this soil concentration represents a worst-case-scenario as the calculated plateau concentration of multi-year maximal annual rate application is at 0.058 mg/kg ($PEC_{soil,plateau}$, see CA 9.1.3).

Taken together, the data obtained with both labels show a consistent picture on the nature of residues of BAS 750 F in rotational crops. The residue in rotational crops was shown to consist of two components, the unchanged parent BAS 750 F (carrying C- and T-label) as well as the TDMs (carrying only the T-label, namely the group of 1,2,4-triazole, TA, TAA and TLA). No further component was identified. Consequently, no compound specific for rotational crops was detected.

The metabolic pathway thus consists of cleavage of the parent backbone structure at the triazole bridge, releasing 1,2,4-triazole, which is further transformed to the derivative metabolites TA, TAA, and TLA. This cleavage is assumed to occur mainly in the soil (see below) but might occur also within the plant. The data obtained shows that parent and cleavage products are taken up by rotational crops from the soil followed by translocation within the plant.

The parent BAS 750 F is present in the plant as a racemic mixture of the S-enantiomer and the R-enantiomer, thus the 1:1 ratio of the test item remained unchanged indicating absence of preferential metabolisation and/or uptake of one of the two enantiomers.

The amount of parent BAS 750 F in rotational crops is generally lower than the amount of TDMs as seen in the metabolic profile of T-labeled samples. Major amounts are only seen in spinach leaf (up to 25% TRR in immature leaf, with TDMs amounting to 65% TRR). In other T-labeled samples, BAS 750 F was present at < 6% TRR. Notably, BAS 750 F was not detected in grain. This was confirmed in C-labeled samples, where BAS 750 F was determined at similar absolute amounts (samples of spinach and radish up to 0.01 mg/kg, samples of forage at 0.015 mg/kg, samples of straw up to 0.10 mg/kg).

The TDM amounts, since generally exceeding the BAS 750 F amounts, explain the different TRRs obtained with both labels. Particular high levels were determined in T-labeled grain (up to 3.2 mg/kg, while levels in C-labeled grain were < 0.04 mg/kg. Note, that plants were grown in plastic boxes with limited drainage. It is assumed that this has enhanced the accessibility of TDM for plant uptake. Consequently, quantitative data obtained in this study is to be considered indicative only. Data on the magnitude of the residue under field conditions, is provided in section 6.6.2 (BAS 750 F field rotational crop study with representative rotational crops).

Extractability by solvents of parent BAS 750 F and TDMs generally was high. With the T-label solvent extracted >90% TRR except for straw/hay (82% or higherTRR). With the C-label extractability was moderate to high, with lower extractability correlating with lower TRR (spinach/radish/forage), high solubilization by enzymes (grain) and matrix type (straw/hay). Further radioactivity was released by solubilization (by treatment with ammonia and enzymes additional amounts of the residue were characterized as being associated with plant constituents, notably starch and cellulose/hemicellulose). The amounts of final residue indicate that to a low extent, radiocarbon was incorporated into insoluble cell constituents. For spinach, radish, forage and grain the final residue was < 0.01 mg/kg (in samples with low total radioactive residue representing higher proportions of the TRR). For hay (corresponding to dried forage) and straw the final residue was 0.05 mg/kg or below. Taken together the residues either identified or characterized, the residue resulting from BAS 750 F soil treatment was quantitatively investigated.

Comparison of metabolic profiles in different rotational crops representing leafy vegetables, root vegetable, and cereals reveals that, subsequent to uptake from soil, parent BAS 750 F and TDMs both are translocated within the “green plant parts” (namely the leaf of spinach, top of radish, and plant of cereal, thus forage/hay/straw). Into the grain, however only the TDM are translocated, while BAS 750 F is detected in grain.

Comparison of metabolic profiles obtained at different replant intervals reveals that BAS 750 F residues tend to decrease during longer soil aging intervals, whereas TFM level remain largely unchanged over the intervals investigated.

In summary, the metabolism of BAS 750 F in rotational crops has been comprehensively studied and is thus well understood. Parent BAS 750 F and the TDMs were identified as the major components of the residue. No components specific to rotational crops were detected. Based on the residue level found a field rotational crop study is required.

CA 6.6.2 Magnitude of residues in rotational crops

Based on the results of the metabolism study (rotational crops, see section CA 6.6.1) a field rotational crop study is required. As part of the field study, residue data has to be generated for the parent BAS 750 F as well as the group of triazole derivative metabolites (TDMs) 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA), triazole lactic acid (TLA). Note that a large data package on TDM residues occurring in succeeding crops has previously been submitted by the *Triazole Derivative Metabolite Group* (TDMG) to UK CRD. Data obtained in the present field study with BAS 750 F is comparable to the data previously submitted and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment:
"*A large residue data package has been submitted to address residues of the TDMs in rotational crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."

Report:	CA 6.6.2/1 Martin T., 2015 a Study on the residue behavior of BAS 750 F on the rotational crops: wheat, carrots or radish, broccoli or cauliflower and spinach or lettuce after one application of BAS 750 01 F to bare soil under field conditions, 2014-2015 2015/1106682
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011), OECD 504
GLP:	yes (certified by ENAC, Entidad Nacional de Acreditación, Madrid, Spain)

EXECUTIVE SUMMARY

A field rotational crop study encompassing four independent trials was conducted in different representative growing areas in Germany, Netherlands, Italy and Spain. Each trial included three plots for different replant intervals (plot 2: 30d, plot 4: 120d, plot 6: 365d), each in parallel with a corresponding untreated plot (plot 1: 30d, plot 3: 120d, plot 5: 365d). The active substance was applied as BAS 750 01 F EC formulation to bare soil at an application rate of 1 x 300 g as/ha. The level of residues (BAS 750 F as well as 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA)) was investigated in cereals, root/tuber, brassica vegetable and leafy vegetable each cultivated after different replant intervals of 30±1, 120±3 and 365±5 days. Plant samples were taken at maturity: wheat grain and straw (BBCH 89), carrot/radish (BBCH49-55), cauliflower/broccoli (BBCH49), lettuce/spinach (BBCH49-55). Additional samples of immature growth stage were taken: wheat forage (BBCH 30-33, BBCH65), carrot/radish (BBCH41), cauliflower/broccoli (BBCH41), lettuce/spinach (BBCH41).

For all four crop groups, cereals, root/tuber, brassica vegetables and leafy vegetables, similar results were obtained: residue levels of parent compound BAS 750 F in samples from treated plots as well as untreated plots were all below the LOQ of 0.01 mg/kg. Residue levels of 1,2,4-triazole in samples from treated plots as well as untreated plots were below the LOQ of 0.01 mg/kg. Regarding the triazole derivative metabolites TA, TAA, and TLA, both residue levels below the LOQ as well as residue levels above the LOQ were determined in samples from treated plots. Comparison with samples from untreated plots shows that these residue levels are to a large extend treatment unrelated. Notably, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment:
"*A large residue data package has been submitted to address residues of the TDMs in rotational crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."
Note, that bare soil application of 300 g BAS 750 F/ha corresponds to a concentration of 0.1 mg/kg of BAS 750 F in the soil (soil depth 20 cm, soil density 1.5 g/cm^3). Consequently, for multi-year application scenarios (of BAS 750 F) which do not result in soil concentrations significantly exceeding 0.1 mg as/kg soil, absence of significant residues can also be expected.

Taken together, data obtained in the field rotational crop study with BAS 750 F shows that residues resulting from the use of BAS 750 F at maximal seasonal application rate of 300 g as/ha according to the cGAP do not result in significant residues of parent BAS 750 F. Residues of the TDMs if exceeding the LOQ of 0.01 mg/kg are within the range of residues included in the ongoing assessment of TDMs.

I. MATERIAL AND METHODS

A. MATERIALS

1.	**Test Material:**	**BAS 750 01 F**
	Description:	100 g BAS 750 F/L, EC
	Lot/Batch #:	FD-140113-0006
	Purity:	*not relevant*
	CAS#:	1417782-03-6
	Development code:	BAS 750 F
	Stability of test compound:	The test item was stable over the test period (expiration date 29 Feb 2016)

2. Test Commodity:

Crop:	1) wheat, 2) radish/carrot, 3) cauliflower/broccoli, 4) lettuce/spinach	
Type:	1) cereal, 2) root and tuber, 3) brassica vegetables, 4) leafy vegetables	
Variety:	wheat:	KWS Chamsin, JB Asano, Tybalt, Winnetou, Palesio, Artur Nick
	radish:	Candela di Ghiaccio, Candela die Fuoco, Largo Rojo
	carrot:	Jeanette, Jubila
	cauliflower:	Panther, Clarina, Freedom F1, Concept F1, Chambord F1, Sirente, Caspe
	broccoli:	Monteco, Parthenon
	lettuce:	Baby leaf (Diablotin, Oaking, Palosta, Lettony, Redlo)
	spinach:	Violin F1, America, Viroflay
Botanical name:	*1) Triticum aestivum, 2) Raphanus sativus, Daucus carota, 3) Brassica oleracea* var. *botrytis, Brassica oleracea* var. *italica, 4) Lactuca sativa, Spinacia oleracea*	
Crop part / processed commodity:		
	wheat:	whole plant (no root), grain, straw
	radish/carrot:	whole plant (no root), root, top
	cauliflower/broccoli:	whole plant without root, inflorescence
	lettuce/spinach:	leaf
Sample size:	**(**see table 6.6.2-1)	

B. STUDY DESIGN

The study was conducted in different representative growing areas in Germany, The Netherlands, Italy and Spain including the growing seasons 2014 and 2015 (four independent trials).

1. Test procedure

Each of the four trial included three plots for different replant intervals (plot 2: 30d, plot 4: 120d, plot 6: 365d), each in parallel with a corresponding untreated plot (plot 1: 30d, plot 3: 120d, plot 5: 365d). The active substance was applied as BAS 750 01 F EC formulation to bare soil once at an application rate of 1 x 300 g as/ha (calculated concentration in the soil 0.1 g as/kg with soil depth 20 cm, soil density 1.5 g/cm^3). The spray volume used was 200 L/ha.

2. Sampling

Sampling parameters are given in Table 6.6.2-1. Control specimens were collected prior to collection of the treated specimens to avoid contamination. Specimen were frozen within 6 hours after h, and remained frozen at or below -18°C until analysis (thus also during transportation).

Table 6.6.2-1: Target sampling parameters of the rotational crop study

		sampling event no.1	*sampling event no.2*	*sampling event no.3*
rotational crop (sampling timing, sample size)	wheat	BBCH 30-33 whole plant no root (1 kg)	BBCH 65 whole plant (no root, 1kg)	BBCH 89 grain (1 kg) straw (0.5 kg)
	carrot / radish	BBCH 41 whole plant (with root, 1 kg)	-	BBCH 49 root (1 kg, 12 pieces) top (1 kg, 12 pieces)
	cauliflower / broccoli	BBCH 41 whole plant (no root, 0.5 kg)	-	BBCH 49 inflorescence (1 kg, 12 pieces)
	lettuce / spinach	BBCH 41 head/leaf (0.5 kg)	-	BBCH 49 head/leaf (1 kg)

3. Description of analytical procedures

Residues of parent compound BAS 750 F were determined according to BASF method No. L0076/09. Residues of the triazole metabolites, 1,2,4-triazole (T), triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA), was according to BASF method L0170/02. The limit of quantitation (LOQ) was 0.010 mg/kg for each analyte. Procedural recoveries are given in the tables (Table 6.6.2-8 - Table 6.6.2-13, with fortification levels between 0.01 and 1.0 mg/kg). Averaged across all plant matrices procedural recoveries were

84% for BAS 750 F (n = 74, RSD = 9.1%),
97% for T (n = 84, RSD = 8.2%),
99% for TA (n = 83, RSD = 6.4%),
99% for TAA (n = 84, RSD = 5.5%)
6% for TLA (n = 84, RSD = 6.6%).

II. RESULTS AND DISCUSSION

BAS 750 F

Residue data is summarized below, for the 30d replant interval (Table 6.6.2-2), for the 120d replant interval (Table 6.6.2-3), and for the 365d replant interval (Table 6.6.2-4).

For all four crop groups, cereals, root/tuber, brassica vegetables and leafy vegetables, BAS 750 F residue levels in samples from treated plots were below the LOQ of 0.01 mg/kg (similarly, samples from untreated plots were below the LOQ).

Table 6.6.2-2: Summary of BAS 750 F residues in representative succeeding crops (replant interval 30 ± 1 days, plot 2)

plot	sub plot	crop	portion analyzed	growth stage	sampling	n	BAS 750 F
				BBCH	*DALA*		*mg/kg*
2	1	wheat	whole plant (no	31 - 33	69 - 195	4	<0.01
			whole plant (no	65	89 - 211	4	<0.01
			grain	89	135 - 257	4	<0.01
			straw	89	135 - 257	4	<0.01
	2	carrots / radish	whole plant	41	52 - 101	4	<0.01
			top	49	65 - 138	4	<0.01
			root	49	65 - 138	4	<0.01
	3	broccoli / cauliflower	whole plant	41	66 - 87	4	<0.01
			inflorescence	49	83 - 135	4	<0.01
	4	spinach / lettuce	leaf	41	58 - 74	4	<0.01
			leaf	49	69 - 88	4	<0.01

Table 6.6.2-3: Summary of BAS 750 F residues in representative succeeding crops (replant interval 120 ± 3 days, plot 4)

plot	sub plot	crop	portion analyzed	growth stage	sampling	n	BAS 750 F
				BBCH	*DALA*		*mg/kg*
4	1	wheat	whole plant (no	31 - 33	196 - 321	4	<0.01
			whole plant (no	65	238 - 370	4	<0.01
			grain	89	301 - 413	4	<0.01
			straw	89	301 - 413	4	<0.01
	2	carrots / radish	whole plant	41	142 - 188	4	<0.01
			top	49	155 - 228	4	<0.01
			root	49	155 - 228	4	<0.01
	3	broccoli / cauliflower	whole plant	41	153 - 177	4	<0.01
			inflorescence	49	184 - 225	4	<0.01
	4	spinach / lettuce	leaf	41	148 - 180	4	<0.01
			leaf	49	162 - 190	4	<0.01

Table 6.6.2-4: Summary of BAS 750 F residues in representative succeeding crops (replant interval 365 ± 5 days, plot 6)

plot	sub plot	crop	portion analyzed	growth stage	sampling	n	BAS 750 F
					DALA		*mg/kg*
6	1	wheat	whole plant (no	31 - 33	403 - 441	4	<0.01
			whole plant (no	65	423 - 455	4	<0.01
			grain	89	467 - 497	4	<0.01
			straw	89	467 - 497	4	<0.01
	2	carrots / radish	whole plant	41	396 - 445	4	<0.01
			top	49-55	410 - 482	4	<0.01
			root	49-55	410 - 482	4	<0.01
	3	broccoli / cauliflower	whole plant	41	404 - 447	4	<0.01
			inflorescence	49	427 - 470	4	<0.01
	4	spinach / lettuce	leaf	41	393 - 426	4	<0.01
			leaf	49-55	407 - 434	4	<0.01

TDM

Residue data is summarized below, for the 30d replant interval (Table 6.6.2-5), for the 120d replant interval (Table 6.6.2-6), and for the 365d replant interval (Table 6.6.2-7).

For all four crop groups (cereals, roots/tubers, brassica vegetables and leafy vegetables), residue levels of 1,2,4-triazole in samples from both treated and untreated plots were below the LOQ of 0.01 mg/kg. Regarding the triazole derivative metabolites TA, TAA, and TLA, both residue levels below the LOQ as well as residue levels above the LOQ were determined in samples from treated plots. Comparison with samples from untreated plots shows that these residue levels are to a large extend treatment unrelated. Notably, data obtained in the present field study with BAS 750 F is comparable to the TDM data previously submitted by the *Triazole Derivative Metabolite Group* (TDMG, see Conclusion below).

Table 6.6.2-5: Summary of TDM residues in representative succeeding crops (replant interval 30±1 days, plot 1 untreated, plot 2 treated)

plot	sub plot	crop	portion analyzed	GS [3] *[mg/kg]*	n	residues *[mg/kg]*			
						T	TA	TAA	TLA
1	1	wheat	whole plant[1]	31-33	4	< 0.01	< 0.01 - 0.021	< 0.01	< 0.01 – 0.037
			whole plant[1]	65	4	< 0.01	< 0.01 - 0.014	< 0.01	< 0.01 – 0.015
			grain	89	4	< 0.01	0.041 - 0.069	< 0.01 - 0.034	< 0.01
			straw	89	4	< 0.01	< 0.01	< 0.01 – 0.023	< 0.01 – 0.020
2	1		whole plant[1]	31-33	4	< 0.01	0.010 - 0.017	< 0.01 – 0.026	< 0.01 - 0.023
			whole plant[1]	65	4	< 0.01	< 0.01 - 0.044	< 0.01 - 0.030	< 0.01 – 0.066
			grain	89	4	< 0.01	0.042 - 0.23	0.019 - 0.13	< 0.01
			straw	89	4	< 0.01	< 0.01 - 0.011	< 0.01 - 0.041	0.012 – 0.037
1	2	carrot / radish	whole plant[2]	41	4	< 0.01	< 0.01 - 0.049	< 0.01	< 0.01 - 0.011
			root	49	4	< 0.01	< 0.01 - 0.014	< 0.01	< 0.01
			top	49	4	< 0.01	< 0.01 - 0.014	< 0.01	< 0.01 – 0.025
2	2		whole plant[2]	41	4	< 0.01	< 0.01 - 0.077	< 0.01	< 0.01 - 0.029
			root	49	4	< 0.01	0.014 - 0.017	< 0.01	< 0.01
			top	49	4	< 0.01	< 0.01 - 0.061	< 0.01	< 0.01 – 0.023
1	3	broccoli / cauliflower	whole plant[1]	41	4	< 0.01	0.012 - 0.026	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	0.020 - 0.084	< 0.01	< 0.01
2	3		whole plant[1]	41	4	< 0.01	0.014 - 0.076	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	0.060 - 0.12	< 0.01	< 0.01
1	4	spinach / lettuce	leaf	41	4	< 0.01	< 0.01 - 0.024	< 0.01	< 0.01 - 0.031
				49	4	< 0.01	< 0.01	< 0.01	< 0.01 - 0.032
2	4		leaf	41	4	< 0.01	< 0.01 - 0.048	< 0.01	< 0.01 - 0.092
				49	4	< 0.01	< 0.01 - 0.026	< 0.01	< 0.01 - 0.084

[1] roots removed, [2] roots included, [3] growth stage (BBCH)

Table 6.6.2-6: Summary of TDM residues in representative succeeding crops (replant interval 120±3 days, plot 3 untreated, plot 4 treated)

plot	sub plot	crop	portion analyzed	GS [3] *[mg/kg]*	n	residues *[mg/kg]*			
						T	TA	TAA	TLA
3	1	wheat	whole plant[1]	31-33	4	< 0.01	< 0.01 - 0.031	< 0.01	< 0.01 – 0.027
			whole plant[1]	65	4	< 0.01	< 0.01 - 0.016	< 0.01 - 0.020	< 0.01 – 0.016
			grain	89	4	< 0.01	0.034 - 0.11	0.018 - 0.072	< 0.01
			straw	89	4	< 0.01	< 0.01	< 0.01 – 0.027	< 0.01 – 0.032
4	1		whole plant[1]	31-33	4	< 0.01	0.026 - 0.12	< 0.01 – 0.036	0.010 - 0.29
			whole plant[1]	65	4	< 0.01	0.017 - 0.093	0.020 – 0.070	0.019 - 0.10
			grain	89	4	< 0.01	0.093 - 0.50	0.066 - 0.21	< 0.01 – 0.012
			straw	89	4	< 0.01	< 0.01 - 0.035	0.020 – 0.13	0.022 – 0.16
3	2	carrot / radish	whole plant[2]	41	4	< 0.01	< 0.01 - 0.014	< 0.01	< 0.01 - 0.017
			root	49	4	< 0.01	< 0.01 - 0.013	< 0.01	< 0.01
			top	49	4	< 0.01	< 0.01 - 0.015	< 0.01	< 0.01 – 0.017
4	2		whole plant[2]	41	4	< 0.01	0.011 - 0.090	< 0.01	< 0.01 - 0.018
			root	49	4	< 0.01	< 0.01 - 0.039	< 0.01	< 0.01
			top	49	4	< 0.01	< 0.01 - 0.056	< 0.01	< 0.01 – 0.038
3	3	broccoli / cauliflower	whole plant[1]	41	4	< 0.01	0.021 - 0.034	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	0.029 - 0.10	< 0.01	< 0.01
4	3		whole plant[1]	41	4	< 0.01	0.034 - 0.18	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	0.064 - 0.35	< 0.01	< 0.01
3	4	spinach / lettuce	leaf	41	4	< 0.01	< 0.01	< 0.01	< 0.01 - 0.012
				49	4	< 0.01	< 0.01	< 0.01	< 0.01 - 0.016
4	4		leaf	41	4	< 0.01	< 0.01 - 0.048	< 0.01	0.014 - 0.042
				49	4	< 0.01	< 0.01 - 0.043	< 0.01	0.017 - 0.050

[1] roots removed, [2] roots included, [3] growth stage (BBCH)

Table 6.6.2-7: Summary of TDM residues in representative succeeding crops (replant interval 365±1 days, plot 5 untreated, plot 6 treated)

plot	sub plot	crop	portion analyzed	GS [3] *[mg/kg]*	n	residues *[mg/kg]*			
						T	TA	TAA	TLA
5	1	wheat	whole plant[1]	31-33	4	< 0.01	< 0.01 - 0.02	< 0.01	< 0.01
			whole plant[1]	65	4	< 0.01	< 0.01 - 0.024	< 0.01 - 0.01	< 0.01 – 0.014
			grain	89	4	< 0.01	0.040 - 0.13	0.019 - 0.055	< 0.01
			straw	89	4	< 0.01	< 0.01	< 0.01 - 0.022	< 0.01 – 0.060
6	1		whole plant[1]	31-33	4	< 0.01	0.026 - 0.13	< 0.01 - 0.010	< 0.01 – 0.11
			whole plant[1]	65	4	< 0.01	0.010 - 0.10	< 0.01 - 0.068	< 0.01 – 0.017
			grain	89	4	< 0.01	0.048 - 0.52	0.021 - 0.35	< 0.01 - 0.015
			straw	89	4	< 0.01	< 0.01 - 0.037	< 0.01 - 0.15	< 0.01 – 0.090
5	2	carrot / radish	whole plant[2]	41	4	< 0.01	< 0.01	< 0.01	< 0.01 - 0.015
			root	49-55	4	< 0.01	< 0.01	< 0.01	< 0.01
			top	49-55	4	< 0.01	< 0.01 - 0.024	< 0.01	< 0.01 – 0.015
6	2		whole plant[2]	41	4	< 0.01	< 0.01 - 0.040	< 0.01	< 0.01 - 0.014
			root	49-55	4	< 0.01	< 0.01 - 0.020	< 0.01	< 0.01
			top	49-55	4	< 0.01	< 0.01 - 0.047	< 0.01	< 0.01 – 0.043
5	3	broccoli / cauliflower	whole plant[1]	41	4	< 0.01	< 0.01 - 0.022	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	< 0.01	< 0.01	< 0.01
6	3		whole plant[1]	41	4	< 0.01	0.019 - 0.12	< 0.01	< 0.01
			inflorescence	49	4	< 0.01	0.054 - 0.17	< 0.01	< 0.01
5	4	spinach / lettuce	leaf	41	4	< 0.01	< 0.01 - 0.011	< 0.01	< 0.01 - 0.014
				49-55	4	< 0.01	< 0.01 - 0.012	< 0.01	< 0.01 - 0.024
6	4		leaf	41	4	< 0.01	< 0.01 - 0.015	< 0.01	< 0.01 - 0.017
				49-55	4	< 0.01	< 0.01 - 0.025	< 0.01	< 0.01 - 0.045

[1] roots removed, [2] roots included, [3] growth stage (BBCH)

III. CONCLUSION

A field rotational crop study of 4 independent trials (N-EU, S-EU) conducted with BAS 750 F at a rate of 1x300 g as/ha (applied to bare soil) including replant intervals of 30d, 120d and 365d shows that BAS 750 F residues do not exceed the LOQ of 0.01 mg/kg for different representative succeeding crops (namely wheat, radish/carrot, broccoli/cauliflower, and spinach/lettuce). When applying the maximal seasonal application rate directly to bare soil, residues in succeeding crops are generally overestimated since in practice, crop interception is reducing the amount of active substance reaching the soil. Thus, results obtained in this study indicate that in practice no significant residues in rotational crops are expected.

Note, that bare soil application of 300 g BAS 750 F/ha corresponds to a concentration of 0.1 mg/kg of BAS 750 F in the soil. Considering crop interception, BAS 750 F concentration in soil during the first year of application of the maximal yearly rate of BAS 750 F, is calculated at maximum 0.08 mg/kg ($PEC_{soil, max}$, see section CA 9.1.3). For a multi-year application of the maximal yearly rate, a plateau will be reached at 0.058 mg/kg ($PEC_{soil, plateau}$). The worst case scenario (extended regular yearly application of the maximal seasonal rate followed by cultivation of a crop treated with the maximal yearly rate prior to cultivation of a rotational crop) is highly unlikely. For this scenario, soil concentration is calculated not to exceed 0.137 mg/kg. In conclusion, also for multi-year application of BAS 750 F according to the use supported in the present dossier, absence of significant residues of BAS 750 F can be expected.

Regarding TDMs, residues of 1,2,4-triazole were also below the LOQ in all crops at all replant intervals, whereas detectable residues were repeatedly found for the triazole derivative metabolites TA, TAA and TLA. Comparison with corresponding samples from untreated plots indicates that these residues are to a large extend treatment unrelated.

Note that a large data package on TDM residues occurring in succeeding crops has previously been submitted by the *Triazole Derivative Metabolite Group* (TDMG) to UK CRD. Data obtained in the present field study with BAS 750 F is comparable to the data previously submitted and thereby can be considered covered by the assessment published in November 2015 as the RMS's initial, draft addendum (UK CRD, 2015: Addendum – Confirmatory Data, addressing sections B.5, B.6, B.7). This assessment includes the following statement on residues and risk assessment:
"*A large residue data package has been submitted to address residues of the TDMs in rotational crops […]. The outcome of the consumer intake assessment, based on the data provided, raises no concerns*."

Taken together, data obtained in the field rotational crop study with BAS 750 F shows that residues resulting from the use of BAS 750 F at maximal seasonal application rate of 300 g as/ha according to the cGAP do not result in significant residues of parent BAS 750 F. Residues of the TDMs if exceeding the LOQ of 0.01 mg/kg are within the range of residues included in the ongoing assessment of TDMs.

Table 6.6.2-8: Residues in succeeding crops (30 ± 1 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1)]	Residues (mg/kg)						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 2: 30 ± 1 DAA replanting interval															
Study code: 727902	BAS 750 01 F		Germany			<0.01	<0.01	0.017	<0.01	<0.01	**Method No.:**	**L0076/09**			
Doc ID: 2015/1106682	1 x 3.0 L/ha		Netherlands	31-33		<0.01	<0.01	0.010	<0.01	<0.01	**analysed as:**	**BAS 750 F**			
GLP: Yes	to bare soil		Italy			<0.01	<0.01	0.105	0.026	0.23	plant	0.01, 0.1, 1.0	74	83.9	9 1
Year: 2014-2015			Spain		whole plant without root	<0.01	<0.01	0.016	<0.01	<0.01	**Method No.:**	**L0170/02**			
			Germany			<0.01	<0.01	0.014	<0.01	<0.01	**analysed as:**	**T**			
			Netherlands	65		<0.01	<0.01	<0.01	<0.01	<0.01	plant	0.01, 0 1, 1.0	84	96.6	8.2
			Italy			<0.01	<0.01	0.044	0.0301	0.066	**analysed as:**	**TA**			
			Spain			<0.01	<0.01	0.017	0.013	0.012	plant	0.01, 0 1, 1.0	83	99.3	6.4
		Wheat	Germany			<0.01	<0.01	0.0901	0.028	<0.01	**analysed as:**	**TAA**			
			Netherlands	89	grain	<0.01	<0.01	0.042	0.019	<0.01	plant	0.01, 0 1, 1.0	84	98.7	5.5
			Italy			<0.01	<0.01	0.226	0.13	<0.01	**analysed as:**	**TLA**			
			Spain			<0.01	<0.01	0.089	0.054	<0.01	plant	0.01, 0 1, 1.0	84	96.1	6.6
			Germany			<0.01	<0.01	<0.01	0.016	0.012					
			Netherlands	89	straw	<0.01	<0.01	<0.01	<0.01	0.013					
			Italy			<0.01	<0.01	0.011	0.041	0.037					
			Spain			<0.01	<0.01	<0.01	0.026	0.033					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	0.029					
			Netherlands	41	whole plant with roots	<0.01	<0.01	<0.01	<0.01	<0.01					
		Radish	Italy			<0.01	<0.01	0.077	<0.01	0.028					
			Spain			<0.01	<0.01	0.049	<0.01	<0.01					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	0.023					
			Netherlands	49	tops	<0.01	<0.01	<0.01	<0.01	0.014					
		Radish	Italy			<0.01	<0.01	0.045	<0.01	0.017					
			Spain			<0.01	<0.01	0.061	<0.01	<0.01					
		Carrots	Germany			<0.01	<0.01	0.014	<0.01	<0.01					
			Netherlands	49	roots	<0.01	<0.01	0.014	<0.01	<0.01					
		Radish	Italy			<0.01	<0.01	0.014	<0.01	<0.01					
			Spain			<0.01	<0.01	0.017	<0.01	<0.01					
		Broccoli	Germany			<0.01	<0.01	0.035	<0.01	<0.01					
			Netherlands	41	whole plant without root	<0.01	<0.01	0.014	<0.01	<0.01					
		Cauliflower	Italy			<0.01	<0.01	0.064	<0.01	<0.01					
			Spain			<0.01	<0.01	0.076	<0.01	<0.01					

Table 6.6.2-8: Residues in succeeding crops (30 ± 1 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1)]	Residues (mg/kg)						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.085	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.060	<0.01	<0.01					
			Italy			<0.01	<0.01	0.087	<0.01	<0.01					
			Spain			<0.01	<0.01	0.12	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	<0.01	<0.01	0.017					
		Spinach	Netherlands			<0.01	<0.01	0.016	<0.01	0.014					
			Italy			<0.01	<0.01	0.048	<0.01	0.092					
			Spain			<0.01	<0.01	0.040	<0.01	0.030					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	0.017					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.014					
			Italy			<0.01	<0.01	0.020	<0.01	0.084					
			Spain			<0.01	<0.01	0.026	<0.01	0.017					

[1)] Growth stage as planned (BBCH)

Table 6.6.2-9: Residues in succeeding crops (120 ± 3 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1)]	Residues (mg/kg)						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 4: 120 ± 3 DAA replanting interval															
Study code: 727902 **Doc ID:** 2015/1106682 **GLP:** Yes **Year:** 2014-2015	BAS 750 01 F 1 x 3.0 L/ha to bare soil	Wheat	Germany	31-33	whole plant without root	<0.01	<0.01	0.026	<0.01	0.010	**Method No.:**	**L0076/09**			
			Netherlands			<0.01	<0.01	0.044	<0.01	0.017	**analysed as:**	**BAS 750 F**			
			Italy			<0.01	<0.01	0.12	0.036	0.29	plant	0.01, 0.1, 1.0	74	83.9	9 1
			Spain			<0.01	<0.01	0.038	<0.01	0.010	**Method No.:**	**L0170/02**			
			Germany	65		<0.01	<0.01	0.017	0.0203	0.019	**analysed as:**	**T**			
			Netherlands			<0.01	<0.01	0.024	0.024	0.024	plant	0.01, 0 1, 1.0	84	96.6	8.2
			Italy			<0.01	<0.01	0.093	0.0703	0.100	**analysed as:**	**TA**			
			Spain			<0.01	<0.01	0.026	0.025	0.029	plant	0.01, 0 1, 1.0	83	99.3	6.4
			Germany	89	grain	<0.01	<0.01	0.093	0.066	<0.01	**analysed as:**	**TAA**			
			Netherlands			<0.01	<0.01	0.19	0.094	<0.01	plant	0.01, 0 1, 1.0	84	98.7	5.5
			Italy			<0.01	<0.01	0.503	0.21	0.012	**analysed as:**	**TLA**			
			Spain			<0.01	<0.01	0.13	0.0930	<0.01	plant	0.01, 0 1, 1.0	84	96.1	6.6
			Germany	89	straw	<0.01	<0.01	<0.01	0.020	0.022					
			Netherlands			<0.01	<0.01	0.014	0.034	0.039					
			Italy			<0.01	<0.01	0.035	0.13	0.16					
			Spain			<0.01	<0.01	0.011	0.048	0.052					
		Carrots	Germany	41	whole plant with roots	<0.01	<0.01	0.014	<0.01	0.016					
			Netherlands			<0.01	<0.01	0.011	<0.01	0.015					
		Radish	Italy			<0.01	<0.01	0.0601	<0.01	0.018					
			Spain			<0.01	<0.01	0.0902	<0.01	<0.01					
		Carrots	Germany	49	tops	<0.01	<0.01	<0.01	<0.01	0.021					
			Netherlands			<0.01	<0.01	<0.01	<0.01	0.026					
		Radish	Italy			<0.01	<0.01	0.056	<0.01	0.038					
			Spain			<0.01	<0.01	0.031	<0.01	<0.01					
		Carrots	Germany	49	roots	<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands			<0.01	<0.01	0.016	<0.01	<0.01					
		Radish	Italy			<0.01	<0.01	0.037	<0.01	<0.01					
			Spain			<0.01	<0.01	0.039	<0.01	<0.01					
		Broccoli	Germany	41	whole plant without root	<0.01	<0.01	0.034	<0.01	<0.01					
			Netherlands			<0.01	<0.01	0.059	<0.01	<0.01					
		Cauliflower	Italy			<0.01	<0.01	0.057	<0.01	<0.01					
			Spain			<0.01	<0.01	0.18	<0.01	<0.01					

Table 6.6.2-9: Residues in succeeding crops (120 ± 3 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1)]	Residues (mg/kg)						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.064	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.26	<0.01	<0.01					
			Italy			<0.01	<0.01	0.089	<0.01	<0.01					
			Spain			<0.01	<0.01	0.35	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	0.012	<0.01	0.022					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.015					
			Italy			<0.01	<0.01	0.027	<0.01	0.042					
			Spain			<0.01	<0.01	0.048	<0.01	0.040					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	0.017					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.019					
			Italy			<0.01	<0.01	0.018	<0.01	0.050					
			Spain			<0.01	<0.01	0.043	<0.01	0.030					

[1)] Growth stage as planned (BBCH)

Table 6.6.2-10: Residues in succeeding crops (365 ± 5 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 6: 365 ± 5 DAA replanting interval															
Study code: 727902 **Doc ID:** 2015/1106682 **GLP:** Yes **Year:** 2014-2015	BAS 750 01 F 1 x 3.0 L/ha to bare soil	Wheat	Germany Netherlands Italy Spain	31-33	whole plant without root	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.013 0.026 0 1301 0.031	<0.01 <0.01 0.0104 <0.01	<0.01 0.014 0.11 0.013	**Method No.:** **L0076/09** **analysed as:** **BAS 750 F** plant	0.01, 0.1, 1.0	74	83.9	9 1
			Germany Netherlands Italy Spain	65		<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.0101 0.030 0.100 0.0203	<0.01 0.019 0.068 0.017	<0.01 0.017 0.15 <0.01	**Method No.:** **L0170/02** **analysed as:** **T** plant **analysed as:** **TA** plant	0.01, 0 1, 1.0 0.01, 0 1, 1.0	84 83	96.6 99.3	8.2 6.4
			Germany Netherlands Italy Spain	89	grain	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.065 0.048 0.52 0.14	0.021 0.024 0.35 0.062	<0.01 <0.01 0.015 <0.01	**analysed as:** **TAA** plant **analysed as:** **TLA** plant	0.01, 0 1, 1.0 0.01, 0 1, 1.0	84 84	98.7 96.1	5.5 6.6
			Germany Netherlands Italy Spain	89	straw	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 0.037 <0.01	<0.01 <0.01 0.15 <0.01	<0.01 0.021 0.090 0.015					
		Carrots Radish	Germany Netherlands Italy Spain	41	whole plant with roots	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 0.040 0.021	<0.01 <0.01 <0.01 <0.01	0.014 0.014 <0.01 <0.01					
		Carrots Radish	Germany Netherlands Italy Spain	49 49 55 49	tops	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 0.035 0.047	<0.01 <0.01 <0.01 <0.01	0.0432 0.0417 <0.01 0.0102					
		Carrots Radish	Germany Netherlands Italy Spain	49 49 55 49	roots	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.013 0.020 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01					
		Broccoli Cauliflower	Germany Netherlands Italy Spain	41	whole plant without root	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.019 0.057 0.12 0.033	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01					

Table 6.6.2-10: Residues in succeeding crops (365 ± 5 DAA replanting interval, treated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.054	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.11	<0.01	<0.01					
			Italy			<0.01	<0.01	0.17	<0.01	<0.01					
			Spain			<0.01	<0.01	0.087	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	<0.01	<0.01	0.010					
		Spinach	Netherlands			<0.01	<0.01	0.013	<0.01	0.017					
			Italy			<0.01	<0.01	0.014	<0.01	0.065					
			Spain			<0.01	<0.01	0.015	<0.01	0.017					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	0.011					
		Spinach	Netherlands	49		<0.01	<0.01	<0.01	<0.01	0.036					
			Italy	55		<0.01	<0.01	0.025	<0.01	0.045					
			Spain	49		<0.01	<0.01	0.015	<0.01	0.012					

1) Growth stage as planned (BBCH)

Table 6.6.2-11: Residues in succeeding crops (30 ± 1 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 1: 30 ± 1 DAA replanting interval															
Study code: 727902 **Doc ID:** 2015/1106682 **GLP:** Yes **Year:** 2014-2015	BAS 750 01 F 1 x 3.0 L/ha to bare soil	Wheat	Germany Netherlands Italy Spain	31-33	whole plant without root	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.021 0.012 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.011 0.037 <0.01	**Method No.:** **L0076/09** **analysed as:** **BAS 750 F** plant	0.01, 0.1, 1.0	74	83.9	9 1
			Germany Netherlands Italy Spain	65		<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.014 0.011 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 0.015 <0.01	**Method No.:** **L0170/02** **analysed as:** **T** plant **analysed as:** **TA** plant	0.01, 0 1, 1.0 0.01, 0 1, 1.0	84 83	96.6 99.3	8.2 6.4
			Germany Netherlands Italy Spain	89	grain	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.041 0.069 0.053 0.050	<0.01 0.034 0.021 0.022	<0.01 <0.01 <0.01 <0.01	**analysed as:** **TAA** plant **analysed as:** **TLA** plant	0.01, 0 1, 1.0 0.01, 0 1, 1.0	84 84	98.7 96.1	5.5 6.6
			Germany Netherlands Italy Spain	89	straw	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.023 <0.01 0.016	<0.01 0.020 <0.01 0.017					
		Carrots Radish	Germany Netherlands Italy Spain	41	whole plant with roots	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.011 <0.01 0.049	<0.01 <0.01 <0.01 <0.01	0.010 0.011 <0.01 <0.01					
		Carrots Radish	Germany Netherlands Italy Spain	49	tops	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 0.014 <0.01	<0.01 <0.01 <0.01 <0.01	0.0112 0.025 <0.01 <0.01					
		Carrots Radish	Germany Netherlands Italy Spain	49	roots	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 0.014 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01					
		Broccoli Cauliflower	Germany Netherlands Italy Spain	41	whole plant without root	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.012 0.026 0.017 0.013	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01					

Table 6.6.2-11: Residues in succeeding crops (30 ± 1 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.0457	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.084	<0.01	<0.01					
			Italy			<0.01	<0.01	0.030	<0.01	<0.01					
			Spain			<0.01	<0.01	0.019	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands			<0.01	<0.01	0.024	<0.01	0.016					
			Italy			<0.01	<0.01	0.015	<0.01	0.031					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.020					
			Italy			<0.01	<0.01	<0.01	<0.01	0.032					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					

[1] Growth stage as planned (BBCH)

Table 6.6.2-12: Residues in succeeding crops (120 ± 3 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 3: 120 ± 3 DAA replanting interval															
Study code: 727902	BAS 750 01 F		Germany			<0.01	<0.01	<0.01	<0.01	<0.01	**Method No.:**	**L0076/09**			
Doc ID: 2015/1106682	1 x 3.0 L/ha		Netherlands	31-33		<0.01	<0.01	0.031	<0.01	0.0146	**analysed as:**	**BAS 750 F**			
GLP: Yes	to bare soil		Italy			<0.01	<0.01	0.012	<0.01	0.0273	plant	0.01, 0.1, 1.0	74	83.9	9 1
Year: 2014-2015			Spain		whole plant without root	<0.01	<0.01	0.011	<0.01	<0.01	**Method No.:**	**L0170/02**			
			Germany			<0.01	<0.01	<0.01	<0.01	<0.01	**analysed as:**	**T**			
			Netherlands	65		<0.01	<0.01	0.017	0.020	0.016	plant	0.01, 0 1, 1.0	84	96.6	8.2
			Italy			<0.01	<0.01	0.011	<0.01	0.0122	**analysed as:**	**TA**			
		Wheat	Spain			<0.01	<0.01	<0.01	<0.01	<0.01	plant	0.01, 0 1, 1.0	83	99.3	6.4
			Germany			<0.01	<0.01	0.034	0.018	<0.01	**analysed as:**	**TAA**			
			Netherlands	89	grain	<0.01	<0.01	0.109	0.072	<0.01	plant	0.01, 0 1, 1.0	84	98.7	5.5
			Italy			<0.01	<0.01	0.068	0.028	<0.01	**analysed as:**	**TLA**			
			Spain			<0.01	<0.01	0.084	0.040	<0.01	plant	0.01, 0 1, 1.0	84	96.1	6.6
			Germany			<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands	89	straw	<0.01	<0.01	<0.01	0.027	0.032					
			Italy			<0.01	<0.01	<0.01	0.0121	0.011					
			Spain			<0.01	<0.01	<0.01	0.022	0.027					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	0.011					
			Netherlands	41	whole plant with roots	<0.01	<0.01	<0.01	<0.01	0.017					
		Radish	Italy			<0.01	<0.01	0.013	<0.01	<0.01					
			Spain			<0.01	<0.01	0.014	<0.01	<0.01					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	0.010					
			Netherlands	49	tops	<0.01	<0.01	<0.01	<0.01	0.0174					
		Radish	Italy			<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain			<0.01	<0.01	0.015	<0.01	<0.01					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands	49	roots	<0.01	<0.01	0.013	<0.01	<0.01					
		Radish	Italy			<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					
		Broccoli	Germany			<0.01	<0.01	0.021	<0.01	<0.01					
			Netherlands	41	whole plant without root	<0.01	<0.01	0.034	<0.01	<0.01					
		Cauliflower	Italy			<0.01	<0.01	0.026	<0.01	<0.01					
			Spain			<0.01	<0.01	0.024	<0.01	<0.01					

Table 6.6.2-12: Residues in succeeding crops (120 ± 3 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.043	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.101	<0.01	<0.01					
			Italy			<0.01	<0.01	0.0305	<0.01	<0.01					
			Spain			<0.01	<0.01	0.029	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.0119					
			Italy			<0.01	<0.01	<0.01	<0.01	0.0102					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	<0.01					
			Italy			<0.01	<0.01	<0.01	<0.01	0.016					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					

[1] Growth stage as planned (BBCH)

Table 6.6.2-13: Residues in succeeding crops (365 ± 5 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
PLOT 5: 365 ± 5 DAA replanting interval															
Study code: 727902	BAS 750 01 F		Germany			<0.01	<0.01	<0.01	<0.01	<0.01	**Method No.:**	**L0076/09**			
Doc ID: 2015/1106682	1 x 3.0 L/ha		Netherlands	31-33		<0.01	<0.01	0.024	<0.01	<0.01	**analysed as:**	**BAS 750 F**			
GLP: Yes	to bare soil		Italy			<0.01	<0.01	0.014	<0.01	<0.01	plant	0.01, 0.1, 1.0	74	83.9	9 1
Year: 2014-2015			Spain		whole plant without root	<0.01	<0.01	0.0120	<0.01	<0.01	**Method No.:**	**L0170/02**			
			Germany			<0.01	<0.01	<0.01	<0.01	<0.01	**analysed as:**	**T**			
			Netherlands	65		<0.01	<0.01	0.024	0.015	0.013	plant	0.01, 0 1, 1.0	84	96.6	8.2
			Italy			<0.01	<0.01	0.011	<0.01	0.014	**analysed as:**	**TA**			
		Wheat	Spain			<0.01	<0.01	<0.01	<0.01	<0.01	plant	0.01, 0 1, 1.0	83	99.3	6.4
			Germany			<0.01	<0.01	0.0402	0.018	<0.01	**analysed as:**	**TAA**			
			Netherlands	89	grain	<0.01	<0.01	0.10	0.039	<0.01	plant	0.01, 0 1, 1.0	84	98.7	5.5
			Italy			<0.01	<0.01	0.065	0.039	<0.01	**analysed as:**	**TLA**			
			Spain			<0.01	<0.01	0.13	0.055	<0.01	plant	0.01, 0 1, 1.0	84	96.1	6.6
			Germany			<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands	89	straw	<0.01	<0.01	<0.01	0.017	0.0103					
			Italy			<0.01	<0.01	<0.01	0.016	<0.01					
			Spain			<0.01	<0.01	<0.01	0.022	0.060					
		Carrots	Germany			<0.01	<0.01	<0.01	<0.01	0.015					
			Netherlands	41	whole plant with roots	<0.01	<0.01	<0.01	<0.01	0.015					
		Radish	Italy			<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain			<0.01	<0.01	<0.01	<0.01	<0.01					
		Carrots	Germany	49		<0.01	<0.01	<0.01	<0.01	0.014					
			Netherlands	49	tops	<0.01	<0.01	<0.01	<0.01	0.015					
		Radish	Italy	55		<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain	49		<0.01	<0.01	0.024	<0.01	<0.01					
		Carrots	Germany	49		<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands	49	roots	<0.01	<0.01	0.020	<0.01	<0.01					
		Radish	Italy	55		<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain	49		<0.01	<0.01	<0.01	<0.01	<0.01					
		Broccoli	Germany			<0.01	<0.01	<0.01	<0.01	<0.01					
			Netherlands	41	whole plant without root	<0.01	<0.01	0.022	<0.01	<0.01					
		Cauliflower	Italy			<0.01	<0.01	0.018	<0.01	<0.01					
			Spain			<0.01	<0.01	0.012	<0.01	<0.01					

Table 6.6.2-13: Residues in succeeding crops (365 ± 5 DAA replanting interval, untreated samples)

Study details	Formulation, Appl. rate (kg as/ha)	Crop	Country	GS [1]	Residues (mg/kg) [2]						Recovery data				
					Matrix	BAS 750 F	T	TA	TAA	TLA	Matrix	Fortification range (mg/kg)	n	Mean (%)	RSD (%)
		Broccoli	Germany	49	inflorescences	<0.01	<0.01	0.022	<0.01	<0.01					
		Cauliflower	Netherlands			<0.01	<0.01	0.061	<0.01	<0.01					
			Italy			<0.01	<0.01	0.061	<0.01	<0.01					
			Spain			<0.01	<0.01	0.027	<0.01	<0.01					
		Lettuce	Germany	41	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands			<0.01	<0.01	<0.01	<0.01	0.014					
			Italy			<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain			<0.01	<0.01	0.011	<0.01	<0.01					
		Lettuce	Germany	49	leaves	<0.01	<0.01	<0.01	<0.01	<0.01					
		Spinach	Netherlands	49		<0.01	<0.01	<0.01	<0.01	0.024					
			Italy	55		<0.01	<0.01	<0.01	<0.01	<0.01					
			Spain	49		<0.01	<0.01	0.012	<0.01	<0.01					

[1] Growth stage as planned (BBCH)

OVERALL CONCLUSION

Concerning residues in rotational crops, both a *nature of the residue* study and a *magnitude of the residue* study have been conducted in different crops representing three different crop categories, namely leafy vegetables, root and tuber vegetables and cereals. BAS 750 F was applied at 300 g ai/ha to bare soil, corresponding to a BAS 750 F concentration in soil of 0.1 mg/kg (soil depth 20 cm, soil density 1.5 g/cm^3) and thus well below the calculated plateau concentration of 0.058 mg/kg ($PEC_{accu,plateau}$ for a multiyear maximal annual rate application scenario, CA 9.1.3). The rotational crops were cultivated after soil aging intervals of 30d, 120d and 365 days, samples were taken at both mature and immature growth stages.
Based on results obtained in the *nature of the residue* study conducted with two labels (C-label, T-label), the pertinent residue in rotational crops is identified as unchanged parent BAS 750 F as well as the triazole derivative metabolites (TDM). The ratio of R- and S-enantiomers of BAS 750 F residue in plant remained unchanged compared with the test substance, indicating absence of preferential metabolism or uptake. Overall, the metabolism in rotational crops is similar to metabolism in primary crops (see section CA 6.2). The proposed definition of the relevant residue in rotational crops is parent BAS 750 F (see section 6.7). The magnitude of both BAS 750 F and TDM was investigated under field conditions. Based on the results obtained in the *magnitude of the residue* study, absence of significant residues of BAS 750 F are expected for the use of BAS 750 F supported in the present dossier. The residue data obtained for the TDM, common to a range of triazole-containing fungicides, is comparable to the data package previously submitted by the *Triazole Derivative Metabolite Group* (TDMG) to UK CRD.
In conclusion, for the use of BAS 750 F supported in the present dossier, no replant restrictions are required. As no significant residues of BAS 750 F are expected, the default MRL of 0.01 mg/kg is appropriate for rotational crops.

CA 6.7 Proposed residue definitions and maximum residue levels

CA 6.7.1 Proposed residue definitions

Plant matrices

I. GENERAL CONSIDERATIONS

For the purpose of proposing **residue definitions** both for **risk assessment** and for **monitoring,** the conclusions of metabolism studies in primary plants and confined rotational crops as well as a standard hydrolysis study are provided below.

1.1 Metabolism in plant

Metabolism was investigated using two radiolabels (BAS 750 F labeled in the C-ring or in the T-ring). Results obtained with both labels show a consistent picture of BAS 750 F metabolism. Investigations were done in three plant species, wheat (cereal crop group), soybean (pulses and oilseed crop group), and grapevine (fruits/fruiting vegetable crop group) after application of BAS 750 F according to the critical GAP. Altogether, three different EU crop groups are represented. These three crops are all target crops for intended BAS 750 F uses either in Europe (wheat, grape) or in Latin-America / NAFTA (soybean, grape). Comparable results were obtained for all three crop groups (see Figure 6.7.1) allowing therefore to extrapolate these results generally to BAS 750 F foliar-treated crops.

In plants, the metabolic pathway is largely based on:

- conjugation of uncleaved parent BAS 750 F

as well as the following key transformation steps (followed by species-typical conjugation) :

- hydroxylation at the C-ring (chlorophenyl-ring)
- cleavage at the T-bridge (triazole bridge)

and the following minor transformation step (followed by species-typical conjugation)

- cleavage at the ether-bridge

Both, the unchanged parent BAS 750 F as well as the (C-ring) hydroxy metabolite (a proposed since necessary intermediate) are conjugated further, as typical for plant, by sugars, resulting in a range of O-glycosylated metabolites (Figure 6.7-1). Cleavage at the T-bridge generates 1,2,4-triazole, which together with its conjugation products TA, TAA and TLA are a group of metabolites (TDM) common to other triazole containing fungicides. In contrast, cleavage at the ether bridge is only a minor transformation step in plants.

1.2 Metabolism in succeeding crops

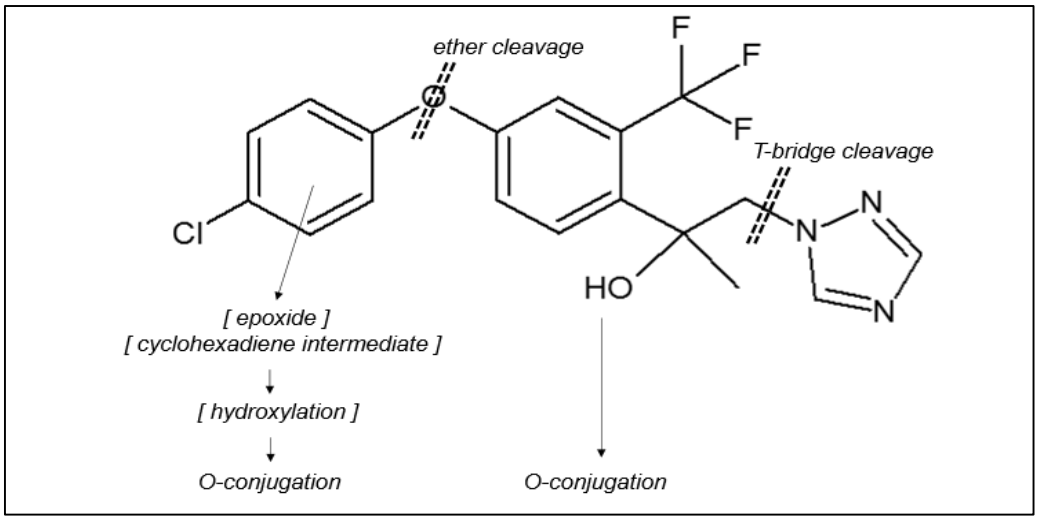
Metabolism of BAS 750 F was investigated in three representative succeeding crops, spinach (leafy), radish (root) and wheat (cereal). The study was performed at the maximum annual use rate of 300 g ai/ha applied to bare soil. Each label, namely C-label and T-label, was applied separately followed by soil aging intervals of 30 days, 120/122 days and 365/364 days. The investigation included both mature crops and immature growth stages.

The metabolic pathway is highly comparable in the different follow crops investigated. The resulting metabolite pattern is qualitatively very similar to the one identified for foliar treated plants. No compounds specific for rotational crops were found. Similar as in primary crops, the unchanged parent was the predominant component of the residue in most matrices and the group of triazole derivative metabolites (TDM) was the predominant component of the residue in grain.

1.3 Hydrolytic stability at high temperatures

The high temperature hydrolysis with BAS 750 F was conducted at three representative processing conditions representing baking/brewing/boiling, pasteurization as well as sterilization processes. Under all three conditions applied BAS 750 F was found to be stable. Therefore no additional degradation products need to be considered for the definition of the relevant residue.

Figure 6.7.1-1: BAS 750 F transformation reactions in plant



Taken together, in most matrices the unchanged parent is the predominant component of the residue (>60% of the radioactive residue), notably in forage (wheat, soybean), leaf/stalk (grapevine), straw/hull/chaff (wheat, soybean), green pod (soybean) and grape (grapevine). The enantiomer ratio of the two BAS 750 F remains unchanged (racemic mixture). Sugar conjugates of the hydroxylated parent are found in major amounts (up to 16% of the radioactive residue in wheat straw, up to 22% in grape leaf), while sugar conjugates of the unchanged parent are found <5% of the radioactive residue.

In contrast, in wheat grain and soybean seed, the predominant component of the residue is the group of TDM with triazole alanine as the most abundant component. Unchanged parent is absent from wheat grain, and found only in lower amounts in soybean seed (up to 4% radioactive residue).

The cleavage products resulting from cleavage of the ether bridge followed by conjugation with sugars, are present both only in straw and only in minor amounts (in sum <3% of radioactive residue).

Both for rotational crops as well as processed fractions, absence of any further residue components is confirmed.

II. RESIDUE COMPONENTS IN PLANT COMMODITIES

The following section discusses the relevance of the residue components identified in plant food items and feed items (for details on toxicology refer to section CA 5.8.1):

II.1 Residue components occurring >10% TRR

(a) parent & conjugates
(b) C-ring hydroxy metabolites & conjugates
(c) cleavage products & conjugates

II.2 Residue components occurring <10% TRR

(a) parent & conjugates
(b) C-ring hydroxy metabolites & conjugates
(c) cleavage products & conjugates

II.3 Non-extractable residue

Table 6.7.1-1: Residue components identified in plant commodities

group definition	(unconjugated) group metabolite members in PLANTS	
	≥ 10% TRR	< 10% TRR
a) parent & conjugates		
	BAS 750 F	M750F011 M750F012 M750F013 M750F014 M750F028
b) "C-ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates		
	M750F018 M750F019 M750F020 M750F026 M750F027	-
c) cleavage products & downstream metabolites / conjugates		
metabolites without the C-ring	-	M750F009 M750F010
metabolites without 1,2,4-T-ring	-	-
1,2,4-triazole and triazole-derived metabolites (TDM)	**1,2,4-T** **TA** **TAA**	**TLA**

1) underlined metabolites account for significant proportions of the residue

II.1 Residue components occurring at > 10% TRR in plants

(a) parent BAS 750 F & conjugates

In most matrices the unchanged parent is the predominant component of the residue (>60% of the radioactive residue), notably in forage (wheat, soybean), leaf/stalk (grapevine), straw/hull/chaff (wheat, soybean), green pod (soybean) and grape (grapevine).

→ *Thus, parent BAS 750 F is relevant for the definition of the residue.*

(b) "C-ring" hydroxy metabolites & conjugates

The **conjugation products** (O-glycosylation) derived from sugar conjugation of a (proposed since necessary) intermediate (group of regio-isomers resulting from the hydroxylation of the C-ring at different ring positions) while contributing to the complexity of the metabolite profile, in fact form a group of structurally highly comparable compounds only differing in the conjugated sugar moiety:

M750F018 O-malonyl-glucosyl-conjugate with a second hydroxylation of the C-ring
M750F019 O-glucosyl-conjugate
M750F020 O-malonyl-glucosyl-conjugate
M750F026 O-glycosyl-glucosyl-conjugate
M750F027 O-pentosyl-glucosyl-conjugate

Taken together, these (C-ring-) O-glycosylated metabolites account for up to 16% TRR in wheat straw, 22.3% TRR in grapevine leaf, less in wheat forage and soybean matrices (<5% TRR).

The expected hydrolysis in the human digestive system will release a (C-ring) hydroxy-metabolite. These compounds are common metabolites of BAS 750 F, also abundantly found in animal metabolism (see section CA 6.7.1.2 Animal matrices). In the rat metabolism study, the hydroxy metabolites M750F016 and M750F017 and their glucuronide conjugates are found in significant amounts (typically >50% of dose, see Table 6.7.1-2). It can therefore be assumed the toxicity of this metabolite group is adequately covered by existing toxicity studies with BAS 750 F. Due to the fact, that this group of metabolites not present in wheat grain at detectable levels, exposure of the consumer is not expected.

In feed commodities, notably in cereal straw, this group of metabolites, together accounting for up to 14-16% TRR, is present at significantly lower amounts than parent (59-69% TRR). Taking into account that feed burden (see section CA 6.7.2.2) is calculated with several worst-case assumptions (i.e. 100% of crop treated, containing residues at highest residue etc.), under realistic conditions these O-conjugates are not expected to significantly contribute to the livestock feed burden for the uses supported in the present dossier.

→ *Thus, these (C-ring) O-glycosylated metabolites are not relevant for the definition of the residue.*

(c) *cleavage products & conjugates*

Cleavage products resulting from cleavage at the T-bridge, are the most abundant component in wheat grain and soybean seed (account for 68% and 82% TRR, respectively).
M750F029 is generated by cleavage at the T-bridge resulting in 1,2,4-triazole, followed by conjugation to triazole alanine (TA). It occurs in wheat grain up to 46% TRR, in soybean seed up to 48% of TRR. Other metabolites of the TDM group were also found, albeit at lower amounts, namely M750F030 (TAA) up to 21% TRR (wheat grain), while M750F001 (1,2,4-triazole) and M750F031 (TLA) were present <2% TRR. The group of TDM are common to other triazole containing fungicides, within the frame of the ongoing evaluation of these common metabolites, it is proposed to set a separate residue definitions for TDM.

→ *As part of this dossier, TDM are not included in BAS 750 F specific residue definitions.*

II.2 Residue components occurring at < 10% TRR in plants

(a) parent BAS 750 F & conjugates

Conjugation products (O-glycosylation) derived from sugar conjugation of the unchanged parent backbone form a group of structurally related metabolites:
M750F011 O-glucosyl-conjugate
M750F012 O- malonyl-glucosyl-conjugate
M750F013 O-glucosyl-glucosyl-conjugate
M750F014 O- malonyl-glucosyl-glucosyl-conjugate
M750F028 O- pentosyl-glucosyl-conjugate

M750F012 found at level up to 5% TRR while the other conjugates were present at non-quantifiable levels. The expected hydrolysis in the human digestive system will release the parent BAS 750 F. It is therefore considered that the toxicity of this metabolite group is adequately covered by existing toxicity studies with BAS 750 F. Due to the fact, that this group of metabolites is not present in detectable amounts in the food item of this study (wheat grain) exposure of the consumer is not expected. In feed commodities, notably in cereal straw, this group of metabolites, together accounting for up to 5% TRR, is present at significantly lower amounts than parent BAS 750 F (59-69% TRR) and therefore not contributing significantly to the livestock feed intake.

→ *Thus, these sugar conjugates of parent are not relevant for the definition of the residue.*

(b) "C-ring" hydroxy metabolites & conjugates
- none -

(c) cleavage products & conjugates

Cleavage products resulting from cleavage at the ether bridge followed by sugar conjugation, namely M750F009 and M750F010, are found only in minor amounts (together accounting for <3% TRR in straw). In the rat metabolism study structurally related metabolites (M750F003, M750F054, M750F071, differing only in the conjugated moiety) are found in significant amounts (typically >11% of dose, see Tables 5.1.1-52-56). It can therefore be assumed that the toxicity of this metabolite group is adequately covered by existing toxicity studies with BAS 750 F.
Due to the fact, that these two metabolites are not present in detectable amounts in the food item of this study (wheat grain) exposure of the consumer is not expected. In feed commodities, notably in cereal straw, their level is significantly lower than parent BAS 750 F (59-69% TRR). Thus, their contribution to feed intake is insignificant.

→ *Thus, M750F009 and M750F010 are not relevant for the definition of the residue.*

II.3 Non-extractable residue

Generally, the RRR and/or final residue remaining after solvent extraction and solubilization treatments was low. Only in C-labeled wheat grain, a significant proportion of the residue was non-extractable (see below).

(1) wheat (cereals)
The wheat study shows that from forage only <5% TRR is non-extracted. In straw, solvent extraction resulted in a residual residue (RRR) of 14-17% TRR, and therefore was followed by further sequential solubilization treatments allowing reduction of the final residue to 6-8% TRR. These additional treatments indicated that most of the non-extractable residue consists of parent, and sugar conjugates of hydroxylated parent covalently linked to plant cell constituents. The remaining final residue indicates incorporation of the BAS 750 F derived radiocarbon into plant cell constituents.

In grain, solvent extraction resulted in a RRR of 56% TRR (C-label, TRR=0.06 mg/kg) and 22.1% TRR (T-label, 0.63 mg/kg). Treatment of the RRR with 1% ammonia had released further 17% TRR (0.011 mg/kg, C-label) and 15.3% TRR (0.095 mg/kg, T-label). It is assumed that the ammonia released T-labeled residue of 0.095 mg/kg reflects the components identified in the ERR, namely triazole derivative metabolites, while for the C-label the released amount was only 0.011 mg/kg.

In addition, with both C- and T-label, the non-extractable residue was reduced by enzyme-treatment to a low and similar level (0.010-0.012 mg/kg) indicating similar amounts of radioactivity incorporated into plant constituents corresponding to 1.9% TRR of T-labeled grain (TRR=0.6 mg/kg) and 15.4% TRR of C-labeled grain (TRR=0.06 mg/kg). Incorporation into plant constituents specifically was shown by treatment with starch-degrading enzymes: amylase/amyloglucosidase treatment did release 4.6% TRR of T-labeled grain and 24.5% TRR of C-labeled grain (corresponding to similar absolute levels of 0.02-0.03 mg/kg with both labels).

Taken together, it can be assumed that the non-extractable residue in wheat grain consists of triazole derivative metabolites as well as BAS 750 F derived radiocarbon which after entering primary metabolism was incorporated as sugar into the starch fraction. In conclusion, grain does not contain any additional residue component in significant amounts, therefore does not need to be considered for the purpose of defining the relevant residue in plant matrices.

(2) soybean (pulses and oilseeds)
The soybean study shows that the non-solvent extractable residue (RRR) accounts for up to 9% in forage and up to 13% in desiccated plant (rest-of-plant). Further solubilization treatment did reduce the final residue to <6% TRR for both matrices. In (desiccated) hull, RRR of up to 13% TRR was reduced by solubilization treatments to a final residue of up to 11% TRR.

In seed, solvent extraction resulted in RRR of 43% TRR (C-label, TRR=0.13 mg/kg) and 33% TRR (T-label, TRR=3.06 mg/kg) which was further reduced by solubilization treatments. The residue components released by solubilization are characterized by HPLC as hydrophilic components, which in T-labeled grain, did not exceed (individual) amounts of 0.01 mg/kg while in T-labeled grain, the predominant proportion was identified as triazole alanine (TA, M750F029, 17% TRR, 0.012 mg/kg) released by macerozyme/cellulase treatment. M750F029 is also the predominant component of the extractable residue, indicating that the structural identity of radioactive residue associated with plant cell wall constituents reflects the identity of extractable portion of the residue in seed. Notably, solubilization resulted in similar residue level (<0.01 mg/kg) for both labels indicating similar extent of incorporation of radiocarbon into plant primary metabolism. In conclusion, soybean seed does not contain any additional residue component in significant amounts, therefore does not need to be considered for the purpose of defining the relevant residue in plant matrices.

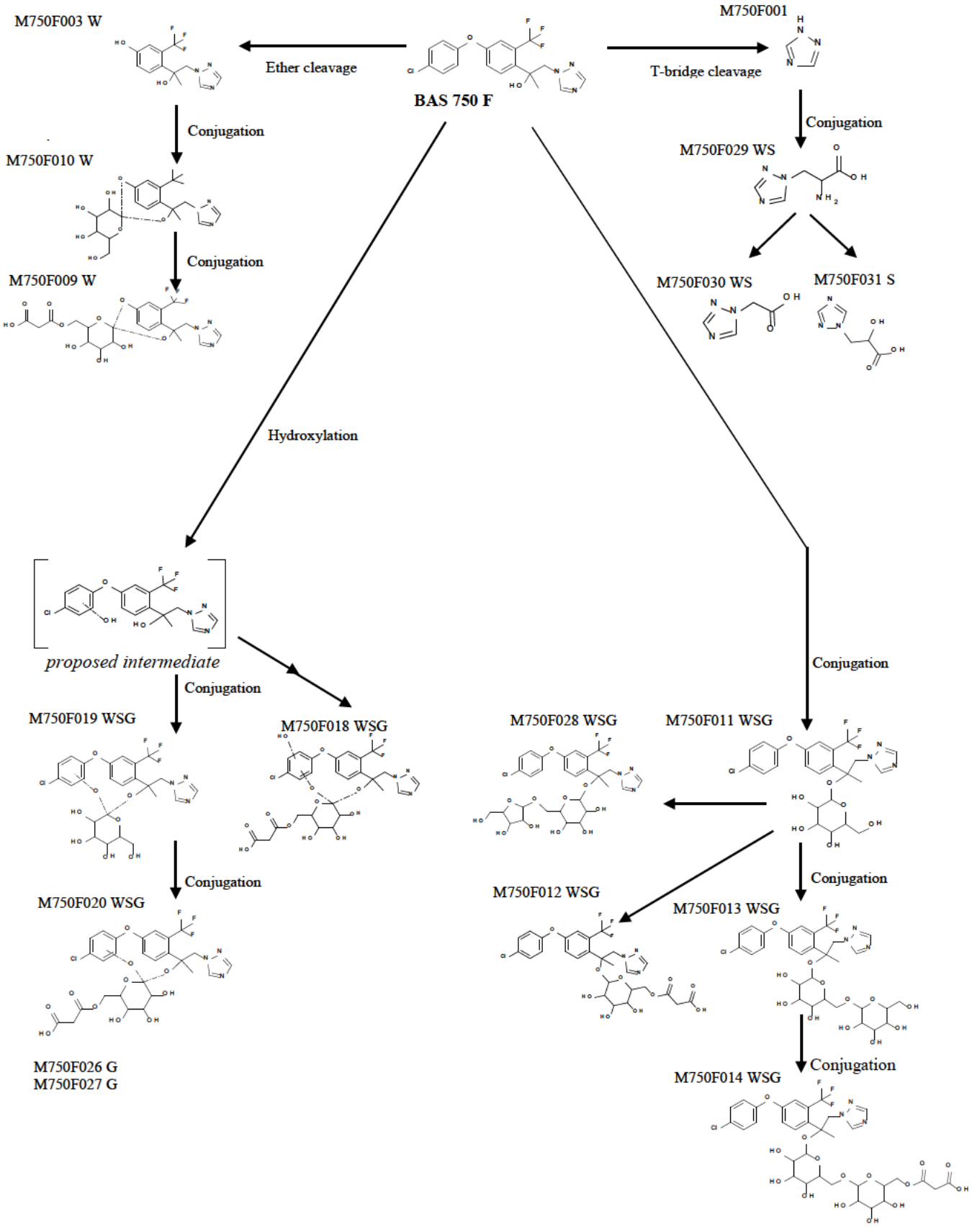
(3) grapevine (fruits and fruiting vegetables)

The grapevine study shows that non-solvent extractable residue (RRR) accounts for grape, leaf and stalk up to 11% TRR, and therefore was subjected to solubilization treatments, reducing the final residue to 3-6% for leaf and stalk. For grape, the final residue was reduced to 0.03 mg/kg (C-label 9% TRR, T-label 8% TRR) indicating similar amounts of radiocarbon incorporated into plant cell constituents. In conclusion, grape matrices do not contain any additional residue component in significant amounts, therefore do not need to be considered for the purpose of defining the relevant residue in plant matrices.

In summary, the non-extractable residue in the investigated crop parts representative of cereals, pulses and oilseeds as well as fruits/fruiting vegetables, can be considered not to contain any metabolites of importance beyond the list of metabolites identified in the extractable residue.

→ *Thus, non-extractable residue of plant is not relevant for the definition of the residue.*

Figure 6.7.1-2: BAS 750 F: metabolic routes in plant (W wheat, S soybean, G grape)



III. DEFINITON OF THE RELEVANT RESIDUE (PLANT)

For commodities of plant origin, including processed fractions, the following residue definitions are proposed:

Residue definition for MRL enforcement/monitoring (RD-Mo):

- *parent BAS 750 F*

Residue definition for risk assessment (RD-RA):

- *parent BAS 750 F*

Based on the evaluation of study results provided in the present dossier, the residue components in plant commodities relevant for the residue definitions include parent BAS 750 F, as well as the group of triazole derivative metabolites (TDM) while sugar conjugates and cleavage products are considered not relevant for the residue definition (see section CA 6.7 and CA 5.8).

RD-Mo

According to *OECD Guidance on the definition of residue (ENV/JM/MONO(2009)30*), the residue definition for tolerance/MRL enforcement (RD-Mo) should focus on those residue components suitable as analyte of multi-residue methods, suitable to indicate possible pesticide misuse, as well as suitable as general marker compound in food commodities concerned.

Parent BAS 750 F fulfills these criteria since its compatibility with multi-residue methods has been confirmed. Analysis of BAS 750 F allows to detect misuse of BAS 750 F containing formulated products. Also, BAS 750 F is a characteristic component of the residue typically accounting for a large proportion of the residue. As an exception in wheat grain, BAS 750 F is not detected, and in soybean seed BAS 750 F is present only in minor proportions of the residue. In these commodities, the predominant component of the residue are TDM which however are not considered suitable as BAS 750 F specific marker molecules. In conclusion, the proposed residue definition in grain and seed is parent BAS 750 F by default.

RD-RA

According to *OECD Guidance on the definition of residue (ENV/JM/MONO(2009)30),* the residue definition for risk assessment (RD-RA) should take into account the contribution of residue components to the potential dietary risk considering both the potential for exposure as well as the toxicity relative to the parent compound.

Parent BAS 750 F is a characteristic component of the residue and typically represents a predominant proportion of the residue. In conclusion, BAS 750 F is suitable to define the relevant residue in plant commodities.

In addition, a further large proportion of the residue is represented by the group of TDM, which are metabolites common to a range of azole fungicides. Based on different toxicological properties, a separate definition of residue relevant for risk assessment is expected as part of a common approach for azole fungicides. Therefore, TDMs are not considered for defining the relevant residue as part of the present dossier.

Animal matrices

I. GENERAL CONSIDERATIONS

For the purpose of proposing residue definitions for **risk assessment** as well as for **monitoring,** the conclusions of animal studies with BAS 750 F are provided below. Metabolism was investigated with in rat, goat, hen and fish showing rapid elimination of the residue (notably excretion and depuration upon dose withdrawal), rapid establishment of a plateau concentration in milk and egg (5-7 days) as well as transformation by metabolic routes comparable to rat. A linear dose-residue relationship confirmed in livestock feeding studies (cow and hen) allows to estimate residue levels for realistic feed intake of BAS 750 F.

In animals, the metabolic pathway is largely based on:

- conjugation of uncleaved parent BAS 750 F

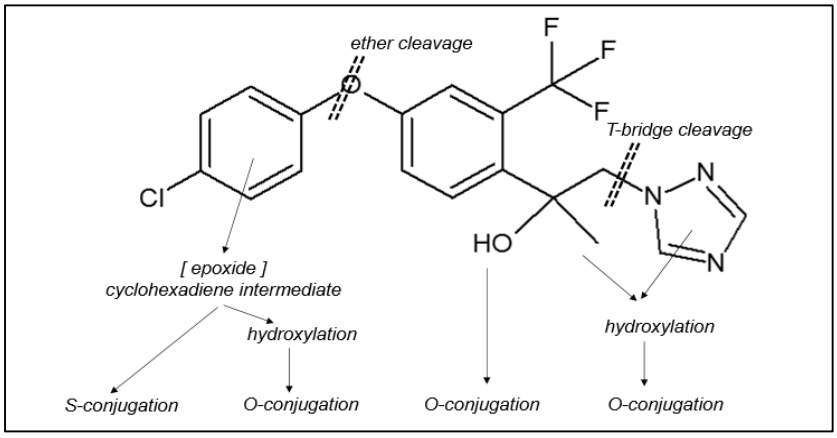
as well as the following key transformation steps (followed by species-typical conjugation) :

- hydroxylation at the C-ring (chlorophenyl-ring)
- cleavage at the T-bridge (triazole bridge)
- cleavage at the ether bridge

and the following minor transformation steps (followed by species-typical conjugation) :

- hydroxylation at the T-ring
- hydroxylation of the methyl group (at quaternary C-atom)

Figure 6.7.1-3: BAS 750 F transformation reactions in animal



The three key transformation steps, repeated and combined with minor transformation steps and diverse species-typical conjugation reactions (sulfatation, glucuronidation, glucosidation, methylation, glutathione conjugation), generate several large groups of structurally related metabolites. The animal metabolism studies taken together, confirm that basic metabolic routes in livestock animals (ruminant, poultry, fish) are comparable to rat. While in rat a large number of transformation products could be identified, the number of significant metabolites in commodities of animal origin is much smaller (see Table 6.7.1-3).

Key transformation reactions of BAS 750 F metabolism in animals

(1) conjugation reactions

Unchanged parent BAS 750 F is detected consistently in livestock commodities. In addition metabolites result from conjugation at the hydroxyl group of the quaternary C-atom (M750F068).

(2) C-ring hydroxylation

A main group of metabolite results from hydroxylation at the aromatic C-ring (Table 6.7.1-2). This transformation of the C-ring is observed both for uncleaved parent backbone as well as cleavage products consisting of two rings (C- and TFMP-ring) or one ring (C-ring). The cytochrome P450 mediated enzymatic oxygenation of aromatic substrates to corresponding phenol structures (see Figure 6.7.1-4), implicates hydration to form an epoxide structure, thereby converting the aromatic ring into a cyclohexadiene ring (e.g. *Jerina et al.1970, Biochemistry 9(1):147-56*). The instable epoxide is converted to a dihydrodiol structure (-OH/-OH, mediated by epoxide hydrolase) or to a corresponding glutathione conjugate -(OH/-SR) by conjugation with glutathione. These cyclohexadiene structures are intermediates which, by re-aromatization, are converted to the hydroxylated or S-conjugated C-ring. A wide array of such C-ring substituted metabolites is generated by repeated oxygenation combined with further O-conjugation or modification of the sulfide substituent (see Table 6.7.1-2, for details see section CA 5.1/1).

Figure 6.7.1-4: BAS 750 F transformation reactions: C-ring hydroxylation

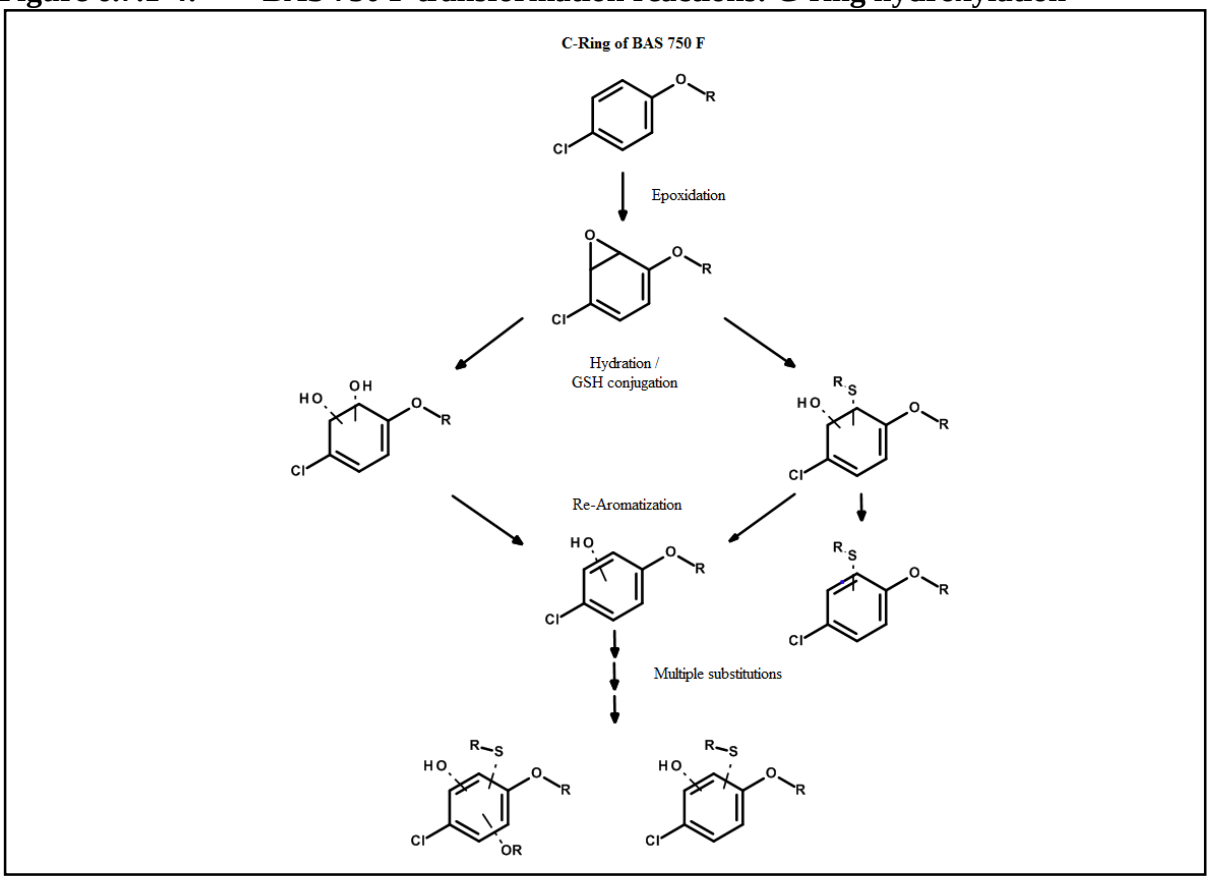
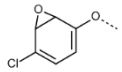
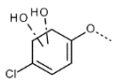
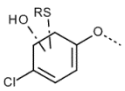
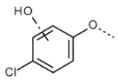
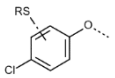
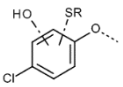
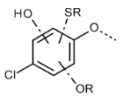


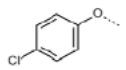
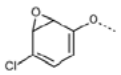
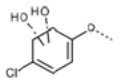
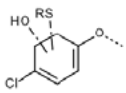
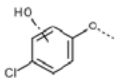
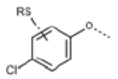
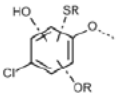
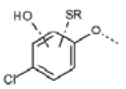
Table 6.7.1-2: Abundance of C-ring metabolites of BAS 750 F in rat metabolism

Note: Data on occurrence of individual metabolites are given in section MCA 5.1.1 (see tables 5.1.1-52 to -56). Coverage was calculated within a dose group (high dose/female, high dose/male, low dose/female, low dose /male) considering amounts detected in urine and bile. Additional amounts in tissues were not included due to differences in sampling time points.

RAT C-ring metabolites		**maximal dose recovery (per metabolite group)**					
		3-ring molecules: C , TFMP, T		2-ring molecules: C , TFMP		1-ring molecule: C	
		Metabolite code	*1)* % dose	Metabolite code	*1)* % dose	Metabolite code	*1)* % dose
ether intermediate		-	n.d.	-	n.d.	-	n.d.
cyclohexadiene intermediates		-	n.d.	-	n.d.	-	n.d.
		**F087** F102 F085 F084 F091 F069	>10 (41)	F065 F106	<1	-	n.d.
C-ring hydroxylation		**F015 F035** F005 **F016 F044** F092 **F017 F045**	>12 (53)	F082 F105 F077 F078 F066	<1%	F094 F081	n.d.
C-ring conjugate		F053 F075 F104	>11	-	n.d.	F050 F079 F052 F083 F055	2
Multiple substitutions	 	**F049** F089 F108 F059 F110 F090 F062 F093 F098 F057 F088 F096 F063 F060 F097 F107	<1	F099 F067 F100 F061 F046	1.9	F048 F080	<1
Entire metabolic branch [2)]		Σ (F085… F107)	**72**	-	-	-	-

1) the maximal value in any dose group, 2) the maximal value in any dose group (Σ F085 – F107, sum of all "downstream metabolites"). Metabolite codes are abbreviated, e.g. "F085" denotes "M750F085" etc., particularly abundant metabolites are indicated by bold typing. The recovery for the metabolic branch (C-ring hydroxylation) was typically >50% of dose. The highest recovery was 71.9% of dose (dose group: T-label, female, low dose) considering urine and bile including metabolites M750F049, M750F075, M750F087, M750F044, M750F045, M750F035, M750F015, M750F016, M750F017, M750F059.

Table 6.7.1-3: C-ring metabolites in livestock commodities

C-ring metabolites in metabolism		*maximal dose recovery (per metabolite group)*		*occurrence in livestock commodities (per metabolite: C- / TFMP- / T -label)*			
chlorophenyl-ring 		**RAT** 5)	dose % 1)	**GOAT** 3)	% TRR 2)	**HEN** 3)	% TRR 2)
ether intermediate		-	n.d.	-	n.d.	-	n.d.
cyclohexadiene intermediates		-	n.d.	F041	Mk: 6 / 7 / 0	-	n.d.
		**F087** F102 F085 F084 F091 F069	**>10 (41)**	-	n.d.	F034	L: 4 / 20 / 7
C-ring hydroxylation		**F015 F035** F005 **F016 F044** F092 **F017 F045**	**>12 (53)**	F015 F016 F016 F063	L: 0 / 3 / 0 L: 12 / 15 / 10 K: 0 / 4 / 0 all: <7 / 0 / 0	-	n.d.
C-ring conjugate		F053 F075 F104	**>11**	-	n.d.	-	n.d.
multiple substitutions	 	**F049** F089 F108 F059 F110 F090 F062 F093 F098 F057 F088 F096 F063 F060 F097 F107	**<1%**	-	n.d.	-	n.d.
metabolic branch 5)	sum	Σ (F085 …F107)	**72**				

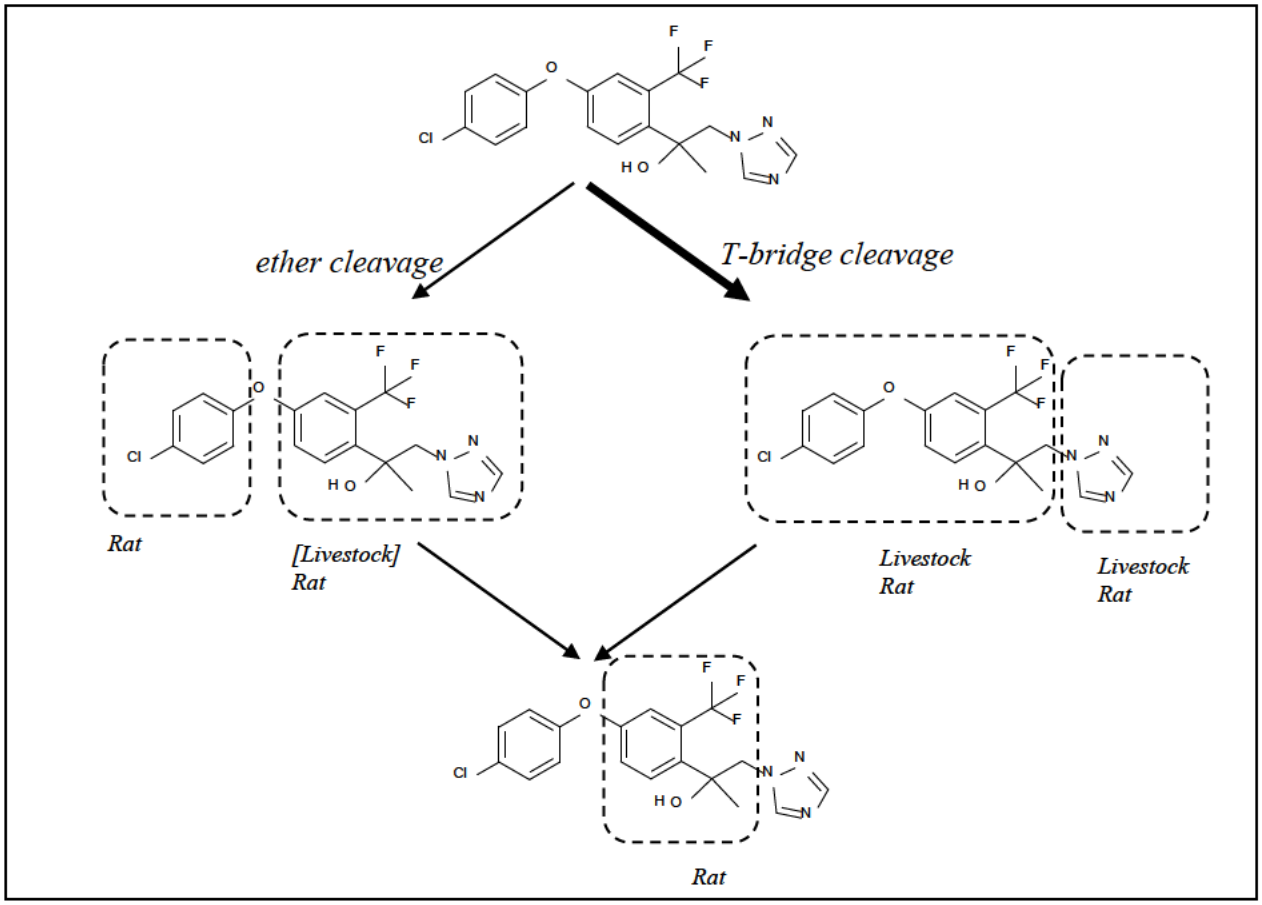
1) the maximal value in any dose group (high dose, male; high dose, female; low dose/male; low dose female) , 2) TRR = total radioactive residue for C-label, TFMP-label and T-label (C/TFMP/T), 3) tissues are abbreviated: "L" for liver, "K" for kidney, "Mk" for milk, "all" for milk, muscle, liver, kidney, fat, Metabolite codes are abbreviated, e.g. "F085" denotes "M750F085" etc., particularly abundant metabolites are indicated by bold typing. The recovery for the metabolic branch (C-ring hydroxylation) was typically >50% of dose. The highest recovery was 71.9% of dose (dose group: T-label, female, low dose) considering urine and bile including metabolites M750F049, M750F075, M750F087, M750F044, M750F045, M750F035, M750F015, M750F016, M750F017, M750F059.

(3) Cleavage of the molecular backbone

As one key transformation reaction, cleavage of the parent backbone at the triazole bridge generates 1,2,4-triazole, a metabolite common to a range of triazole-containing fungicides. In addition, a two-ring cleavage product is a characteristic component of the residue, namely M750F022 and downstream metabolites such as M750F038 as well as its fatty conjugates (M750F023, M750F024, M750F025), sulfate conjugate (M750F043) and glucuronic acid conjugate M750F064.

Cleavage at the ether bridge, followed by conjugation and C-ring hydroxylation, generates a wide array of metabolites seen in rat, and appears to occur also in livestock, albeit only to minor extent, as indicated by the presence of one metabolite in detectable amounts (M750F003 in liver). The detailed investigations in rat show that both T-bridge and ether bridge can be subject to successive cleavage, as indicated by the presence of urine metabolite M750F101, albeit only in traces, and absent from livestock.

Figure 6.7.1-5: BAS 750 F transformation reactions : cleavage of molecular backbone
[Note, parenthesis indicate animal with only low occurrence]



(4) minor transformation reactions

In rat metabolism, hydroxylation was also observed in the T-ring, namely metabolite M750F097, albeit only at non-quantifiable amounts. A related metabolite was seen in non-edible matrix of fish (liver). Furthermore, hydroxylation was observed in trace amounts at the methyl-group of BAS 750 F, namely metabolite M750F062. A related metabolite was seen in goat (M750F072 in liver, kidney).

II. RESIDUE COMPONENTS IN LIVESTOCK COMMODITIES

The following section discusses relevance of metabolites occurring in edible commodities of livestock (for details on toxicology refer to section CA 5.8).

II.1 Residue components occurring >10% TRR
(a) parent and conjugates
(b) C-ring hydroxy metabolites and conjugates
(c) cleavage products and conjugates

II.2 Residue components occurring <10% TRR
(a) parent and conjugates
(b) C-ring hydroxy metabolites and conjugates
(c) cleavage products and conjugates

II.3 Non-extractable residue

An overview over the residue components discussed is provided in the following table.

Table 6.7.1-4: Residue components identified in edible livestock commodities

group definition	(unconjugated) group metabolite members in LIVESTOCK	
	≥ 10% TRR	**< 10% TRR**
a) parent and conjugates		
	BAS 750 F M750F068	M750F072
b) "C-ring"-hydroxylation of non-cleaved molecule & downstream metabolites / conjugates		
	M750F016 M750F034	**M750F015** **M750F041** M750F063
c) cleavage products & downstream metabolites / conjugates		
Metabolites without the C-ring		**M750F003**
Metabolites without 1,2,4-T-ring	**M750F022** M750F023 M750F024 M750F025 **M750F038** M750F043 M750F064	-
1,2,4-triazole and triazole-derived metabolites (TDM)	**1,2,4-T**	-

II.1. Residue components occurring at > 10% TRR in livestock commodities

(a) parent and conjugates

BAS 750 F, its unchanged molecular structure is detected consistently in livestock commodities, typically in amounts >10% TRR.

→ *Thus, parent BAS 750 F is relevant for the definition of the residue.*

M750F068 is a glucuronide conjugate of the unchanged parent backbone (O-conjugation at the hydroxyl group of the quaternary C-atom. Hydrolytic removal of the glucuronide group in the human digestive system is expected to release the unchanged parent molecule. It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies with BAS 750 F (section CA5.8). In the livestock metabolism study, this metabolite was determined in quantifiable amounts in goat kidney (C-label, 17.8% TRR, 0.06 mg/kg while being absent in the other two labels). Taking into account an overdosing factor of 11X for the metabolism study, (based on mean estimated burden for beef cattle, see section CA6.9), the level of M750F068 in animal commodities, including liver, are expected to be <0.01 mg/kg.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

(b) C-ring hydroxy metabolites and conjugates

M750F016 is a C-ring hydroxylated metabolite with parent backbone unchanged. It is the predominant member of a group of regio-isomers, including M750F015 (minor metabolite, see below) and M750F017 (quantified only in excreta, see section CA6.2). These (C-ring)-hydroxy-metabolites are abundantly present in rat (representing up to 12% of dose). Together with conjugated products and intermediate transformation products of this C-ring hydroxylation branch, they account for up to 72% of dose in rat metabolism (see Table 6.7.1-3). It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies with BAS 750 F (section CA5.8).
In livestock this metabolite was determined in quantifiable amounts only in liver and kidney. In liver amounts of 12% TRR, 15% TRR, and 10%TRR (C-, TFMP-, T-label) were found corresponding to 0.06-0.20 mg/kg, while for kidney only in the TFMP-labeled sample detectable amounts were found (3.7% TRR, 0.016 mg/kg). Taking into account an overdosing factor of 11X for the metabolism study (based on mean estimated burden for beef cattle, see section CA6.9), these M750F016 occurrences correspond in practice to level of <0.01 mg/kg for kidney and <0.02 mg/kg for liver. Overall, these amounts are not contributing in significant amounts to the human dietary exposure resulting from BAS 750 F residues.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

M750F034 is an intermediate in the CYP mediated oxygenation of the C-ring (similar to goat metabolite M750F041, see below). The cyclohexadiene structure of the C-ring is the precursor preceding the re-aromatization to more stable hydroxy metabolites, such as the regio-isomers M750F015 and M750F016 ("C-OH metabolites", see Figure 6.7.1-3). Dihydrodiol cyclohexadiene structures as such, are unstable under acid condition (e.g. *Jerina et al.1970, Biochemistry 9(1):147-56*), known to re-arrange non-enzymatically to the hydroxylated aromatic ring. Likewise, M750F034 is considered unstable under dietary conditions, and to re-arrange, upon exposure to stomach acid, to a "C-OH metabolite" (namely M750F015, M750F016 or M750F017), thereby eliminating actual dietary exposure to metabolite M750F034.

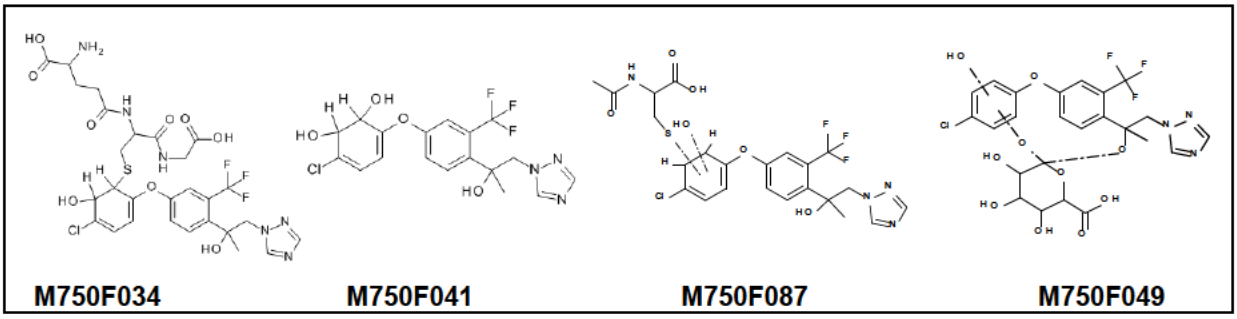
Furthermore, such cyclohexadiene intermediates, namely M750F087 (cyclohexadiene structure with -OH/-SR, see Figure 6.7.1-6) are also abundantly present in rat as a group amounting to at least 10.4% of dose and amounting to up to 53% of dose when considering also amounts co-eluting with structurally related metabolites (i.e. downstream metabolites of the "C-OH group", see below).

The latter "C-ring hydroxy metabolites" and their glucuronic acid conjugates M750F035, M750F044, M75F045 (designated "C-OH group"), are found abundantly in rat amounting to at least 12% of dose. If structurally realted co-elutes are additionally taken into account, namely M750F087 and M750F049 (C-ring di-hydroxylated metabolite, see Figure 6.7.1-6) significantly higher dose coverages are obtained.
For example:

- 22% of dose for M750F044 co-eluting with M750F087 (high dose TFMP-label, male)
- 23% of dose for "C-OH group" co-eluting with M750F049 (high T-label, male, high dose)
- 49-53% of dose for "C-OH group" co-eluting with M750F087/M750F049 (low dose, C-label, male/female)

When taken together, structurally related metabolites of this metabolic branch (C-ring oxygenation branch) amount to 72% of dose. It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies in rat dosed with BAS 750 F.

Figure 6.7.1-6: Related structures of M750F034, M750F041, M750F049 and M750F087



In dietary commodities of animal origin, i.e. poultry liver (hen metabolism study), metabolite M750F034 was found with 4.3% TRR, 20.1% TRR and 6.7% TRR (C-, TFMP-, and T-label) corresponding to up to 0.12 mg/kg. Considering that the hen metabolism study was conducted with highly exaggerated doses (216N for turkey, 154N for broiler, 30N for layer), residue level of M750F034 in practice will be well below 0.01 mg/kg, and thus not contributing in significant amounts to the human dietary exposure to BAS 750 F related residues.

→ *Thus, abundant coverage in rat metabolism, occurrence in edible livestock commodities only at very low level and only in poultry liver, as well as instantaneous elimination under dietary conditions (stomach acid) indicate that this metabolite both from a toxicological as well as exposure point of view does not have any relevance for human consumption.*

(c) cleavage products and conjugates

M750F022 is generated by cleavage of the T-bridge. Elimination of the T-ring leaves the ether structure (i.e. C-ring and TFMP-ring) unchanged, therefore resulting in a cleavage product structurally closely related to the parent molecule. Notably, in rat metabolism for both parent and M750F022, comparable downstream transformation products are seen, including conjugates with glucuronic acid, sulfate and C-ring hydroxy metabolites (see Table 6.7.1-3). T-bridge cleavage is a key transformation step of BAS 750 F metabolism as reflected by numerous downstream metabolites, which however were not measured at appreciably high quantities at the chosen sampling time points. Since not detectable under the study conditions, M750F022 was subjected to toxicological testing. The resulting data confirms a toxicological profile comparable to BAS 750 F, including absence of genotoxicity.

In ruminant livestock, M750F022 is occurring only in kidney in major amounts (up to 10.7% TRR), otherwise in milk, muscle, liver and fat at <7.6% TRR. In contrast, in hen matrices this metabolite generally accounts for the predominant proportion of the residue. In fat, M750F022 together with its fatty acid conjugates (M750F023, M750F024, M750F025, see below) accounts for almost the complete residue. As a metabolite potentially relevant in livestock commodities, feeding studies, in addition to BAS 750 F analysis, included data generation for M750F022 (cow 3 animals per dose group, and hen study 10 animals per dose group). The following tables (Table 6.7.1-5 and Table 6.7.1-6) provide representative data (each study, highest dose group) on M750F022 amounts relative to BAS 750 F amounts. In addition, the amounts of M750F022 fatty acid conjugates relative to the free metabolite M750F022 were determined in representative samples providing the basis for a proposal of correction factors.

Table 6.7.1-5: Relative amounts of BAS 750 F and M750F022 in cow matrices (representative data)

COW (150 mg/kg feed)	*group mean (mg/kg)*	
	BAS 750 F (MW 397.8)	**M750F022 (MW 346.7)**
milk [1)]	0.21	0.02
cream [2)]	1.23	0.06
skim milk [2)]	0.07	<0.01
muscle	0.16	0.02
liver	3.03	0.04
kidney	1.29	0.04
fat (mesenterial)	1.71	0.16

Table 6.7.1-6: Relative amounts of BAS 750 F and M750F022 in hen matrices (representative data)

HEN (15 mg/kg feed)	*group mean (mg/kg)*	
	BAS 750 F (MW 397.8)	**M750F022 (MW 346.7)**
egg	0.03	0.06
yolk	0.08	0.02
white	<0.01	<0.01
muscle	0.02	0.03
liver	0.10	0.15
fat	0.17	0.31
skin with fat	0.01	0.18

In summary, the comparability of toxicological properties of M750F022 and BAS 750 F was confirmed by toxicological investigations. This justifies to consider residues of M750F022 and BAS 750 F together (expressed as the sum, in BAS 750 F equivalents). Levels of M750F022 in ruminants are only low, while levels in poultry commodities are in the same order of magnitude as BAS 750 F, often somewhat higher. Therefore, M750F022 and its fatty acid conjugates, noteably in fat, egg and muscle, contributes quantitatively to the BAS 750 F related residue in poultry commodities and needs to be addressed in dietary exposure assessments.

→ *Thus, from an exposure point of view, metabolite M750F022 (and its fatty acid conjugates) is relevant for the definition of the residue for dietary risk assessment.*

M750F023, M750F024 M750F025 are fatty acid conjugates of M750F022, which upon hydrolytic removal of the fatty acid group will release metabolite M750F022. Consequently, it is appropriate to apply the toxicological reference values of M750F022 to this group of conjugates. While non-detectable in fish and goat, this group of metabolites accounted for a significant proportion of the residue in the hen metabolism study (>25% TRR in fat, egg yolk, less in liver and kidney, and not more than 0.01 mg/kg in muscle). This observation is confirmed by *magnitude of the residue* study in hen (see section CA6.4/3) showing that the residue level for "*sum of M750F022 and fatty acid conjugates*" is higher than the residue level of "*M750F022*" (in fat by a factor of 4, in egg and muscle by a factor of 1.5, see the following table). Therefore, fatty acid conjugates are, from a toxicological perspective considered equivalent to M750F022, and from an exposure perspective considered to contribute to M750F022 residue in several hen matrices, namely fat (including skin), muscle and egg.

Table 6.7.1-7: Proposed correction factor for residues of M750F022 and fatty acid conjugates in hen matrices (detailed data provided in section CA6.4/3)

matrix	correction factor
liver	1.0
muscle	1.5
egg	1.5
fat, skin with fat	4.0

M750F038 is generated by conversion of the hydroxyl group of M750F022 to a carboxy group, leaving the remaining molecular structure unchanged. Consequently, toxicological properties equivalent to M750F022 can be assumed. By increasing hydrophilicity, enhanced excretability of the molecule can be assumed (in comparison to M750F022). This is consistent with the fact, that M750F038 occurrence in livestock is not only restricted to excretory organs (goat liver, kidney) but also found in significant amounts in excreta of these animals (noteably in bile up to 11% TRR, in feces up to 6% TRR). Additional amounts are to be assumed for urine, where co-elution with M750F042 accounts for up to 28-47% of dose. Based on the overall high comparability of metabolic routes in ruminants and rat, a similar excretion behaviour is to be expected for rat (and thus also for humans). Therefore, it is appropriate to apply toxicological reference values of M750F022 to M750F038.
In edible livestock commodities, M750F038 is found in goat liver (up to 11% TRR, 0.15 mg/kg). In goat kidney up to 14% TRR (0.06 mg/kg) were found, additional amounts are likely to be present in a fraction of 26.6% TRR (0.09 mg/kg) co-eluting with M750F064 (glucuronide of M750F022). Considering that the goat metabolism study was conducted at exaggerated dose (overdosing factor of 11N based on median feed burden for beef cattle), the concentration of M750F038 in practice is expected not to exceed 0.01 mg/kg in liver and kidney, and thus to not contribute in significant amounts to the human dietary exposure resulting from BAS 750 F residues.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

M750F043 is a sulfate conjugate of M750F022. Hydrolytic removal of the sulfate group in the human digestive system is expected to release M750F022. Consequently, toxicological properties equivalent to M750F022 can be assumed. M750F043 is occurring only in goat milk at 14.2% TRR (C-label) and 25% TRR (T-label) albeit corresponding to only very low amounts (0.004 mg/kg and 0.016 mg/kg respectively). Taking into account an overdosing factor of 7N for the goat metabolism study (based on the median feed burden of dairy cattle), in practice, level of M750F043 in milk, is expected to be far below <0.01 mg/kg and contribution to dietary exposure non-significant.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption*

M750F064 is a glucuronide conjugate of M750F022. Hydrolytic removal of the glucuronide group in the human digestive system is expected to release M750F022, thus the toxicological reference values of M750F022 can be applied to M750F064. M750F064 is occurring in goat kidney (C-label), co-eluting with M750F038 (also a metabolite structurally closely related to M750F022, see above). Together, both metabolites are accounting for 26.6% TRR (corresponding to 0.09 mg/kg). Taking into account an overdosing factor of 11N for the goat metabolism study (based on the median feed burden of beef cattle), in practice, level of M750F064 in kidney, is expected not to exceed 0.01 mg/kg and therefore not to contribute in significant amounts to dietary exposure.

M750F001 (1,2,4-T) is generated by cleavage of the T-bridge. It is a metabolite common to other triazole containing fungicides. It was found in major amounts in most livestock tissues, namely in goat (>60% TRR in milk, muscle, kidney, lower in liver and fat), hen (>60% TRR in egg white, muscle, liver, kidney and fat), fish (>80% TRR in filet and filet-skin) as well as in rat (up to 20% of dose).

II.2 Residue components occurring at < 10% TRR in livestock commodities

(a) parent and conjugates

M750F072 is a sulfate conjugate with the intact parent backbone being hydroxylated at the methyl group. Hydrolytic removal of the sulfate group in the human digestive system is expected to release the (methyl)-hydroxylated parent molecule. Note, that a similar “diol” structural motif is present in the metabolite M750F022 which upon genotoxicity testing did not give rise to toxicological concern (see above). It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies with BAS 750 F (section CA5.8).
In livestock this metabolite was determined in quantifiable amounts in only goat milk (C- and TFMP-label <6% TRR, <0.04 mg/kg) while being absent in T-label. Taking into account an overdosing factor of 7N for the goat metabolism study (based on the median feed burden of dairy cattle), in practice, level of M750F072 in milk, is expected to be well below 0.01 mg/kg and therefore not to contribute in significant amounts to dietary exposure.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

(b). C-ring hydroxy metabolites and conjugates

M750F015 is a regio-isomer, thus closely structurally related, of the C-ring hydroxylated metabolites M750F016 and M750F017, where as part of the transformation reaction at the C-ring, the chlorine was shifted to a neighboring position ("*NIH shift*"). As detailed above, these C-ring hydroxylated metabolites as a group, are abundantly present in rat metabolism (typically >50% of dose, see part on M750F016) and thus are considered covered by the existing toxicological studies with BAS 750 F. M750F015 is occurring in goat only in low amounts (<3% TRR, 0.011 mg/kg, only TFMP-label). Taking into account an overdosing factor of 11N for the goat metabolism study (based on the median feed burden of beef cattle), in practice, level of M750F015, is expected to be well below 0.01 mg/kg and therefore not to contribute in significant amounts to dietary exposure.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

M750F041 is an intermediate in the CYP mediated oxygenation of the C-ring. Similar to hen metabolite M750F034 (discussed above), the dihydrodiol cyclohexadiene structure of the C-ring of M750F041 is inherently unstable and will, notably under acidic conditions, non-enzymatically re-arrange to a hydroxylated aromatic ring structure. Thus, under dietary conditions, upon exposure to stomach acid, M750F041 will re-arrange to to a "C-OH metabolite" (namely M750F015, M750F016 and M750F017) thereby eliminating actual dietary exposure to M750F041. As discussed above, such cyclohexadiene intermediates, namely M750F087, are abundantly present in rat (as a group amounting to up to 53% of dose when considering also amounts co-eluting with structurally related metabolites).

In dietary commodities of animal origin, i.e. ruminant milk (goat metabolism study), metabolite M750F041 was found at non-major amounts (6.0 – 7.2 % TRR, corresponding to <0.005 mg/kg). Considering an overdosing factor of 7N (for goat metabolism study compared with median feed burden for dairy cattle), the amounts in practice is expected to be <0.001 mg/kg in milk, and thus not contributing significantly to dietary exposure.

→ *Thus, abundant coverage in rat metabolism, occurrence in edible livestock commodities only at trace levels and only in milk, as well as instantaneous elimination under dietary conditions (stomach acid) indicate that this metabolite both from a toxicological as well as exposure point of view does not have any relevance for human consumption.*

M750F063 is a glucuronide conjugate of the group of C-ring hydroxylated metabolites, thus hydrolytic removal of the glucuronide group in the human digestive system is expected to release the corresponding C-ring hydroxy-metabolite (M750F016 or M750F015). As detailed above, these C-ring hydroxylated metabolites as a group, are abundantly present in rat metabolism (typically >50% of dose, see part on M750F016) and thus are considered covered by the existing toxicological studies with BAS 750 F. M750F063 was found in C-labeled goat samples co-eluting with M750F022, together accounting for less than 7% TRR (or less than 0.05 mg/kg). Other C-ring hydroxy-metabolites, notably M750F016, are much more abundant, therefore the quantitative contribution of M750F063 is non-significant.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

(c) cleavage products and conjugates

M750F003 is a two ring cleavage product (TFMP and T-ring) resulting from cleavage of the ether bridge. This cleavage product has been identified in rat at a minimum of 1.9% dose. Together with sulfate conjugate (M750F071) and a glucuronide conjugate (M750F054) it represents up to 12.5% of dose in BAS 750 F treated rats. It can therefore be considered that the toxicity of this metabolite is covered by the existing toxicology studies with BAS 750 F (section CA 5.8). In livestock, this metabolite was determined in quantifiable amounts in goat kidney (only with TFMP-label at 3.2% TRR, 0.14 mg/kg) while being absent in the T-label. Considering that the goat metabolism study was conducted at exaggerated dose (overdosing factor of 11N based on median feed burden for beef cattle), the concentration of M750F003 in practice is expected to not exceed 0.01 mg/kg in liver, and thus to not contribute in significant amounts to the human dietary exposure resulting from BAS 750 F residues.

→ *Thus, both from a toxicological as well as exposure point of view this metabolite does not have any relevance for human consumption.*

II.3 non-extractable residue in livestock commodities

The hen metabolism study shows that the non-extractable/non-solubilizable residue in edible matrices is low (<5% TRR, except for liver with a final residue of up to 7% TRR, corresponding to 0.02 mg/kg and muscle with a final residue of 8% TRR corresponding to <0.01 mg/kg).

The goat metabolism study shows that the non-extractable/non-solubilizable residue in edible matrices is low (<5% TRR, except for milk with a final residue of up to 7.5% TRR, corresponding to 0.002 mg/kg).

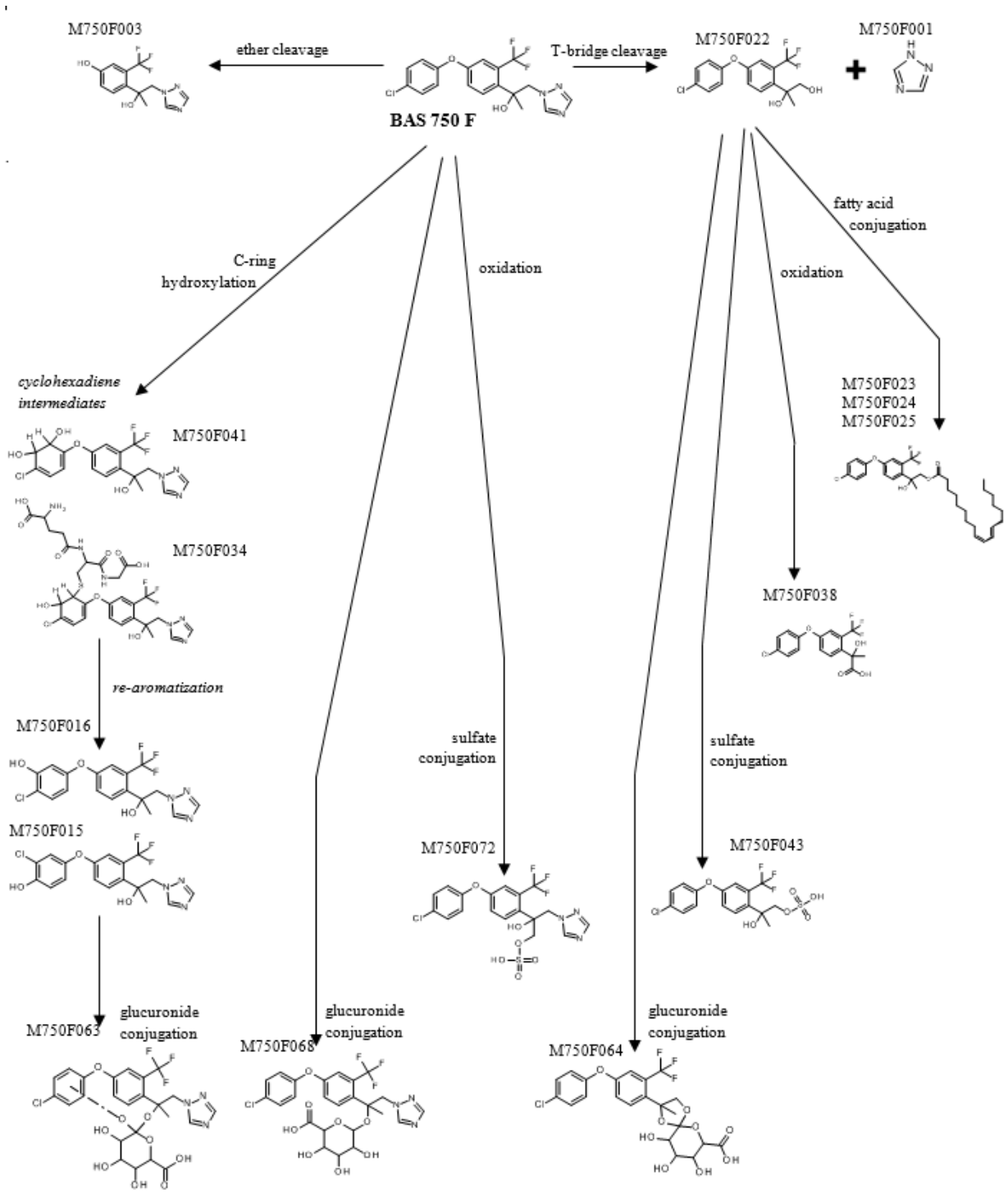
The fish metabolism study shows that the non-extractable/non-solubilizable residue in edible matrices is low (<0.01 mg/kg).

Overall, for hen, goat and fish, a predominant proportion of the TRR was accessible to identification and/or characterization of residue components.

→ *Thus, non-extractable/non-solubilizable residue is not relevant for the definition of the residue.*

Figure 6.7.1-7: BAS 750 F: metabolic routes in livestock animals (food commodities)

Note, arrows indicate metabolic routes, not separate biochemical reactions. The group of M750F023, M750F024, and M750F025 are acyl-conjugates of M750F022, differing only in the fatty acid side chain, thus only one representative molecular structure is provided. Metabolites occurring only in non-edible commodities are not considered.



III. DEFINITON OF THE RELEVANT RESIDUE (ANIMAL)

For commodities of animal origin, the following residue definitions are proposed:

Residue definition for MRL enforcement/monitoring (RD-Mo):

- *parent BAS 750 F*

Residue definition for risk assessment (RD-RA):

- *animal except poultry:* *parent BAS 750 F*
- *poultry:* *sum of parent BAS 750 F, metabolite M750F022 and fatty acid conjugates of M750F022, expressed as parent equivalents*

Based on the evaluation of study results provided in the present dossier, the residue components in animal commodities relevant for the residue definitions include parent BAS 750 F, metabolite M750F022 and its fatty acid conjugates as well as the group of triazole derivative metabolites (TDM) while several other metabolite are considered not relevant for the residue definition (see section CA6.7 and CA5.8).

Residue definition for MRL enforcement/monitoring (RD-Mo)

According to *OECD Guidance on the definition of residue (ENV/JM/MONO(2009)30)* the residue definition for tolerance/MRL enforcement (RD-Mo) should focus on those residue components suitable as analyte of multi-residue methods, suitable to indicate possible pesticide misuse, as well as suitable as general marker compound in food commodities concerned.

Parent BAS 750 F fulfills these criteria since its compatibility with multi-residue methods has been confirmed. Analysis of BAS 750 F allows to detect misuse of BAS 750 F containing formulated products. Also, BAS 750 F is a characteristic component of the residue typically accounting for a significant proportion of the residue.

M750F022 is also present in significant amounts. However, M750F022 not suitable as analyte for multi-residue methods (Due to insufficient ionization properties, residue analysis has to rely on GC/MS. Resulting from sensitivity to matrix interference a time-intensive methodology is needed to achieve a quantitation limit of 0.01 mg/kg).

TDMs, also present in significant amounts, however are not considered suitable as BAS 750 F specific marker molecules.

Residue definition for risk assessment (RD-RA)

According to *OECD Guidance on the definition of residue (ENV/JM/MONO(2009)30),* the residue definition for risk assessment (RD-RA) should take into account the contribution of residue components to the potential dietary risk considering both the potential for exposure as well as the toxicity relative to the parent compound.

In commodities of ruminant origin, and by extrapolation of swine origin, parent BAS 750 F is a characteristic component of the residue and typically represents a predominant proportion of the residue. In conclusion, BAS 750 F is suitable to define the relevant residue in these commodities.

In commodities of poultry origin, in addition to parent BAS 750 F residues, significant amounts of metabolite M750F022 are typically present. In some commodities, namely fat, egg and muscle, a significant proportion of M750F022 is present as fatty acid conjugates. Release of M750F022 by hydrolytic cleavage of fatty acid conjugates in the human digestive tract might contribute significantly to the amount of M750F022. Toxicological investigations show that M750F022 (see section CA5.8) has comparable toxicological properties. Therefore, the toxicological reference values derived for BAS 750 F can be applied to M750F022. In order to account for a large proportion of the residue it is therefor proposed to define the sum of BAS 750 F, metabolite M750F022 and its fatty acid conjugates as the relevant component of the residue in poultry commodities for risk assessment. The proposed matrix-specific conversion factors (muscle 6.20, fat 16.33, liver 4.94, egg 4.86) take into account both the matrix-specific contribution of fatty acid conjugates as well as the molecular weight difference between M750F022 and BAS 750 F (details are provided in section CA 6.7.2).

In addition, a predominant proportion of the residue is represented by the group of TDM, which are metabolite common to a range of azole fungicides. Based on different toxicological properties, a separate definition of residue relevant for risk assessment is expected as part of a common approach for azole fungicides. Therefore, TDMs are not considered for defining the relevant residue as part of the present dossier.

CA 6.7.2 Proposed maximum residue levels (MRLs) and justification of the acceptability of the levels proposed

Plant matrices

For both target crops, wheat and barley, sufficient supervised residue trials were conducted. BAS 750 01 F or BAS 750 00 F (EC formulations) was applied twice at a rate of 0.150 kg/ha BAS 750 F, according to the recommended use pattern, to generate residue results and MRL proposals for the cereal crops wheat, triticale, rye, barley and oat, harvested at BBCH89. The trials conducted fulfill the requirements concerning the geographical and the seasonal distribution, and can therefore serve as basis for deriving MRL values for cereal grain and straw. Based on the residue definition (RD-Mo), the MRL proposals refer to the parent compound BAS 750 F only.

(1) Wheat

In support of the representative use in wheat, data from a total of 17 cGAP compliant new field trials on wheat is provided in the present dossier.

Table 6.7.2-1: Critical GAP for the proposed use in wheat

crop	outdoor/ protected	growth stage (BBCH)	maximum number of applications	minimum application interval (days)	maximum		minimum PHI (days)
					rate (kg as/ha)	water (L/ha)	
wheat	outdoor	49, 69	2	14	0.15	200	35

The residue trials were performed in various European Member States in both European regions (N-EU, 8 trials; S-EU 9 trials) during two growing seasons (2013, 8 trials; 2014, 9 trials) and thereby fulfill the requirements of seasonal and geographical distribution.
Note, that field trial data obtained for the formulated product BAS 750 01 F is available for the season 2014 (4 trials N-EU trials, 5 trials S-EU). In addition, for season 2013, field trial data is available obtained with the formulated product BAS 750 00 F (4 trials N-EU trials, 4 trials S-EU). Generally, equivalent residue data is obtained with both formulated products as confirmed by BAS 750 00 F residue data generated in parallel with the formulated product BAS 750 01 F in 2014.

Table 6.7.2-2: Summary BAS 750 F residue data for the proposed use in wheat

European region	BAS 750 F residues in grain *(mg/kg)*	HR *(mg/kg)*	STMR *(mg/kg)*	MRL *(mg/kg)*
N-EU	6x <0.01, 0.01, 0.02	0.024	0.010	0.04
S-EU	7x <0.01, 0.02, 0.03	0.026	0.010	0.04

Table 6.7.2-3: Summary BAS 750 F residue data for the proposed use in wheat

European region	BAS 750 F residues in straw *(mg/kg)*	HR *(mg/kg)*	STMR *(mg/kg)*	MRL *(mg/kg)*
N-EU	1.9, 2.3, 2.6, 3.6, 3.9, 4.3, 5.5, 10	10	3.75	15
S-EU	0.50, 0.56, 1.6, 2.9, 3.1, 3.8, 4.6, 8.5, 18	18	3.10	30

Calculations were done using the OECD calculator, in cases residues are <LOQ, a value of 0.01 mg/kg was used for the calculation. The MRL proposal is extrapolated from wheat grain to triticale and rye grain.

In conclusion, it is proposed to establish a MRL of

0.04 mg/kg BAS 750 F for grain of wheat, triticale and rye
30 mg/kg for straw of wheat, triticale and rye

(2) Barley

In support of the representative use in barley, data from a total of 18 cGAP compliant new field trials on barley is provided in the present dossier.

Table 6.7.2-4: Critical GAP for the proposed use in barley

crop	**outdoor/ protected**	**growth stage (BBCH)**	**maximum number of applications**	**minimum application interval (days)**	**maximum**		**minimum PHI (days)**
					rate (kg as/ha)	**water (L/ha)**	
barley	outdoor	49, 69	2	14	0.15	200	35

The residue trials were performed in various European Member States in both European regions (N-EU, 9 trials; S-EU 9 trials) during two growing seasons (2013, 8 trials; 2014, 10 trials) and thereby fulfill the requirements of seasonal and geographical distribution.
Note, that field trial data obtained with the formulated product BAS 750 01 F is available for the season 2014 (4 trials N-EU trials, 5 trials S-EU). In addition, for season 2013, field trial data is available obtained with the formulated BAS 750 00 F (4 trials N-EU trials, 4 trials S-EU).

Generally, equivalent residue data is obtained with both formulated products as confirmed by BAS 750 00 F residue data generated in parallel with the formulated product BAS 750 01 F in 2014.

Table 6.7.2-5: Summary BAS 750 F residue data for the proposed use in barley

European region	BAS 750 F residues in grain *(mg/kg)*	HR *(mg/kg)*	STMR *(mg/kg)*	MRL *(mg/kg)*
N-EU	0.01, 0.06, 0.07, 2x0.09, 0.11, 0.15, 0.19, 0.28	0.28	0.087	0.6
S-EU	0.02, 0.03, 0.06, 0.07, 0.09, 0.10, 0.14, 0.16, 0.41	0.41	0.088	0.6

Table 6.7.2-6: Summary BAS 750 F residue data for the proposed use in barley

European region	BAS 750 F residues in straw *(mg/kg)*	HR *(mg/kg)*	STMR *(mg/kg)*	MRL *(mg/kg)*
N-EU	1.0, 1.7, 3.1, 3.6, 3.9, 4.3, 5.6, 6.8, 15	15	3.9	30
S-EU	0.39, 2.1, 2.2, 2.4, 4.2, 4.6, 6.4, 11, 18	18	4.2	30

Calculations were done using the OECD calculator, in cases residues are <LOQ, a value of 0.01 mg/kg was used for the calculation. The MRL proposal is extrapolated from barley grain to oat grain.

In conclusion, it is proposed to establish a MRL of

0.6 mg/kg BAS 750 F for grain of barley and oat
30 mg/kg for straw of barley and oat

Animal matrices

TO support the cereal use in the present dossier, MRLs for commodities of animal origin are proposed, based on the following calculations:

(1) Calculation of livestock feed intake
(2) Calculation of residue level in commodities of ruminant and swine origin
(3) Calculation of residue level in commodities of poultry origin
(4) MRL proposals for livestock commodities

The proposed residue definition for MRL and enforcement (RD-Mo) is BAS 750 F (see section CA6.7.1.2). Residue data of BAS 750 F in animal commodities is estimated taking into account results of crop field trials (feed items, see Table 6.7.2-4), results of processing studies (transfer factors for feed items, see Table 6.7.2-4.) to calculate the livestock feed intake as well as taking into account the linear dose-residue-relationship confirmed in livestock feeding studies. Feed intake calculations (see Table 6.7.2-8) as well as proposals for MRL (see Table 6.7.2-13) and are obtained using the Excel calculator *Animal model.2015.xls* developed by EFSA.

(1) Calculation of livestock feed intake

Table 6.7.2-7: BAS 750 F residue level in feed items (input for feed intake estimation)

Feed item		commodity	BAS 750 F		
			STMR *mg/kg*	**HR** *mg/kg*	**PF**
forages	barley	straw	4.20	18	-
	oat	straw	4.20	18	-
	rye	straw	3.75	18	-
	triticale	straw	3.75	18	-
	wheat	straw	3.75	18	-
cereal grains	barley	grain	0.088	-	-
	oat	grain	0.088	-	-
	rye	grain	0.010	-	-
	triticale	grain	0.010	-	-
	wheat	grain	0.010	-	-
by-products	brewer's grain	dried	0.088	-	2.31
	distiller's grain	dried	0.088	-	2.31
	wheat gluten	meal	0.010	-	0.39
	wheat	milled by-products	0.010	-	0.72

Feed intake level was estimated using the Excel calculator *Animal model 2015a.xls* developed by EFSA. The output is summarized in Table 6.7.2-8. When considering residue data as it results from the cereal use supported in the present dossier, exceedance of the feed intake value of 0.004 mg/kg bw is observed for cattle, sheep and poultry, while feed intake below the trigger is calculated for pig.

Table 6.7.2-8: Estimated feed intake (Regulation (EU) No 283/2013)

livestock animal	**BAS 750 F**				**highest contributing commodities**	
	median burden *(mg/kg bw)*	**maximum burden** *(mg/kg bw)*	*above 0.004 mg/kg bw*	**maximum burden** *(mg/kg DM)*		
beef cattle	0.036	0.148	yes	6.15	barley	straw
dairy cattle	0.057	0.236	yes	6.14	barley	straw
ram/ewe	0.097	0.407	yes	12.21	barley	straw
lamb	0.123	0.518	yes	12.19	barley	straw
pig (breeding)	0.003	0.003	no	0.12	distiller's grain	dried
pig (finishing)	0.004	0.004	no	0.12	distiller's grain	dried
poultry broiler	0.007	0.007	yes	0.09	brewer's grain	dried
poultry layer	0.036	0.147	yes	2.15	wheat	straw
turkey	0.005	0.005	yes	0.07	brewer's grain	dried

(2) Calculation of residue level in commodities of ruminant and swine origin

Residue data for BAS 750 F is derived from a cow feeding study (see section CA6.4/2) which includes three independent determinations (three animals) for each of the four feeding levels. According to the Excel calculator *Animal model 2015a.xls*, the individual data was entered (see Table 6.7.2-9), determinations at LOQ are marked with an asterisk, *). Note, that data for all three fat types was entered separately (data for “perirenal fat” was entered in column “fat”, while “mesenterial fat” and “subcutaneous fat” was entered in additional columns). Significantly different residue data was observed for the three fat types. Thus, separate calculations allow to take worst case scenario into account (see below, Table 6.7.2-10).

Table 6.7.2-9: BAS 750 F data derived from cow feeding study (see CA6.4/2)

Active substance:	BAS 750 F						highest intakes	
					feeding levels		N Rate	N Rate
	Estimated intakes		expressed		mg/kg bw	mg/kg DM	Dairy cattle	Beef cattle
	Maximum	Median	as	Level 1	0.035	1.6	0.1	0.2
Beef cattle	0.148	0.036	mg/kg bw	Level 2	0.193	7.5	0.8	1.3
Dairy cattle	0.236	0.057	mg/kg bw	Level 3	1.042	49.0	4.4	7.1
				Level 4	3.740	141.3	15.8	25.3

RD Monitoring	BAS 750 F													
matrices	Muscle		Fat		Liver		Kidney		Milk		fat mesenterial		fat subcutaneous	
0.035	0.010	*	0.016		0.029		0.010	*	0.010	*	0.018		0.012	
0.035	0.010	*	0.017		0.031		0.013		0.010	*	0.018		0.016	
0.035	0.010	*	0.018		0.034		0.014		0.010	*	0.018		0.017	
0.193	0.010	*	0.029		0.112		0.028		0.010	*	0.030		0.010	*
0.193	0.010	*	0.058		0.155		0.043		0.010	*	0.051		0.017	
0.193	0.010	*	0.059		0.182		0.074		0.010	*	0.077		0.041	
1.042	0.051		0.461		0.643		0.047		0.060		0.456		0.171	
1.042	0.063		0.586		0.936		0.320		0.071		0.563		0.493	
1.042	0.105		0.900		1.400		0.505		0.110		0.566		0.784	
3.740	0.128		0.942		2.500		0.944		0.184		0.652		0.019	
3.740	0.141		1.900		3.010		1.060		0.192		0.961		0.562	
3.740	0.221		2.290		3.580		1.880		0.273		1.870		1.200	

**, determinations below LOQ are marked with an asterisk.*

Based on feed intake estimations resulting from the cereal use supported in the present dossier, the Excel calculator *Animal model 2015a.xls* provides MRL proposals for bovine, sheep and swine commodities (see Table 6.7.2-10).

Table 6.7.2-10: Ruminant and swine commodities: MRL proposals

Bovine					
Closest level[a] 0.193 mg/kg bw 0.8 N Dairy C. 1.3 N Beef C.	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.014**	**0.028**	0.03
Fat	0.049	0.059	**0.059**	**0.193**	0.2
Liver	0.150	0.182	**0.086**	**0.336**	0.4
Kidney	0.048	0.074	**0.020**	**0.104**	0.1
Milk	0.010	0.010	**0.012**	**0.022**	0.03
fat mesenterial	0.053	0.077	**0.075**	**0.130**	
fat subcutaneous	0.023	0.041	**0.025**	**0.079**	
Sheep					
Closest level[a] 0.193 mg/kg bw 0.4 N Lamb 0.5 N Ewe	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.017**	**0.052**	0.06
Fat	0.049	0.059	**0.089**	**0.381**	0.4
Liver	0.150	0.182	**0.139**	**0.648**	0.7
Kidney	0.048	0.074	**0.032**	**0.246**	0.3
Milk	0.010	0.010	**0.014**	**0.031**	0.04
fat mesenterial	0.053	0.077	**0.095**	**0.265**	
fat subcutaneous	0.023	0.041	**0.019**	**0.325**	
Swine					
Closest level[a] 0.035 mg/kg bw 9.4 N Finishing 12.2 N Breeding	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.012**	**0.015**	0.015
Fat	0.017	0.018	**0.035**	**0.050**	
Liver	0.031	0.034	**0.043**	**0.115**	0.15
Kidney	0.012	0.014	**0.001**	**0.001**	0.0015
Milk	0.010	0.010	**0.010**	**0.009**	0.009
fat mesenterial	0.018	0.018	**0.059**	**0.059**	0.06
fat subcutaneous	0.015	0.017	**0.002**	**0.002**	

(3) Calculation of residue level in commodities of poultry origin

Residue data for BAS 750 F is derived from a hen feeding study (see section CA6.4/1) which includes three independent determinations for each of the four feeding levels. According to the Excel calculator *Animal model 2015a.xls*, the individual data was entered (see Table 6.7.2-11, determinations at LOQ are marked with an asterisk, *).

Table 6.7.2-11: BAS 750 F data (derived from hen feeding study, see CA6.4/1)

Active substance:	BAS 750 F										(highest intake)	
						feeding levels		N rate	N rate			
	Estimated intakes		expressed			mg/kg bw	mg/kg DM	Layer	Broiler			
	Maximum	Median	as		Level 1	0.010	0.2	0.1	1.5			
Broiler	0.007	0.007	mg/kg bw		Level 2	0.096	1.7	0.7	14.8			
Layer	0.147	0.036	mg/kg bw		Level 3	0.296	5.1	2.0	45.5			
					Level 4	0.984	17.2	6.7	151.4			
RD Monitoring	BAS 750 F											
matrices	Muscle		Fat		Liver		Kidney		Eggs			
0.010	0.010	*	0.010	*	0.010	*			0.010	*		
0.010	0.010	*	0.010	*	0.010	*			0.010	*		
0.010	0.010	*	0.010	*	0.010	*			0.010	*		
0.096	0.010	*	0.010	*	0.017				0.010	*		
0.096	0.010	*	0.010	*	0.010	*			0.010	*		
0.096	0.010	*	0.010	*	0.011				0.010	*		
0.296	0.010	*	0.019		0.021				0.010	*		
0.296	0.010	*	0.021		0.012				0.010	*		
0.296	0.010	*	0.025		0.013				0.010	*		
0.984	0.027		0.250		0.020				0.042			
0.984	0.010		0.150		0.060				0.034			
0.984	0.010	*	0.100		0.035				0.030			

**, determinations below LOQ are marked with an asterisk.*

Based on feed intake estimations resulting from the cereal use supported in the present dossier, the Excel calculator *Animal model 2015a.xls* provides MRL proposals for poultry commodities (see Table 6.7.2-12).

Table 6.7.2-12: Poultry commodities: MRL proposals

Poultry									
Closest level[a]	Residues at closest level (mg/kg)		Estimated value at 1N level[b]		MRL proposal (mg/kg)	CF	**STMR (mg/kg)**	**HR (mg/kg)**
0.096 mg/kg bw 0.7 N Layer 14.8 N Layer	Mean	Highest	$STMR_{Mo}$ (mg/kg)	HR_{Mo} (mg/kg)				
Meat	-	-	-	-	-	-	**0.07**	**0.12**
Muscle	0.01	0.01	0.01	0.01	0.015	6.2	**0.06**	**0.07**
Fat	0.01	0.01	0.01	0.02	0.03	16.3	**0.16**	**0.36**
Liver	0.01	0.02	0.01	0.03	0.03	4.9	**0.05**	**0.13**
Egg	0.01	0.01	0.01	0.01	0.015	4.9	**0.05**	**0.05**

(4) MRL proposals for livestock commodities

MRL values as derived from the Excel calculator *Animal model.2015.xls* developed by EFSA are proposed as basis for enforcement and monitoring. MRL proposals include extrapolations according to EFSA, namely from swine (breeding/finishing) to swine (1011000), from cattle (beef/dairy) to bovine (1012000) and equine (1015000), from ram/ewe to sheep (1013000) and goat (1014000), from broiler, layer, turkey to poultry (1017000). Similarly, MRL proposals in milk were derived from dairy cattle for cattle (1020010), horse (1020040) and others (1020990), from ewe for sheep (1020020) and goat (1020030).

In the absence of specific data for edible offal, MRL proposal is based on corresponding liver data as worst case scenario. Similarly, in the absence of specific data for kidney of poultry origin, the MRL proposal for poultry kidney is based on poultry liver as worst case.

For fish commodities no MRL was proposed based on data obtained in the fish metabolism study (see section CA 6.2). Fish feed burden resulting from the cereal uses supported in the present dossier are estimated to be well below 0.5 mg/kg DM feed. Taking into consideration that the highest residue level for BAS 750 F in edible fish matrices of 0.027 mg/kg (filet skin of C-labeled 10d-fish) represents the plateau level resulting from a daily dose of 5 mg/kg, it can be concluded that for fish feed burden of <1.8 mg/kg no significant BAS 750 F residues (0.01 mg/kg) are expected in dietary commodities of fish.

Proposed MRL for livestock commodities are summarized in the following table.

Table 6.7.2-13: BAS 750 F MRLs proposed for commodities of animal origin

code number	commodity	proposed MRL *(* denotes MRL at the LOQ) mg/kg*
1011010	Swine: Meat	0.015
1011020	Swine: Fat free of lean meat	0.06
1011030	Swine: Liver	0.15
1011040	Swine: Kidney	0.01*
1011050	Swine: Edible offal	0.15
1012010	Bovine: Meat	0.03
1012020	Bovine: Fat	0.2
1012030	Bovine: Liver	0.4
1012040	Bovine: Kidney	0.1
1012050	Bovine: Edible offal	0.4
1013010	Sheep: Meat	0.06
1013020	Sheep: Fat	0.4
1013030	Sheep: Liver	0.7
1013040	Sheep: Kidney	0.3
1013050	Sheep: Edible offal	0.7
1014010	Goat: Meat	0.06
1014020	Goat: Fat	0.4
1014030	Goat: Liver	0.7
1014040	Goat: Kidney	0.3
1014050	Goat: Edible offal	0.7
1015010	Horse: Meat	0.03
1015020	Horse: Fat	0.2
1015030	Horse: Liver	0.4
1015040	Horse: Kidney	0.1
1015050	Horse: Edible offal	0.4
1016010	Poultry: Meat	0.015
1016020	Poultry: Fat	0.03
1016030	Poultry: Liver	0.03
1016040	Poultry: Kidney	0.03
1016050	Poultry: Edible offal	0.03
1020010	Milk and milk products: Cattle	0.03
1020020	Milk and milk products: Sheep	0.04
1020030	Milk and milk products: Goat	0.04
1020040	Milk and milk products: Horse	0.03
1030000	Birds' eggs	0.015

CA 6.7.3 Proposed maximum residue levels (MRLs) and justification of the acceptability of the levels proposed for imported products (import tolerance)

No import tolerances are proposed in the present dossier. To support the cereal use of BAS 750 F it is proposed to establish the following MRLs in commodities of plant and animal origin:

Table 6.7.3-1: Proposed MRLs for BAS 750 F

code number	commodity	proposed MRL *(* denotes MRL at the LOQ) mg/kg*
500010	Barley: grain	0.6
500050	Oat: grain	0.6
500070	Rye: grain	0.04
500090	Wheat: grain	0.04
1011010	Swine: Meat	0.015
1011020	Swine: Fat free of lean meat	0.06
1011030	Swine: Liver	0.15
1011040	Swine: Kidney	0.01*
1011050	Swine: Edible offal	0.15
1012010	Bovine: Meat	0.03
1012020	Bovine: Fat	0.2
1012030	Bovine: Liver	0.4
1012040	Bovine: Kidney	0.1
1012050	Bovine: Edible offal	0.4
1013010	Sheep: Meat	0.06
1013020	Sheep: Fat	0.4
1013030	Sheep: Liver	0.7
1013040	Sheep: Kidney	0.3
1013050	Sheep: Edible offal	0.7
1014010	Goat: Meat	0.06
1014020	Goat: Fat	0.4
1014030	Goat: Liver	0.7
1014040	Goat: Kidney	0.3
1014050	Goat: Edible offal	0.7
1015010	Horse: Meat	0.03
1015020	Horse: Fat	0.2
1015030	Horse: Liver	0.4
1015040	Horse: Kidney	0.1
1015050	Horse: Edible offal	0.4
1016010	Poultry: Meat	0.015
1016020	Poultry: Fat	0.03
1016030	Poultry: Liver	0.03
1016040	Poultry: Kidney	0.03
1016050	Poultry: Edible offal	0.03
1020010	Milk and milk products: Cattle	0.03
1020020	Milk and milk products: Sheep	0.04
1020030	Milk and milk products: Goat	0.04
1020040	Milk and milk products: Horse	0.03
1030000	Birds' eggs	0.015

CA 6.8 Proposed safety intervals

The target formulation BAS 750 01 F is an EC formulation containing 100 g BAS 750 F / L. It is intended to be used in cereal crops.

CA 6.8.1 Pre-harvest interval (in days) for each relevant crop

This dossier supports a safe use with two applications. The second application should be carried out latest at BBCH growth 69 (end of flowering). Under usual climatic conditions, these use directions correspond to a minimum preharvest interval of 35 - 42 DAT.

CA 6.8.2 Re-entry period (in days) for livestock, to areas to be grazed

Because BAS 750 F is not intended to be used in areas to be grazed, no re-entry period for livestock has to be defined.

CA 6.8.3 Re-entry period for man to crops, buildings or spaces treated

The use of the target formulation BAS 750 01 F in cereals does not create any relevance with regard to worker re-entry. (The relevant assessment is provided in section CA7.2.3. The label should include the information that treated fields should not be re-entered before the spray has completely dried.)

CA 6.8.4 Withholding period (in days) for animals feeding stuffs

The withholding period for animal feeding stuff is fixed by the use recommendations with last application at BBCH growth stage 69. In order to avoid residues above the MRL values proposed for products of animal origin, a withholding period of 35 - 42 days (after application) for grains and 28 days for other plant parts to be used as feeding stuff is recommended.

CA 6.8.5 Waiting period between last application and sowing or planting

This is not relevant here since a pre-emergence use is not intended and application of BAS 750 01 F is directed to the crop only.

CA 6.8.6 Waiting periods between application and handling treated products

This is not relevant here since a post-harvest treatment is not intended. The typical procedures for cereal harvesting are covered under “re-entry” (see CA/7.2.3).

CA 6.8.7 Waiting period before sowing/planting succeeding crops

No replant restriction is needed since no significant residues are expected for the uses supported in the present dossier (see section CA6.6).

CA 6.9 Estimation of the potential and actual exposure through diet and other sources

For the purpose of assessing dietary risk due to exposure to residues resulting from the cereal use of BAS 750 F supported in the present dossier, TMDI, IEDI and IESTI calculations were conducted. Based on the results, a large margin of safety was identified, showing that chronic or acute intake of BAS 750 F residues are unlikely to present a public health concern.

I. GENERAL CONSIDERATIONS

(1) Definitions of relevant residue

Proposals for the definition of the relevant residue both for MRL enforcement/monitoring (RD-Mo) as well as for risk assessment (RD-RA) are derived in section CA6.7.1.

- Commodities of plant origin
  RD-Mo: parent BAS 750 F
  RD-RA: parent BAS 750 F

- Commodities of animal origin except poultry
  RD-Mo: parent BAS 750 F
  RD-RA: parent BAS 750 F

- Commodities of poultry origin
  RD-Mo: parent BAS 750 F
  RD-RA: sum of parent BAS 750 F, metabolite M750F022 and fatty acid conjugates of M750F022, expressed in parent equivalents

(2) Toxicological reference values

The endpoints for assessment of chronic risk (ADI) and acute risk (ARfD) used are provided in the table below. Detailed information is provided in section CA3.1.

Table 6.9-1: Toxicological reference values used in the exposure assessments

Endpoint	Value	Study	Safety factor	Reference
Acceptable Daily Intake (ADI)	0.05 mg/kg bw/d	rat, 2-year	100	CA3.1, 3.6
Acute Reference Dose (ARfD)	0.25 mg/kg bw	rabbit, maternal tox.	100	CA3.1, 3.6

(3) Residue data for food items (input for exposure calculations)

Exposure to BAS 750 F residues is based on the cereal use supported in the present dossier.

- Residue level in food items of plant origin are derived from residue field trials in wheat and barley (see section CA6.3). Residues in rotational crops as well as processed fractions are not significantly contributing to the dietary intake, thus were not considered.
- Residue level in animal food items are derived from livestock feeding studies (see section CA6.4) taking into account BAS 750 F feed burden calculated based on field residue trial data (wheat and barley feed items, section CA6.3) as well as processing transfer factors (section CA6.5). To account for the proposed residue definition for poultry (RD-RA, see above), derivation of residue level in poultry commodities includes first, the conversion of M750F022 residue data into BAS 750 F equivalents and second, the multiplication with a correction factor to account for contributing amounts of M750F022 fatty acid conjugates.
- For other commodities, namely plant food items as well as ruminant and swine commodities, RD-RA and RD-Mo do not differ, thus recalculation of residue data is not necessary.

The derivation of input residue data is described in detail below for plant commodities (a), for ruminant and swine commodities (b) and for poultry commodities (c). The input residue data, used for the dietary exposure calculations, namely HR and STMR, is listed in Table 6.9-14.

(4) Exposure calculation model

The EFSA model for chronic and acute risk assessment - rev. 2_0 (Model PRIMo) was used to conduct assessments of the potential chronic and acute dietary risk for the EU consumer. The EFSA model was used as it considers all the different diets in the EU and all consumer groups.

II. DERIVATION OF INPUT VALUES FOR EXPOSURE CALCULATIONS

For the purpose of estimating dietary exposure to BAS 750 F residues, residue data in dietary commodities of plant origin, namely (a) *cereals*, and animal origin, namely (b) *ruminants/swine* and (c) *poultry*, is derived based on *magnitude of the residue* studies conducted with BAS 750 F (see below). A summary of the residue data used as input for exposure calculations (STMR, HR) is provided in Table 6.9-16. MRLs for BAS 750 F in food items are derived in section CA6.7.2. A summary of BAS 750 F MRLs proposed for plant commodities is provided in Table 6.9-16 and of BAS 750 F MRLs proposed for animal commodities is provided in Table 6.9-16

Commodities of plant origin

(a) Cereal commodities

Input data for plant matrices takes into account potential residues in grain of wheat and barley grain, and by extrapolation of rye, triticale and oat. Based on field residue data comprising 17 independent residue trials in wheat and 18 independent residue trials in barley, MRL proposals (see section CA6.7) as well as STMR and HR were calculated (OECD calculator, see section CA6.3).

Table 6.9-2: BAS 750 F MRLs proposed for commodities of plant origin

code number	commodity	proposed MRL *mg/kg*
0500010	barley	0.6
0500050	oat	0.6
0500070	rye	0.04
0500090	wheat/triticale	0.04

Commodities of animal origin

Proposed MRL for livestock commodities are summarized in the following table.

Table 6.9-3: BAS 750 F MRLs proposed for commodities of animal origin

code number	commodity	proposed MRL *(* denotes MRL at the LOQ)* *mg/kg*
1011010	Swine: Meat	0.015
1011020	Swine: Fat free of lean meat	0.06
1011030	Swine: Liver	0.15
1011040	Swine: Kidney	0.01*
1011050	Swine: Edible offal	0.15
1012010	Bovine: Meat	0.03
1012020	Bovine: Fat	0.2
1012030	Bovine: Liver	0.4
1012040	Bovine: Kidney	0.1
1012050	Bovine: Edible offal	0.4
1013010	Sheep: Meat	0.06
1013020	Sheep: Fat	0.4
1013030	Sheep: Liver	0.7
1013040	Sheep: Kidney	0.3
1013050	Sheep: Edible offal	0.7
1014010	Goat: Meat	0.06
1014020	Goat: Fat	0.4
1014030	Goat: Liver	0.7
1014040	Goat: Kidney	0.3
1014050	Goat: Edible offal	0.7
1015010	Horse: Meat	0.03
1015020	Horse: Fat	0.2
1015030	Horse: Liver	0.4
1015040	Horse: Kidney	0.1
1015050	Horse: Edible offal	0.4
1016010	Poultry: Meat	0.015
1016020	Poultry: Fat	0.03
1016030	Poultry: Liver	0.03
1016040	Poultry: Kidney	0.03
1016050	Poultry: Edible offal	0.03
1020010	Milk and milk products: Cattle	0.03
1020020	Milk and milk products: Sheep	0.04
1020030	Milk and milk products: Goat	0.04
1020040	Milk and milk products: Horse	0.03
1030000	Birds' eggs	0.015

Magnitude of the residue studies in cow and hen confirmed a linear relationship of BAS 750 F feed intake and residue level in animal matrices. Residue level in animal matrices (according to RD-RA) for realistic feed intake were calculated based on estimated feed intake considering residue data from field residue trials (feed items, CA6.3), transfer factors from processing studies (CA6.5) while residues in rotational crops, since <LOQ, were not considered. The input data used for feed burden estimation is summarized in Table 6.9-4.

Table 6.9-4: BAS 750 F residue level in feed items (input for feed intake estimation)

Feed item		**commodity**	**BAS 750 F**		
			STMR *mg/kg*	**HR** *mg/kg*	**PF**
forages	barley	straw	4.20	18	-
	oat	straw	4.20	18	-
	rye	straw	3.75	18	-
	triticale	straw	3.75	18	-
	wheat	straw	3.75	18	-
cereal grains	barley	grain	0.088	-	-
	oat	grain	0.088	-	-
	rye	grain	0.010	-	-
	triticale	grain	0.010	-	-
	wheat	grain	0.010	-	-
by-products	brewer's grain	dried	0.088	-	2.31
	distiller's grain	dried	0.088	-	2.31
	wheat gluten	meal	0.010	-	0.39
	wheat	milled by-products	0.010	-	0.72

Feed intake level was estimated using the Excel calculator *Animal model 2015a.xls* developed by EFSA. The output is summarized in Table 6.9-5. When considering residue data as it results from the cereal use supported in the present dossier, exceedance of the feed intake value of 0.004 mg/kg bw is observed for cattle, sheep and poultry, while feed intake below the trigger is calculated for pig.

Table 6.9-5: Estimated feed intake (Regulation (EU) No 283/2013)

livestock animal	**BAS 750 F**				**highest contributing commodities**	
	median burden *(mg/kg bw)*	**maximum burden** *(mg/kg bw)*	*above 0.004 mg/kg bw*	**maximum burden** *(mg/kg DM)*		
beef cattle	0.036	0.148	yes	6.15	barley	straw
dairy cattle	0.057	0.236	yes	6.14	barley	straw
ram/ewe	0.097	0.407	yes	12.21	barley	straw
lamb	0.123	0.518	yes	12.19	barley	straw
pig (breeding)	0.003	0.003	no	0.12	distiller's grain	dried
pig (finishing)	0.004	0.004	no	0.12	distiller's grain	dried
poultry broiler	0.007	0.007	yes	0.09	brewer's grain	dried
poultry layer	0.036	0.147	yes	2.15	wheat	straw
turkey	0.005	0.005	yes	0.07	brewer's grain	dried

(b) Ruminant and swine commodities

Since the proposed residue definitions (RD-RA and RD-Mo) coincide, conversion factors do not need to be calculated. Residue data for BAS 750 F is derived from a cow feeding study (see section CA6.4/2) which includes three independent determinations (three animals) for each of the four feeding levels. According to the Excel calculator *Animal model 2015a.xls*, the individual data was entered (see Table 6.9-6, determinations at LOQ are marked with an asterisk *). Note, that data for all three fat types was entered separately, to account for the significantly different residue data observed (data for "perirenal fat" was entered in column "fat", while "mesenterial fat" and "subcutaneous fat" was entered in additional columns).

Table 6.9-6: BAS 750 F residue data derived from cow feeding study (section CA 6.4/2)

Active substance:	BAS 750 F							highest intakes	
					feeding levels		N Rate	N Rate
	Estimated intakes		expressed		mg/kg bw	mg/kg DM	Dairy cattle	Beef cattle
	Maximum	Median	as	Level 1	0.035	1.6	0.1	0.2
Beef cattle	0.148	0.036	mg/kg bw	Level 2	0.193	7.5	0.8	1.3
Dairy cattle	0.236	0.057	mg/kg bw	Level 3	1.042	49.0	4.4	7.1
				Level 4	3.740	141.3	15.8	25.3

RD Monitoring	BAS 750 F															
matrices	Muscle		Fat		Liver		Kidney		Milk		fat mesenterial		fat subcutaneous			
0.035	0.010	*	0.016		0.029		0.010	*	0.010	*	0.018		0.012			
0.035	0.010	*	0.017		0.031		0.013		0.010	*	0.018		0.016			
0.035	0.010	*	0.018		0.034		0.014		0.010	*	0.018		0.017			
0.193	0.010	*	0.029		0.112		0.028		0.010	*	0.030		0.010	*		
0.193	0.010	*	0.058		0.155		0.043		0.010	*	0.051		0.017			
0.193	0.010	*	0.059		0.182		0.074		0.010	*	0.077		0.041			
1.042	0.051		0.461		0.643		0.047		0.060		0.456		0.171			
1.042	0.063		0.586		0.936		0.320		0.071		0.563		0.493			
1.042	0.105		0.900		1.400		0.505		0.110		0.566		0.784			
3.740	0.128		0.942		2.500		0.944		0.184		0.652		0.019			
3.740	0.141		1.900		3.010		1.060		0.192		0.961		0.562			
3.740	0.221		2.290		3.580		1.880		0.273		1.870		1.200			

Based on feed intake estimations resulting from the cereal use supported in the present dossier, the Excel calculator *Animal model 2015a.xls* provides values for STMR and HR for bovine, sheep and swine commodities (see Table 6.9-7).

Table 6.9-7: Ruminant and swine commodities: input data for bovine, sheep, swine

Bovine

Closest level[a] 0.193 mg/kg bw 0.8 N Dairy C. 1.3 N Beef C.	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.014**	**0.028**	0.03
Fat	0.049	0.059	**0.059**	**0.193**	0.2
Liver	0.150	0.182	**0.086**	**0.336**	0.4
Kidney	0.048	0.074	**0.020**	**0.104**	0.1
Milk	0.010	0.010	**0.012**	**0.022**	0.03
fat mesenterial	0.053	0.077	**0.075**	**0.130**	
fat subcutaneous	0.023	0.041	**0.025**	**0.079**	

Sheep

Closest level[a] 0.193 mg/kg bw 0.4 N Lamb 0.5 N Ewe	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.017**	**0.052**	0.06
Fat	0.049	0.059	**0.089**	**0.381**	0.4
Liver	0.150	0.182	**0.139**	**0.648**	0.7
Kidney	0.048	0.074	**0.032**	**0.246**	0.3
Milk	0.010	0.010	**0.014**	**0.031**	0.04
fat mesenterial	0.053	0.077	**0.095**	**0.265**	
fat subcutaneous	0.023	0.041	**0.019**	**0.325**	

Swine

Closest level[a] 0.035 mg/kg bw 9.4 N Finishing 12.2 N Breeding	Residues at the closest feeding level (mg/kg)		Estimated value at 1N level		MRL proposal (mg/kg)
	Mean	Highest	**STMR (mg/kg)**	**HR (mg/kg)**	
Meat	-	-	**0.018**	**0.020**	-
Muscle	0.010	0.010	**0.012**	**0.015**	0.015
Fat	0.017	0.018	**0.035**	**0.050**	
Liver	0.031	0.034	**0.043**	**0.115**	0.15
Kidney	0.012	0.014	**0.001**	**0.001**	0.0015
Milk	0.010	0.010	**0.010**	**0.009**	0.009
fat mesenterial	0.018	0.018	**0.059**	**0.059**	0.06
fat subcutaneous	0.015	0.017	**0.002**	**0.002**	

(c) Poultry commodities

For poultry the proposed residue definition for risk assessment (RD-RA) and the proposed residue definition for monitoring (RD-Mo) do differ (see section CA 6.7.1).

RD-Mo: *parent BAS 750 F*

RD-RA: *sum of parent BAS 750 F, metabolite M750F022 and fatty acid conjugates of M750F022, expressed in parent equivalents*

Calculation of conversion factors includes the following steps:

- Step 1: M750F022 residue data is converted to BAS 750 F equivalents.
  Multiplication of M750F022 residue level (mg/kg) with a factor of 1.15 accounts for the difference in molecular weight of M750F022 with 346.7 g/mol and BAS 750 F with 397.8 g/mol.

- Step 2: M750F022 residue data (mg/kg BAS 750 F equivalents) is multiplied with a matrix-specific correction factor. The correction factor accounts for the matrix-specific amounts of fatty acid-conjugates of M750F022 (see section CA6.7.1).

- Step 3: Conversion factors are derived using the Excel calculator *Animal model 2015a.xls* based on residue data according to RD-Mo (BAS 750 F, see Table 6.9-10) and residue data according to RD-RA (sum of BAS 750 F, M750F022&FA conjugates, expressed as BAS 750 F equivalents, Table 6.9-11).

Residue data for BAS 750 F and M750F022 is derived from a hen feeding study (see section CA6.4/1), which included three independent determinations for each of the four feeding levels.

Table 6.9-8: M750F022 residue data as derived from hen feeding study (section CA6.4/1)

feeding level (mg/kg bw/d)	M750F022 (mg/kg)							
	Muscle		Liver		Fat		Eggs	
0.010	0.010	*	0.010	*	0.010	*	0.010	*
	0.010	*	0.010	*	0.010	*	0.010	*
	0.010	*	0.010	*	0.010	*	0.010	*
0.096	0.010	*	0.018		0.024		0.010	*
	0.010	*	0.017		0.044		0.010	*
	0.010	*	0.019		0.030		0.010	*
0.296	0.010	*	0.033		0.064		0.015	*
	0.010	*	0.020		0.070		0.015	*
	0.010	*	0.030		0.071		0.015	*
0.984	0.037		0.200		0.360		0.094	
	0.030		0.130		0.270		0.073	
	0.031		0.120		0.300		0.071	

**, determinations below LOQ are marked with an asterisk*

Step 1: Expression of M750F022 as BAS 750 F equivalents

Residue data in Table 6.9-8 was multiplied with a factor of 1.15 resulting in data as of Table 6.9-9

Table 6.9-9: Residue data of M750F022 converted to BAS 750 F equivalents

feeding level (mg/kg bw/d)	M750F022 (expressed in BAS 750 F equivalents, mg/kg)			
	Muscle	Liver	Fat	Eggs
	0.012 *	0.012 *	0.012 *	0.012 *
0.010	0.012 *	0.012 *	0.012 *	0.012 *
	0.012 *	0.012 *	0.012 *	0.012 *
	0.012 *	0.021	0.028	0.012 *
0.096	0.012 *	0.020	0.051	0.012 *
	0.012 *	0.022	0.035	0.012 *
	0.012 *	0.038	0.074	0.017 *
0.296	0.012 *	0.023	0.081	0.017 *
	0.012 *	0.035	0.082	0.017 *
	0.043	0.230	0.414	0.108
0.984	0.035	0.150	0.311	0.084
	0.036	0.138	0.345	0.082

**, determinations below LOQ are marked with an asterisk*

Step 2: Correction for FA conjugates of M750F022 (expressed as BAS 750 F equivalents)

Residue data in Table 6.9-9 was multiplied with correction factor for fatty acid conjugates (see section CA6.7). The correction factors used are 4X (fat), 1.5X (muscle, egg), and 1X (liver).

Table 6.9-10: Residue data of M750F022 & FA conjugates

feeding level (mg/kg bw/d)	M750F022 & conjugates (expressed in BAS 750 F equivalents, mg/kg)			
	Muscle	Liver	Fat	Eggs
	0.017 *	0.012 *	0.046 *	0.017 *
0.010	0.017 *	0.012 *	0.046 *	0.017 *
	0.017 *	0.012 *	0.046 *	0.017 *
	0.017 *	0.021	0.110	0.017 *
0.096	0.017 *	0.020	0.202	0.017 *
	0.017 *	0.022	0.138	0.017 *
	0.017 *	0.038	0.294	0.026 *
0.296	0.017 *	0.023	0.322	0.026 *
	0.017 *	0.035	0.327	0.026 *
	0.064	0.230	1.656	0.162
0.984	0.052	0.150	1.242	0.126
	0.053	0.138	1.380	0.122

**, determinations below LOQ are marked with an asterisk*

Table 6.9-11: Residue as the sum of BAS 750 F, M750F022 & FA conjugates (RD-RA)

feeding level (mg/kg bw/d)	sum of BAS 750 F& M750F022 & conjugates (mg/kg)			
	Muscle	Liver	Fat	Eggs
	0.027 *	0.022 *	0.056 *	0.027 *
0.010	0.027 *	0.022 *	0.056 *	0.027 *
	0.027 *	0.022 *	0.056 *	0.027 *
	0.027 *	0.038	0.120	0.027 *
0.096	0.027 *	0.030	0.212	0.027 *
	0.027 *	0.033	0.148	0.027 *
	0.027 *	0.059	0.313	0.036 *
0.296	0.027 *	0.035	0.343	0.036 *
	0.027 *	0.048	0.352	0.036 *
	0.091	0.250	1.906	0.204
0.984	0.062	0.210	1.392	0.160
	0.063	0.173	1.480	0.152

**, determinations below LOQ are marked with an asterisk*

Step 3: Calculation of matrix-specific conversion factors

Using the Excel calculator *Animal model 2015a.xls*, matrix-specific conversion factors were derived based on first, residue data according to RD-Mo (BAS 750 F residues, see Table 6.9-12) and second, RD-RA (sum of BAS 750 F, M750F022&FA conjugate residues, expressed as BAS 750 F equivalents, see Table 6.9-13). The conversion factors used to calculate residue data in poultry commodities are provided in Table 6.9-14.

Table 6.9-12: RD-Mo residue data (derived from hen feeding study, see CA6.4/1)

Active substance:	BAS 750 F								(highest intake)
						feeding levels		N rate	N rate
	Estimated intakes		expressed			mg/kg bw	mg/kg DM	Layer	Broiler
	Maximum	Median	as		Level 1	0.010	0.2	0.1	1.5
Broiler	0.007	0.007	mg/kg bw		Level 2	0.096	1.7	0.7	14.8
Layer	0.147	0.036	mg/kg bw		Level 3	0.296	5.1	2.0	45.5
					Level 4	0.984	17.2	6.7	151.4
RD Monitoring	BAS 750 F								
matrices	Muscle		Fat		Liver		Kidney	Eggs				
0.010	0.010 *		0.010 *		0.010 *			0.010 *			
0.010	0.010 *		0.010 *		0.010 *			0.010 *			
0.010	0.010 *		0.010 *		0.010 *			0.010 *			
0.096	0.010 *		0.010 *		0.017			0.010 *			
0.096	0.010 *		0.010 *		0.010 *			0.010 *			
0.096	0.010 *		0.010 *		0.011			0.010 *			
0.296	0.010 *		0.019		0.021			0.010 *			
0.296	0.010 *		0.021		0.012			0.010 *			
0.296	0.010 *		0.025		0.013			0.010 *			
0.984	0.027		0.250		0.020			0.042			
0.984	0.010		0.150		0.060			0.034			
0.984	0.010 *		0.100		0.035			0.030			

**, determinations below LOQ are marked with an asterisk*

Table 6.9-13: RD-RA residue data (as calculated, see table 6.9-11)

feeding level mg/kg bw	RD Risk assessment				sum of BAS 750 F, M750F022 & FA conjugates			
	Muscle		Fat		Liver		Eggs	
	0.027	*	0.056	*	0.022	*	0.027	*
0.010	0.027	*	0.056	*	0.022	*	0.027	*
	0.027	*	0.056	*	0.022	*	0.027	*
	0.027	*	0.120		0.038		0.027	*
0.096	0.027	*	0.212		0.030		0.027	*
	0.027	*	0.148		0.033		0.027	*
	0.027	*	0.313		0.059		0.036	*
0.296	0.027	*	0.343		0.035		0.036	*
	0.027	*	0.352		0.048		0.036	*
	0.091		1.906		0.250		0.204	
0.984	0.062		1.392		0.210		0.160	
	0.063		1.480		0.173		0.152	

**, determinations at LOQ are marked with an asterisk.*

Table 6.9-14: Conversion factors for poultry commodities (RD-RA / RD-Mo)

Conversion Factor for risk assessment (median)							
Muscle		Fat		Liver		Eggs	
n.c.	-	n.c.	-	n.c.	-	n.c.	-
n.c.	-	14.80	#	3.00	#	n.c.	-
n.c.	-	16.33		2.94	#	n.c.	-
6.20	#	9.28		4.94		4.86	
6.20		**16.33**		**4.94**		**4.86**	
#: indicates that residues at or close to the LOQ (x2) were included in CF calculation							

Table 6.9-15: Poultry commodities: input data

Poultry								
Closest level[a] 0.096 mg/kg bw, 0.7 N Layer, 14.8 N Layer	Residues at closest level (mg/kg)		Estimated value at 1N level[b]		MRL proposal (mg/kg)	CF	**STMR (mg/kg)**	**HR (mg/kg)**
	Mean	Highest	$STMR_{Mo}$ (mg/kg)	HR_{Mo} (mg/kg)				
Meat	-	-	-	-	-	-	**0.07**	**0.12**
Muscle	0.01	0.01	0.01	0.01	0.015	6.2	**0.06**	**0.07**
Fat	0.01	0.01	0.01	0.02	0.03	16.3	**0.16**	**0.36**
Liver	0.01	0.02	0.01	0.03	0.03	4.9	**0.05**	**0.13**
Egg	0.01	0.01	0.01	0.01	0.015	4.9	**0.05**	**0.05**

The following table summarizes the input values from plant and animal commodities used for the chronic and acute dietary risk assessment (according to EFSA PRIMO model)

Table 6.9-16: Input values used for dietary risk assessment (BAS 750 F, IEDI, IESTI)

Code no. (1)	Examples of individual products within the groups to which the MRLs apply	BAS 750 F, M750F022 and fatty acid conjugates expressed as BAS 750 F (mg/kg)	
		STMR	HR
500010	Barley	0.088	0.41
500050	Oats	0.088	0.41
500070	Rye	0.010	0.026
500090	Wheat	0.010	0.026
1011010	Swine: Meat	0.018	0.020
1011020	Swine: Fat free of lean meat	0.059	0.059
1011030	Swine: Liver	0.043	0.115
1011040	Swine: Kidney	-	-
1011050	Swine: Edible offal	0.043	0.115
1012010	Bovine: Meat	0.018	0.02
1012020	Bovine: Fat	0.059	0.193
1012030	Bovine: Liver	0.086	0.336
1012040	Bovine: Kidney	0.02	0.104
1012050	Bovine: Edible offal	0.086	0.336
1013010	Sheep: Meat	0.018	0.02
1013020	Sheep: Fat	0.089	0.381
1013030	Sheep: Liver	0.139	0.648
1013040	Sheep: Kidney	0.032	0.246
1013050	Sheep: Edible offal	0.139	0.648
1014010	Goat: Meat	0.018	0.02
1014020	Goat: Fat	0.089	0.381
1014030	Goat: Liver	0.139	0.648
1014040	Goat: Kidney	0.032	0.246
1014050	Goat: Edible offal	0.139	0.648
1015010	Horse: Meat	0.018	0.02
1015020	Horse: Fat	0.059	0.193
1015030	Horse: Liver	0.086	0.336
1015040	Horse: Kidney	0.02	0.104
1015050	Horse: Edible offal	0.086	0.336
1016010	Poultry: Meat	0.07	0.12
1016020	Poultry: Fat	0.16	0.36
1016030	Poultry: Liver	0.05	0.13
1016040	Poultry: Kidney	0.05	0.13
1016050	Poultry: Edible offal	0.05	0.13
1020010	Milk and milk products: Cattle	0.012	0.022
1020020	Milk and milk products: Sheep	0.014	0.031
1020030	Milk and milk products: Goat	0.014	0.031
1020040	Milk and milk products: Horse	0.012	0.022
1030000	Birds' eggs	0.05	0.05
1030010	Eggs: Chicken	0.05	0.05
1030020	Eggs: Duck	0.05	0.05
1030030	Eggs: Goose	0.05	0.05
1030040	Eggs: Quail	0.05	0.05
1030990	Other eggs	0.05	0.05

Note, in absence of specific data, STMR and HR values for liver are also used for edible offal and/or kidney.

III ACCEPTABLE DAILY INTAKE: DIETARY EXPOSURE CALCULATION

TMDI and IEDI calculation

(a) TMDI calculations

The TMDI calculation was performed with the current EFSA model (version 2) using an ADI of 0.05 mg/kg bw/day and (proposed) MRLs as listed in Table 6.9-2 (plant commodities) and Table 6.9-3 (animal commodities).

The summary of the chronic assessment is presented in Table 6.9-17. The ADI utilization ranges from 0.2 to 2.6% ADI. The highest TDMI was 2.6% ADI. For the "IE adult", the highest contributors are barley (1.5% ADI) and sheep liver (0.3% ADI). For the "NL child" the highest contributors are cattle milk/milk products (1.8% ADI) and wheat (0.4% ADI).

Table 6.9-17: TMDI calculation based on proposed BAS 750 F MRLs as listed in table 6.9-3

BAS 750 F

Prepare workbook for refined calculations

Undo refined calculations

Status of the active substance:		Code no.	
LOQ (mg/kg bw):		proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	**0.05**	ARfD (mg/kg bw):	**0.25**
Source of ADI:		Source of ARfD:	
Year of evaluation:	**2016**	Year of evaluation:	

Explain choice of toxicological reference values.
The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
3

No of diets exceeding ADI: ---

Highest calculated TMDI values in % of ADI	MS Diet	contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
2.6	IE adult	1.5	Barley	0.3	Sheep: Liver	0.2	Oats	
2.6	NL child	1.8	Milk and milk products: Cattle	0.4	Wheat	0.1	Oats	
1.9	WHO cluster diet E	1.0	Barley	0.3	Wheat	0.2	Milk and milk products: Cattle	
1.7	WHO Cluster diet F	0.7	Barley	0.3	Wheat	0.2	Milk and milk products: Cattle	
1.7	FR infant	1.5	Milk and milk products: Cattle	0.1	Wheat	0.0	Bovine: Meat	
1.7	WHO Cluster diet B	0.7	Wheat	0.3	Barley	0.2	Milk and milk products: Cattle	
1.6	DE child	0.9	Milk and milk products: Cattle	0.3	Wheat	0.2	Oats	
1.5	WHO cluster diet D	0.5	Wheat	0.3	Milk and milk products: Cattle	0.3	Barley	
1.4	DK child	0.5	Oats	0.4	Wheat	0.4	Rye	
1.4	ES child	0.7	Milk and milk products: Cattle	0.4	Wheat	0.1	Bovine: Meat	
1.4	WHO regional European diet	0.4	Barley	0.3	Milk and milk products: Cattle	0.2	Wheat	
1.2	ES adult	0.6	Barley	0.3	Milk and milk products: Cattle	0.2	Wheat	
1.2	NL general	0.4	Barley	0.4	Milk and milk products: Cattle	0.2	Wheat	
1.0	SE general population 90th percentile	0.7	Milk and milk products: Cattle	0.3	Wheat	0.0	Birds' eggs	
0.7	LT adult	0.2	Milk and milk products: Cattle	0.1	Oats	0.1	Rye	
0.6	UK Infant	0.3	Oats	0.2	Wheat	0.1	Bovine: Liver	
0.5	FR all population	0.3	Wheat	0.2	Milk and milk products: Cattle	0.0	Bovine: Edible offal	
0.5	IT kids/toddler	0.5	Wheat	0.0	Barley	0.0	Oats	
0.5	FR toddler	0.2	Wheat	0.1	Bovine: Edible offal	0.1	Bovine: Meat	
0.4	DK adult	0.2	Wheat	0.1	Oats	0.1	Rye	
0.4	UK Toddler	0.3	Wheat	0.1	Oats	0.0	Birds' eggs	
0.4	PT General population	0.3	Wheat	0.0	Barley	0.0	Barley	
0.3	IT adult	0.3	Wheat	0.0	Barley	0.0	Oats	
0.3	FI adult	0.1	Oats	0.1	Wheat	0.1	Rye	
0.3	UK vegetarian	0.2	Wheat	0.1	Oats	0.0	Barley	
0.2	UK Adult	0.1	Wheat	0.0	Barley	0.0	Oats	
	PL general population		FRUIT (FRESH OR FROZEN)		FRUIT (FRESH OR FROZEN)		FRUIT (FRESH OR FROZEN)	

Conclusion:
The estimated Theoretical Maximum Daily Intakes (TMDI), based on pTMRLs were below the ADI.
A long-term intake of residues of BAS 750 F is unlikely to present a public health concern.

In conclusion, the TMDI is well below the ADI for all European sub-population groups. A long-term intake of residues of BAS 750 F is unlikely to present a public health concern.

(b) IEDI calculations

In addition, a chronic exposure assessment was performed according the proposed residue definition for risk assessment (RD-RA) for plant commodities (BAS 750 F), for animal commodities except poultry (BAS 750 F) and for poultry commodities (sum of BAS 750 F, M750F022 and its FA conjugates expressed as BAS 750 F).

The summary of the calculation using the EFSA model is presented in Table 6.9-18. For the assessment, the ADI of 0.05 mg/kg bw/day was used. According to the EFSA model, the IEDI is being calculated simultaneously for adults, children, toddlers and infants (different age groups), vegetarian and elderly in the various EU countries.

With the current EFSA model the chronic risk assessment ranges from 0.1 to 1.6% of ADI. The diet with the highest IEDI is "NL child" with 1.6% of ADI. For this diet, the highest contributor is cattle milk/milk products with 0.7% of the ADI. The diet with the second highest IEDI is “ES child” with 1.2% of the ADI, in which bovine meat is the major contributor with 0.5% of the ADI.

Table 6.9-18: IEDI calculation (residue input data see table 6.9-16)

BAS 750 F

Prepare workbook for refined calculations

Undo refined calculations

Status of the active substance:		Code no.	
LOQ (mg/kg bw):		proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	0.05	ARfD (mg/kg bw):	0.25
Source of ADI:		Source of ARfD:	
Year of evaluation:	2016	Year of evaluation:	

Explain choice of toxicological reference values.

The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
2

No of diets exceeding ADI: ---

Highest calculated TMDI values in % of ADI	MS Diet	contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
1.6	NL child	0.7	Milk and milk products: Cattle	0.4	Bovine: Meat	0.1	Poultry: Meat	
1.2	ES child	0.5	Bovine: Meat	0.3	Milk and milk products: Cattle	0.2	Poultry: Meat	
1.0	WHO regional European diet	0.4	Bovine: Meat	0.1	Poultry: Meat	0.1	Milk and milk products: Cattle	
1.0	WHO Cluster diet B	0.3	Bovine: Meat	0.2	Wheat	0.1	Poultry: Meat	
1.0	FR infant	0.6	Milk and milk products: Cattle	0.2	Bovine: Meat	0.1	Poultry: Meat	
0.9	WHO cluster diet E	0.3	Bovine: Meat	0.1	Barley	0.1	Poultry: Meat	
0.9	WHO Cluster diet F	0.3	Bovine: Meat	0.1	Barley	0.1	Milk and milk products: Cattle	
0.8	DE child	0.3	Milk and milk products: Cattle	0.1	Bovine: Meat	0.1	Birds' eggs	
0.8	FR toddler	0.5	Bovine: Meat	0.1	Poultry: Meat	0.1	Birds' eggs	
0.7	WHO cluster diet D	0.2	Bovine: Meat	0.1	Wheat	0.1	Milk and milk products: Cattle	
0.7	IE adult	0.2	Barley	0.1	Bovine: Meat	0.1	Milk and milk products: Cattle	
0.7	ES adult	0.3	Bovine: Meat	0.1	Milk and milk products: Cattle	0.1	Poultry: Meat	
0.7	NL general	0.3	Bovine: Meat	0.2	Milk and milk products: Cattle	0.1	Barley	
0.5	SE general population 90th percentile	0.3	Milk and milk products: Cattle	0.1	Birds' eggs	0.1	Wheat	
0.5	FR all population	0.2	Bovine: Meat	0.1	Poultry: Meat	0.1	Wheat	
0.4	LT adult	0.1	Milk and milk products: Cattle	0.1	Bovine: Meat	0.0	Poultry: Meat	
0.4	DK child	0.1	Wheat	0.1	Rye	0.1	Birds' eggs	
0.3	DK adult	0.2	Bovine: Meat	0.0	Wheat	0.0	Birds' eggs	
0.2	UK Infant	0.1	Birds' eggs	0.1	Wheat	0.0	Oats	
0.2	UK Toddler	0.1	Birds' eggs	0.1	Wheat	0.0	Oats	
0.1	IT kids/toddler	0.1	Wheat	0.0	Barley	0.0	Oats	
0.1	UK vegetarian	0.0	Wheat	0.0	Birds' eggs	0.0	Oats	
0.1	PT General population	0.1	Wheat	0.0	Barley	0.0	Barley	
0.1	IT adult	0.1	Wheat	0.0	Barley	0.0	Oats	
0.1	FI adult	0.0	Birds' eggs	0.0	Wheat	0.0	Oats	
0.1	UK Adult	0.0	Wheat	0.0	Birds' eggs	0.0	Barley	
	PL general population		FRUIT (FRESH OR FROZEN)		FRUIT (FRESH OR FROZEN)		FRUIT (FRESH OR FROZEN)	

Conclusion:
The estimated Theoretical Maximum Daily Intakes (TMDI), based on pTMRLs were below the ADI.
A long-term intake of residues of BAS 750 F is unlikely to present a public health concern.

The ADI utilization for both calculations, is well below 100% and thus a further refinement of the chronic risk assessment is not necessary. According to the presented TMDI and IEDI calculations a chronic intake of BAS 750 F residues is unlikely to present a public health concern.

IV ACUTE REFERENCE DOSE: DIETARY EXPOSURE CALCULATION

(c) IESTI calculation

An acute exposure assessment was performed according the proposed residue definition for risk assessment (RD-RA) for plant commodities (BAS 750 F), for animal commodities except poultry (BAS 750 F) and for poultry commodities (sum of BAS 750 F, M750F022 and its FA conjugates expressed as BAS 750 F).

The summary of the calculation using the EFSA model is presented in Table 6.9-19. For the assessment, the ARfD of 0.25 mg/kg bw is used.

For children, the highest ARfD utilization was 1.1% for consumption first, of cattle milk/milk products and second, of liver (bovine). For adults, the highest ARfD utilization was 0.5% for consumption of poultry meat (0.5% ARfD).

Table 6.9-19: IESTI calculation (residue input data see table 6.9-16)

	Acute risk assessment /children						Acute risk assessment / adults / general population					
	The acute risk assessment is based on the ARfD.											
	For each commodity the calculation is based on the highest reported MS consumption per kg bw and the corresponding unit weight from the MS with the critical consumption. If no data on the unit weight was available from that MS an average European unit weight was used for the IESTI calculation.											
	In the **IESTI 1** calculation, the variability factors were 10, 7 or 5 (according to JMPR manual 2002), for lettuce a variability factor of 5 was used.											
	In the **IESTI 2** calculations, the variability factors of 10 and 7 were replaced by 5. For lettuce the calculation was performed with a variabilty factor of 3.											
	Threshold MRL is the calculated residue level which would leads to an exposure equivalent to 100 % of the ARfD.											
Unprocessed commodities	**No of commodities for which ARfD/ADI is exceeded (IESTI 1):**		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 2):**		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 1):**		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 2):**		---
	IESTI 1	*)	**)	**IESTI 2**	*)	**)	**IESTI 1**	*)	**)	**IESTI 2**	*)	**)
	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)
	1.1	Milk and milk products: Cattle	0.022 / -	1.1	Milk and milk products: Cattle	0.022 / -	0.6	Poultry: Meat	0.12 / -	0.6	Poultry: Meat	0.12 / -
	1.1	Bovine: Liver	0.336 / -	1.1	Bovine: Liver	0.336 / -	0.4	Bovine: Edible offal	0.336 / -	0.4	Bovine: Edible offal	0.336 / -
	1.0	Bovine: Edible offal	0.336 / -	1.0	Bovine: Edible	0.336 / -	0.4	Bovine: Liver	0.336 / -	0.4	Bovine: Liver	0.336 / -
	0.5	Poultry: Meat	0.12 / -	0.5	Poultry: Meat	0.12 / -	0.3	Barley	0.088 / -	0.3	Barley	0.088 / -
	0.3	Milk and milk	0.031 / -	0.3	Milk and milk	0.031 / -	0.2	Poultry: Liver	0.13 / -	0.2	Poultry: Liver	0.13 / -
	No of critical MRLs (IESTI 1)			---			**No of critical MRLs (IESTI 2)**			---		
Processed commodities	**No of commodities for which ARfD/ADI is exceeded:**		---				**No of commodities for which ARfD/ADI is exceeded:**		---			
			***)						***)			
	Highest % of ARfD/ADI	Processed commodities	pTMRL/ threshold MRL (mg/kg)				Highest % of ARfD/ADI	Processed commodities	pTMRL/ threshold MRL (mg/kg)			
	0.0	Wheat flour	0.01 / -				0.0	Bread/pizza	0.01 / -			
	*) The results of the IESTI calculations are reported for at least 5 commodities. If the ARfD is exceeded for more than 5 commodities, all IESTI values > 90% of ARfD are reported.											
	**) pTMRL: provisional temporary MRL											
	***) pTMRL: provisional temporary MRL for unprocessed commodity											
	Conclusion:											
	For BAS 750 F IESTI 1 and IESTI 2 were calculated for food commodities for which pTMRLs were submitted and for which consumption data are available.											
	No exceedance of the ARfD/ADI was identified for any unprocessed commodity.											
	For processed commodities, no exceedance of the ARfD/ADI was identified.											

According to the presented IESTI calculation an acute intake of BAS 750 F residues resulting from the use supported in the present dossier is unlikely to present a public health concern.

Overall conclusion: Dietary exposure assessment

Considering, both for commodities of plant and of animal origin, residues resulting from the cereal use supported in the present dossier, estimated dietary exposure is very low compared to the toxicological reference values.

TMDI calculations based on (proposed MRLs) result in an estimated exposure well below the ADI (at maximum 2.6% ADI). IEDI calculations based on residue data according to the (proposed) residue definition for risk assessment (RD-RA) result in an estimated exposure of at maximum 1.6% ADI. IESTI calculations result in acute exposures far below the toxicological reference value (1.1% ARfD or below).

These assessments indicate a large margin of safety for both chronic and acute exposure, and thereby absence of chronic and acute health concerns resulting from the proposed cereal use of BAS 750 F.

CA 6.10 Other studies

The active substance BAS 750 F and its formulation BAS 750 00 F are intended to be used in cereal crops. In the present dossier no “other study” is provided.

CA 6.10.1 Effect on the residue level in pollen and bee products

The objective of these studies would be to determine the residue level in pollen and bee products important for human consumption. In principle, residues could be taken up by honeybees from crops during blossom.

So far, no study addressing the residues of BAS 750 F in honey and bee products was conducted. The representative uses supported in the present dossier, wheat and barley, are not important for honey production. It is unlikely that BAS 750 F residues will be present in honey or bee products for human consumption resulting from use supported in the present dossier.

We create chemistry

BAS 750 F

Document M-CA, Section 7

FATE AND BEHAVIOUR IN THE ENVIRONMENT

Compiled by:

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E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
21/Mar/2016	To fulfil the request of RMS UK, discussion of endpoints in the context of P and vP assessment for soil has been included in the end of chapter 7.1.2.	Document MCA Section 7 Version 1 BASF DocID 2016/1000818 replaced by revised Version BASF DocID 2016/1103853
	To fulfil the request of RMS UK, discussion on potential effects of water treatment procedures on BAS 750F and on its metabolites has been included in the end of chapter 7.2.	
	To fulfil the request of RMS UK, discussion of endpoints in the context of P and vP assessment for water and sediment has been included in the end of chapter 7.2, subsequently.	

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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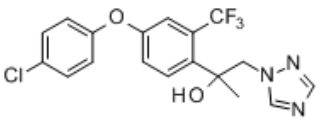
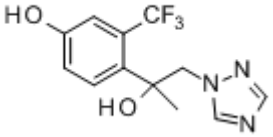
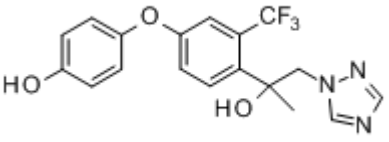
CA 7 FATE AND BEHAVIOUR IN THE ENVIRONMENT

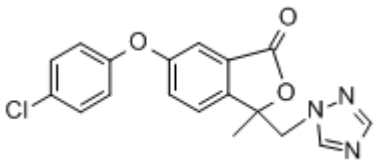
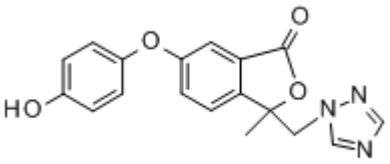
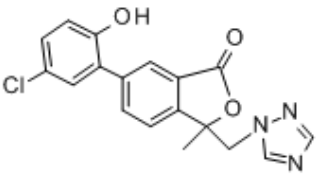
BAS 750 F is a broad spectrum fungicide for use against a range of diseases in cereals. Further evaluation of it's use for the control of harmful diseases in a broad range of additional crops is under development. All studies were conducted in accordance with requirements contained in Regulation (EC) 1107/2009. Studies were performed to investigate the environmentally relevant properties of BAS 750 F using one of three different C-14 labeled compounds representing each ring system of BAS 750 F: chlorophenyl, triazole and trifluoromethylphenyl. With these studies, a full environmentally relevant metabolic profile for BAS 750 F was elucidated and this information used to propose environmentally relevant exposure concentrations.

A literature search was performed on the parent molecule and the newly discovered metabolites from the soil and aquatic compartments. But, being a new active ingredient, no references in the literature were found for BAS 750 F. References were found for 1,2,4-triazole, a common metabolite in azole fungicides, fertilizers and a naturally occurring molecule. However, none were found to be relevant for this dossier submission or add to the overall risk assessment. A full set of the found references and assessment for relevance may be found in an Excel file attached to M-CA Section 9.

An overview of the metabolites discussed in this section is given below Table 7-1.

Table 7-1: Overall Metabolites from E-fate Studies

Metabolite Code	Reg No.	Compartments assessed	Structure
BAS 750 F	5834378	Soil Surface Water Ground Water Air	
M750F001 (1,2,4-triazole)	87084	Soil Surface Water Ground Water	
M750F003	5924326	Surface Water	
M750F005	6003433	Surface Water	

Metabolite Code	**Reg No.**	**Compartments assessed**	**Structure**
M750F006	5863469	Surface Water	
M750F007	6003432	Surface Water	
M750F008	6010286	Surface Water	

CA 7.1.1 Route of degradation in soil

CA 7.1.1.1 Aerobic degradation

The degradation of BAS 750 F in aerobic soil systems shows little mineralization (approximately 9.7% TAR within 121 days, chlorophenyl label). The greatest amount of mineralization was observed with the chlorophenyl labeled test item and was minimal with all other tested labels.

Two minor metabolites of BAS 750 F were observed and characterized in the soil metabolism studies of BAS 750 F. The metabolite M750F001 (1,2,4-triazole) was observed during the aerobic soil metabolism studies in very low amounts. Only in a single soil did the levels reach above 5% TAR at a single measureable time point (5.2% TAR single rep at 90 DAT; 5.1% avg of 2 reps) and then subsequent measurements showed decline of the levels of M750F001 at the end of the study. M750F003 was also observed in low amounts, never reaching higher than 2.2% TAR in any test system. The metabolite is most likely formed due to cleavage of the chlorophenyl moiety, due to the higher degree of mineralization observed with this label. Several other transient and low level metabolites were observed during the studies, but because of the low levels no further characterization was necessary.

The greatest sink observed for BAS 750 F in these systems was bound residues reaching levels of 12.6-26.7% TAR after 121 days. The bound residues increased over the duration of the studies. The majority of the radioactivity was associated with the humins and humic acids though roughly 1/3 was associated with the fulvic acids.

BAS 750 F is applied as a racemic mixture (50:50) of enantiomers. Therefore the enantiomeric ratio was monitored throughout all relevant studies in which enantiospecific degradation and/or enrichment could be envisioned. This analysis resulted in a slight shift of enantiomeric ratio from 50:50 to 45:55 observed in two soils of the aerobic soil metabolism study. This is not deemed a significant change. This is due to the inherent variation of the chiral methodology, which could also be seen in some of the 0 DAT samples, which were observed with ratios of 47:53. A more in depth discussion relative to chirality is presented in chapter N5.

The overall route of degradation in soils is summarized at the end of this section and is shown in the scheme found in Figure 7.1.1-1 at the end of this section.

Report:	CA 7.1.1.1/1 Staudenmaier H., Dalkmann P., 2015a Aerobic soil metabolism of BAS 750 F 2014/1275177
Guidelines:	OECD 307, EPA 835.4100
GLP:	yes (certified by Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz)

EXECUTIVE SUMMARY

The aerobic soil metabolism of BAS 750 F was investigated in two agricultural soils, one originating from the area of Speyer (Germany; soil LUFA 5M) and one from the area of Frenchtown (New Jersey/United States; soil New Jersey).

The soils were freshly collected from the field and passed through a 2 mm sieve before use. The soils were treated separately with two radiolabeled test items, [chlorophenyl-U-^{14}C]-BAS 750 F and [triazole-3(5)-^{14}C]-BAS 750 F, respectively. The test items were applied in batch applications at a nominal rate of 0.4 mg BAS 750 F per kg dry soil which corresponds to a field application rate of 150 g BAS 750 F per hectare, calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}. Soil aliquots of 100 g (dry weight basis) were weighed into test vessels and placed into an incubation cabinet. The incubations were carried out in the dark in the laboratory under aerobic conditions at a soil moisture of 40% of the maximum water holding capacity and a temperature of 20°C. A closed incubation system with continuous aeration (moistened air) was used with an attached trapping system for the determination of volatile compounds. During and at the end of the incubation, the microbial biomass was determined by the substrate induced respiration method, verifying that the soil was viable throughout the incubation period.

Samples from both, the chlorophenyl and triazole label treatment, were taken at 0, 3, 7, 14, 30, 58/62, 90 and 120/121 days after treatment (DAT). At each sampling time, two replicate soil samples were worked up.

Soil portions of 100 g (dry weight) were extracted two times with acetonitrile, two times with acetonitrile/water (80/20, v/v), and two times with acetonitrile/water (50/50, v/v). The individual extracts were analyzed by liquid scintillation counting (LSC). The combined acetonitrile, as well as the combined acetonitrile/water extracts were concentrated and analyzed by radio-HPLC. The remaining soil after extraction was combusted in order to determine the amount of non-extractable soil bound residues (NER). The NER were further characterized by NaOH extraction and subsequent fractionation into fulvic acids, humic acids, and humins. A full material balance was provided for each sampling interval.

Soil LUFA 5M

In soil samples of soil LUFA 5M treated with either chlorophenyl-labeled or triazole-labeled BAS 750 F, the amount of extractable radioactivity (ERR) slightly decreased from an average of 99.0% and 99.1% TAR at day 0 to 82.4% and 81.9 TAR at the end of the study after 121 days of incubation for the respective label. The material balance ranged from 99.3 to 100.5% TAR for both labels.

The most prominent peak present in the extracts of soil LUFA 5M consisted of the parent compound BAS 750 F. During the course of the study, the amounts of BAS 750 F in the total extracts slightly decreased from an average of 98.2% (chlorophenyl-label) and 98.9% TAR (triazole-label) at day 0 down to 80.8% and 81.2% TAR after 121 days of incubation. A number of metabolites were detected in low amounts, none of them exceeding 0.9% and 0.6% TAR any sampling time for the chlorophenyl- and the triazole-labeled test item, respectively. Metabolites M750F001 (1,2,4-(1H)-triazole) and M750F003 were detected when the triazole-labeled test item was applied, reaching maximum amounts of about 0.5% TAR (121 DAT) and 0.6% TAR (14 DAT).

In soil samples treated with the chlorophenyl-labeled test item, non-extractable radioactive residues (NER) were formed in moderate amounts. They increased from an average of 1.0% TAR on day 0 to a maximum of 12.7% TAR. In soil samples treated with the triazole-labeled test item, the formation of NER was slightly higher, increasing from about 0.9% TAR at day 0 to about 17.9% TAR by the end of the study. About half of the NER fraction could be attributed to the humin fraction. Mineralization was low and amounted to 4.7% (chlorophenyl-label) and 0.2% TAR (triazole-label) at the end of the study.

Soil New Jersey

For soil New Jersey, the decrease of the ERR was more pronounced than for soil LUFA 5M, from 99.2% and 99.5% TAR (0 DAT) to 65.7% and 73.3% TAR (120 DAT) for the chlorophenyl- and the triazole-labeled test item, respectively. The material balance ranged from 94.9 to 101.7% TAR for both labels.

Similar to soil LUFA 5M, the most prominent peak present in the soil extracts consisted of the parent compound BAS 750 F. In contrast to soil LUFA 5M, the decline of BAS 750 F was more distinctive, from 98.3% and 99.2% TAR (day 0) down to 63.3% and 67.4% TAR after 120 days for the chlorophenyl- and the triazole-labeled test item, respectively. In soil extracts of samples treated with the triazole-labeled test item, the metabolite M750F001 (1,2,4-(1H)-triazole) was detected in amounts of about 0.2% TAR on day 0 increasing to a maximum of 5.1% TAR at day 90. No further increase was observed at the last sampling event at 120 DAT (4.9% TAR). Metabolite M750F003 was detected in maximum amounts of 1.4% TAR (14 DAT). A number of other metabolites were detected in very low amounts, none of them exceeding 1.4% (chlorophenyl-label) and 0.8% TAR (triazole-label) at any sampling time.

Compared to soil LUFA 5M, NER were formed in slightly higher amounts in samples of soil New Jersey, increasing from an average of 0.8% (chlorophenyl-label) and 0.5% TAR (triazole-label) on day 0 to a maximum of 19.5% and 26.7% TAR. About half of the NER fraction could be attributed to the humin fraction. Mineralization was low to moderate and amounted to 9.7% (chlorophenyl-label) and 0.5% TAR (triazole-label) at the end of the study.

Chiral analysis
Throughout the incubation period, *R*- and *S*-enantiomers of BAS 750 F (both labels) were almost equally present in the pooled acetonitrile as well as acetonitrile/water extracts of soil LUFA 5M. In pooled extracts of soil New Jersey (both labels) the ratio changed from an equal distribution of both enantiomers to a very slightly higher ratio of the S-enantiomer of BAS 750 F at the end of the study (about 45/55 (*R*/*S*)).

Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values for parent BAS 750 F as triggers for additional work was performed following the recommendations of the FOCUS Kinetics workgroup. The analysis was done by non-linear regression methods using the software package KinGUI 2.

The following best-fit $DegT_{50}$ and $DegT_{90}$ values were calculated:

Table 7.1.1.1-1: Best-fit $DegT_{50}$/$DegT_{90}$ values of BAS 750 F in aerobic soil (trigger endpoints)

Soil	Label	$DegT_{50}$ [d]	$DegT_{90}$ [d]	Kinetic model	χ^2 error
LUFA 5M	Chlorophenyl	>1000	>1000	FOMC	0.6
	Triazole	543.5	>1000	DFOP	0.2
New Jersey	Chlorophenyl	201.9	761.3	DFOP	0.8
	Triazole	474.5	>1000	FOMC	0.9

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in two ^{14}C-labeled forms.

Internal code: BAS 750 F
Reg. No.: 5834378
CAS No.: 1417782-03-6
Chemical name (IUPAC): 2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass: 397.78 g mol^{-1}
Molecular formula: $C_{18}H_{15}ClF_3N_3O_2$

1. Chlorophenyl-U-^{14}C-label (in the following referred to as "chlorophenyl"-label)

Batch No.: CFQ41561
Specific radioactivity of a.s.: 7.878 MBq mg^{-1}
Radiochemical purity: 98.9%
Purity: 99.1%

2. Triazole-3(5)-^{14}C-label (in the following referred to as "triazole"-label)

Batch No.: 1062-2001
Specific radioactivity of a.s.: 5.46 MBq mg^{-1}
Radiochemical purity: 98.8%
Purity: 98.9%

2. Soil

The agricultural soils LUFA 5M (BASF soil No. 13/1651/02) from LUFA (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) and New Jersey (BASF soil No. 13/1720/01) from the area of Frenchtown (New Jersey/United States) were used in this study sampled from 0-20 cm (soil LUFA 5M) and 0-15 cm (soil New Jersey) depth. After collecting the soil from the field, the soil was kept at room temperature until sieving. The soil was passed through a 2 mm sieve, remoistened to approximately 7-15% soil moisture and stored at about 4 °C in the dark no longer than 3 months before use. An overview of soil parameters is given in Table 7.1.1.1-2.

Table 7.1.1.1-2: Soil characteristics

Soil designation	**LUFA 5M** **BASF soil No. 13/1651/02** Germany (Origin LUFA Speyer)	**New Jersey** **BASF soil No. 13/1720/01** Unites States (Frenchtown, New Jersey)
DIN 4220 Particle size distribution *[%]* sand 0.063 – 2 mm silt 0.002 – 0.063 mm clay < 0.002 mm textural class	 80.0 13.9 6.1 loamy sand (Sl2)	 n.d. n.d. n.d. n.d.
USDA Particle size distribution *[%]* sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 82.8 11.1 6.1 loamy sand	 29 49 22 loam
Organic C *[%]*	2.03	1.33
pH [H_2O]	7.9	6.9
pH [$CaCl_2$]	7.2	n.d.
cation exchange capacity *[$cmol^+ kg^{-1}$]*	11.4	9.1
Max. water holding capacity *[g /100 g dry weight]*	25.2	37.0
microbial biomass (from certificate) *[mg C/100 g dry soil]*	26.5	51.7
microbial biomass (after 60/62 days of incubation) *[mg C/100 g dry soil]* [a]	23.6	40.4
microbial biomass (after 120/123 days of incubation) *[mg C/100 g dry soil]* [a]	19.8	34.6

n.d. = Not determined
[a] Determined at BASF test facility Limburgerhof

B. STUDY DESIGN

1. Experimental conditions

The test item (both labels) was applied at a nominal concentration of 0.4 mg ^{14}C-BAS 750 per kg dry soil which corresponds to a field application rate of 150 g a.s. ha^{-1} (calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}). Portions of 100 g soil (dry weight basis) were then filled into test vessels.

For incubation, all test vessels were connected in line with aeration tubes in the soil metabolism apparatus. During the study, samples were continuously aerated with a slight stream of moistened synthetic air. For removing carbon dioxide, the air was passed through a bottle with NaOH before passing over the test vessels. For trapping of volatiles potentially evolving from soil during the incubation, test vessels were connected to three gas washing flasks containing ethylene glycol, 0.5 M H_2SO_4, and 0.5 M NaOH. The treated soils were incubated at 40% of the maximum water holding capacity and 20 ± 2 °C in the dark.

To determine the microbial biomass at 60/62 and 120/123 days after treatment (DAT), an additional soil portion was treated with acetonitrile (without test item) and incubated under the same conditions (dark, 20 ± 2 °C) as the treated soils.

2. Sampling

Sampling times for soil New Jersey were 0, 3, 7, 14, 30, 58, 90 and 120 DAT for both label treatments. Soil LUFA 5M was sampled 0, 3, 7, 14, 30, 62, 90 and 121 DAT (both labels). Due to a deviation in the work-up of the 58 DAT samples of soil LUFA 5M (chlorophenyl-label), this sampling was replaced by an additional sampling at 62 DAT.

3. Description of analytical procedures

For the determination of the extractable radioactive residues (ERR), soil samples were consecutively extracted twice with acetonitrile (ACN), twice with ACN/water (80/20; v/v), and twice with ACN/water (50/50; v/v). Each extract was analyzed for radioactivity by liquid scintillation counting (LSC). The ACN-extracts as well as the ACN/water-extracts were pooled and each solution was concentrated. The residues were then re-dissolved in a well-defined volume of solvent and analyzed by radio HPLC. The extracts were also analyzed by chiral HPLC.

The soil residues remaining after extraction were dried, homogenized by means of a small mill, and aliquots were combusted in a biological oxidizer. The evolved $^{14}CO_2$ from each combusted aliquot was trapped and measured by LSC to determine the amount of the non-extractable residues (NER).

From 7 DAT (soil New Jersey) and 14 DAT (soil LUFA 5M) onwards, the non-extractable residues were further characterized by NaOH extraction. The samples were consecutively extracted with NaOH (three times) and water (once). Finally, all extracts were pooled and acidified with concentrated hydrochloric acid to pH 1.5 to precipitate the humic acid fraction. After centrifugation, the supernatant (fulvic acids) was separated from the precipitate. The precipitate (humic acid fraction) was dissolved in NaOH. The humic acid fraction and the fulvic acid fraction were measured for radioactivity. The remaining soil samples after NaOH and water extraction were dried at room temperature. Afterwards, aliquots were combusted. The released $^{14}CO_2$ was trapped and analyzed by LSC to determine the ^{14}C-residues in the humin fraction.

The fulvic acid fraction was partitioned with ethyl acetate. The organic phase was further analyzed by LSC and radio HPLC.

The microbial biomass in the soil samples treated with solvent only was determined at 60/62 and 120/123 DAT. The method was based on the determination of oxygen consumption upon addition of glucose. The microbial biomass declined over the incubation phase. However, the results demonstrate that the soil was still viable and microbially active at days 60/62 and 120/123 (end) of the study.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints). Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006): "Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration" Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 (version control 1.1, Dec 2014), 440 pp.*]. The software package KinGUI (version 2.2014.224.1704) was used for parameter fitting [*SCHÄFER et al. (2007) KinGUI: A new kinetic software tool for evaluations according to FOCUS Degradation Kinetics. In: Del Re, A.A.M. et al. (Eds.): Proceedings of the XIII Symposium on Pesticide Chemistry, Piacenza, 2007, p. 916-923. - BASF DocID 2007/1062781; WITT et al. (2014) KinGUI, Version 2.2014.224.1704 Bayer CropScience AG*]. The error tolerance and the number of iterations of the optimization tool were set to 0.00001 and 100, respectively.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC), are already implemented in KinGUI. The Goodness-of-fit was evaluated by visual assessment, χ^2 minimum error, and type-I-error rate (t-test) [for details see Chapter 6.3 in *FOCUS (2006)*].

Replicate measurements were considered for the parameter estimation. The initial concentration of the applied test item was set to the material balance recovered at day 0. The χ^2 value was calculated for the kinetic model as recommended by FOCUS, considering the average of replicate measurements. For the individual parameters, the t-test statistics were based on number of individual measurements.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Total recoveries of radioactivity extracted from the soils are summarized in Table 7.1.1.1-3 to Table 7.1.1.1-6. The overall mean values for the material balance for both soils and both labels were in the range of 94.9 to 101.7% TAR.

Table 7.1.1.1-3: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with chlorophenyl-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues				NER	Volatiles [a]			Material balance
	ACN	ACN/H_2O (80/20)	ACN/H_2O (50/50)	Total		CO_2	Others [b]	Total	
0	89.8	8.7	0.8	99.3	1.0	n.a.	n.a.	n.a.	100.3
0	88.5	9.4	0.9	98.7	1.0	n.a.	n.a.	n.a.	99.7
0 (mean)	**89.1**	**9.0**	**0.8**	**99.0**	**1.0**	**-**	**-**	**-**	**100.0**
3	87.0	9.5	1.3	97.8	2.6	0.2	0.0	0.2	100.6
3	86.6	9.6	1.3	97.5	2.7	0.2	0.0	0.2	100.3
3 (mean)	**86.8**	**9.5**	**1.3**	**97.6**	**2.7**	**0.2**	**0.0**	**0.2**	**100.4**
7	84.6	10.0	1.6	96.2	3.6	0.4	0.0	0.4	100.2
7	84.9	10.1	1.6	96.6	3.7	0.4	0.0	0.4	100.7
7 (mean)	**84.8**	**10.0**	**1.6**	**96.4**	**3.6**	**0.4**	**0.0**	**0.4**	**100.5**
14	83.7	9.1	1.8	94.6	4.9	0.8	0.0	0.8	100.3
14	82.9	9.2	1.9	94.0	5.0	0.8	0.0	0.8	99.8
14 (mean)	**83.3**	**9.2**	**1.8**	**94.3**	**4.9**	**0.8**	**0.0**	**0.8**	**100.0**
30	79.0	9.4	2.0	90.4	6.9	1.5	0.0	1.5	98.8
30	79.3	10.1	2.1	91.5	7.1	1.5	0.0	1.5	100.0
30 (mean)	**79.2**	**9.7**	**2.0**	**90.9**	**7.0**	**1.5**	**0.0**	**1.5**	**99.4**
62	74.4	9.9	2.4	86.7	9.9	2.6	0.0	2.6	99.3
62	74.5	10.2	2.4	87.1	10.1	2.6	0.0	2.6	99.8
62 (mean)	**74.5**	**10.1**	**2.4**	**86.9**	**10.0**	**2.6**	**0.0**	**2.6**	**99.5**
90	71.8	9.9	2.5	84.2	11.3	3.6	0.0	3.6	99.1
90	71.9	10.1	2.6	84.6	11.6	3.6	0.0	3.6	99.8
90 (mean)	**71.9**	**10.0**	**2.6**	**84.4**	**11.4**	**3.6**	**0.0**	**3.6**	**99.4**
121	69.0	10.3	2.7	82.0	12.8	4.7	0.0	4.7	99.4
121	69.5	10.6	2.8	82.8	12.6	4.7	0.0	4.7	100.0
121 (mean)	**69.2**	**10.4**	**2.8**	**82.4**	**12.7**	**4.7**	**0.0**	**4.7**	**99.7**

TAR = Total applied radioactivity (100% = 0.418 mg kg^{-1})

n.a. = Not available

[a] Cumulative values

[b] Sum of H_2SO_4 and ethylene glycol trap

Table 7.1.1.1-4: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with triazole-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues				NER	Volatiles [a]			Material balance
	ACN	ACN/H_2O (80/20)	ACN/H_2O (50/50)	Total		CO_2	Others [b]	Total	
0	88.2	10.0	0.9	99.1	0.9	n.a.	n.a.	n.a.	100.0
0	89.7	8.6	0.8	99.1	0.9	n.a.	n.a.	n.a.	100.0
0 (mean)	**88.9**	**9.3**	**0.8**	**99.1**	**0.9**	**-**	**-**	**-**	**100.0**
3	87.2	9.4	1.3	97.9	2.8	0.0	0.0	0.0	100.7
3	85.4	9.6	1.3	96.4	2.8	0.0	0.0	0.0	99.2
3 (mean)	**86.3**	**9.5**	**1.3**	**97.2**	**2.8**	**0.0**	**0.0**	**0.0**	**100.0**
7	84.8	10.1	1.6	96.4	3.8	0.1	0.0	0.1	100.3
7	84.4	10.1	1.6	96.1	3.8	0.1	0.0	0.1	100.0
7 (mean)	**84.6**	**10.1**	**1.6**	**96.3**	**3.8**	**0.1**	**0.0**	**0.1**	**100.2**
14	82.7	9.1	1.9	93.8	5.5	0.1	0.0	0.1	99.3
14	82.5	9.2	1.9	93.6	5.5	0.1	0.0	0.1	99.2
14 (mean)	**82.6**	**9.2**	**1.9**	**93.7**	**5.5**	**0.1**	**0.0**	**0.1**	**99.3**
30	78.9	9.9	2.1	91.0	8.1	0.1	0.0	0.1	99.2
30	79.7	10.0	2.2	91.9	8.3	0.1	0.0	0.1	100.3
30 (mean)	**79.3**	**10.0**	**2.1**	**91.5**	**8.2**	**0.1**	**0.0**	**0.1**	**99.8**
58	76.3	9.9	2.4	88.5	12.4	0.1	0.0	0.1	101.0
58	74.4	10.1	2.4	86.9	12.5	0.1	0.0	0.1	99.5
58 (mean)	**75.3**	**10.0**	**2.4**	**87.7**	**12.4**	**0.1**	**0.0**	**0.1**	**100.3**
90	71.6	10.0	2.8	84.4	14.9	0.2	0.0	0.2	99.4
90	72.3	10.1	2.8	85.2	15.0	0.2	0.0	0.2	100.4
90 (mean)	**71.9**	**10.1**	**2.8**	**84.8**	**14.9**	**0.2**	**0.0**	**0.2**	**99.9**
121	68.7	10.4	3.0	82.1	17.5	0.2	0.0	0.2	99.8
121	68.2	10.5	3.0	81.7	18.2	0.2	0.0	0.2	100.2
121 (mean)	**68.4**	**10.5**	**3.0**	**81.9**	**17.9**	**0.2**	**0.0**	**0.2**	**100.0**

TAR = Total applied radioactivity (100% = 0.420 mg kg^{-1})

n.m. = Not available

[a] Cumulative values

[b] Sum of H_2SO_4 and ethylene glycol trap

Table 7.1.1.1-5: Recovery and distribution of radioactivity in soil New Jersey after treatment with chlorophenyl-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues				NER	Volatiles [a]			Material balance
	ACN	ACN/H_2O (80/20)	ACN/H_2O (50/50)	Total		CO_2	Others [b]	Total	
0	90.8	7.3	0.7	98.9	0.8	n.a.	n.a.	n.a.	99.7
0	91.3	7.5	0.7	99.5	0.8	n.a.	n.a.	n.a.	100.3
0 (mean)	**91.0**	**7.4**	**0.7**	**99.2**	**0.8**	**-**	**-**	**-**	**100.0**
3	83.9	9.5	1.5	94.8	3.4	0.3	0.0	0.3	98.6
3	86.4	8.9	1.8	97.1	3.5	0.3	0.0	0.3	100.9
3 (mean)	**85.1**	**9.2**	**1.7**	**96.0**	**3.5**	**0.3**	**0.0**	**0.3**	**99.7**
7	81.3	8.5	1.7	91.5	5.0	0.9	0.0	0.9	97.4
7	80.3	8.7	1.7	90.7	5.1	0.9	0.0	0.9	96.6
7 (mean)	**80.8**	**8.6**	**1.7**	**91.1**	**5.0**	**0.9**	**0.0**	**0.9**	**97.0**
14	77.2	10.2	2.3	89.6	6.9	1.7	0.0	1.7	98.2
14	77.2	10.0	1.7	88.9	6.7	1.7	0.0	1.7	97.3
14 (mean)	**77.2**	**10.1**	**2.0**	**89.2**	**6.8**	**1.7**	**0.0**	**1.7**	**97.7**
30	70.7	9.6	2.8	83.1	10.8	3.3	0.0	3.3	97.2
30	71.6	9.1	2.7	83.4	10.7	3.3	0.0	3.3	97.4
30 (mean)	**71.1**	**9.4**	**2.8**	**83.2**	**10.7**	**3.3**	**0.0**	**3.3**	**97.3**
58	64.2	8.6	2.5	75.3	14.6	5.5	0.0	5.5	95.4
58	66.3	8.9	2.4	77.7	14.8	5.5	0.0	5.5	98.0
58 (mean)	**65.3**	**8.8**	**2.4**	**76.5**	**14.7**	**5.5**	**0.0**	**5.5**	**96.7**
90	59.6	9.5	3.3	72.4	17.1	7.6	0.0	7.6	97.1
90	58.5	9.1	3.1	70.8	16.9	7.6	0.0	7.6	95.3
90 (mean)	**59.1**	**9.3**	**3.2**	**71.6**	**17.0**	**7.6**	**0.0**	**7.6**	**96.2**
120	55.3	8.5	3.0	66.8	19.7	9.7	0.0	9.7	96.2
120	53.0	8.5	3.0	64.5	19.3	9.7	0.0	9.7	93.5
120 (mean)	**54.1**	**8.5**	**3.0**	**65.7**	**19.5**	**9.7**	**0.0**	**9.7**	**94.9**

TAR = Total applied radioactivity (100% = 0.421 mg kg^{-1})

n.m. = Not available

[a] Cumulative values

[b] Sum of H_2SO_4 and ethylene glycol trap

Table 7.1.1.1-6: Recovery and distribution of radioactivity in soil New Jersey after treatment with triazole-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues				NER	Volatiles [a]			Material balance
	ACN	ACN/H_2O (80/20)	ACN/H_2O (50/50)	Total		CO_2	Others [b]	Total	
0	92.1	7.6	0.8	100.5	0.6	n.a.	n.a.	n.a.	101.0
0	90.2	7.5	0.8	98.5	0.5	n.a.	n.a.	n.a.	99.0
0 (mean)	**91.2**	**7.5**	**0.8**	**99.5**	**0.5**	**-**	**-**	**-**	**100.0**
3	85.9	9.9	1.7	97.5	3.3	0.0	0.0	0.0	100.8
3	86.3	10.0	1.9	98.2	3.4	0.0	0.0	0.0	101.6
3 (mean)	**86.1**	**10.0**	**1.8**	**97.8**	**3.3**	**0.0**	**0.0**	**0.0**	**101.2**
7	84.8	9.8	2.1	96.7	5.2	0.1	0.0	0.1	102.0
7	83.2	9.4	1.9	94.5	4.9	0.1	0.0	0.1	99.5
7 (mean)	**84.0**	**9.6**	**2.0**	**95.6**	**5.0**	**0.1**	**0.0**	**0.1**	**100.7**
14	79.5	11.5	2.1	93.1	6.9	0.1	0.0	0.1	100.1
14	82.3	11.4	2.3	96.0	7.1	0.1	0.0	0.1	103.2
14 (mean)	**80.9**	**11.4**	**2.2**	**94.6**	**7.0**	**0.1**	**0.0**	**0.1**	**101.7**
30	72.4	11.0	3.4	86.7	12.6	0.2	0.0	0.2	99.5
30	73.0	10.5	3.5	87.0	12.5	0.2	0.0	0.2	99.7
30 (mean)	**72.7**	**10.7**	**3.4**	**86.9**	**12.6**	**0.2**	**0.0**	**0.2**	**99.6**
58	67.1	10.9	3.5	81.6	19.1	0.3	0.0	0.3	100.9
58	64.9	10.9	3.3	79.1	18.3	0.3	0.0	0.3	97.6
58 (mean)	**66.0**	**10.9**	**3.4**	**80.3**	**18.7**	**0.3**	**0.0**	**0.3**	**99.3**
90	60.6	11.2	4.1	75.8	24.1	0.4	0.0	0.4	100.3
90	58.5	11.4	4.1	74.0	23.3	0.4	0.0	0.4	97.7
90 (mean)	**59.5**	**11.3**	**4.1**	**74.9**	**23.7**	**0.4**	**0.0**	**0.4**	**99.0**
120	58.4	11.2	4.2	73.8	26.6	0.5	0.0	0.5	101.0
120	57.5	11.1	4.2	72.8	26.7	0.5	0.0	0.5	100.0
120 (mean)	**57.9**	**11.2**	**4.2**	**73.3**	**26.7**	**0.5**	**0.0**	**0.5**	**100.5**

TAR = Total applied radioactivity (100% = 0.416 mg kg^{-1})

n.m. = Not available

[a] Cumulative values

[b] Sum of H_2SO_4 and ethylene glycol trap

B. EXTRACTABLE AND BOUND RESIDUES

Soil LUFA 5M

The amount of extractable radioactivity (ERR) slightly decreased in soil samples of soil LUFA 5M treated with either the chlorophenyl- or the triazole-labeled test item, from an average of 99.0% and 99.1% TAR at day 0 to 82.4% and 81.9% TAR, respectively, at the end of the study after 121 days of incubation.

Non-extractable radioactive residues (NER) were formed in moderate amounts. They increased from an average of 1.0% and 0.9% TAR on day 0 to a maximum of 12.7% and 17.9% TAR by the end of the study for the chlorophenyl- and triazole-labeled test item.

Soil New Jersey
In soil New Jersey the amount of ERR decreased from about 99.2% and 99.5% TAR (0 DAT) to about 65.7% and 73.3% TAR (120 DAT) for the chlorophenyl- and the triazole labeled test item respectively.

The amounts of NER increased from 0.8% TAR on day 0 to 19.5% TAR on day 120 for the chlorophenyl-labeled test item and from 0.5% to 26.7% TAR for the triazole-labeled test item.

C. VOLATILIZATION

Soil LUFA 5M
Low mineralization occurred with CO_2 amounts reaching in total 4.7% TAR (chlorophenyl-label) and 0.2% TAR (triazole-label) after 121 days of incubation. No other volatile compounds were observed.

Soil New Jersey
Low to moderate mineralization occurred with CO_2 amounts reaching 9.7% TAR in soil samples treated with the chlorophenyl-labeled test item and 0.5% TAR in soil samples treated with the triazole-labeled test item at the end of the study. No other volatile compounds were observed.

D. TRANSFORMATION OF PARENT COMPOUND

A summary of HPLC results are presented in Table 7.1.1.1-7 and Table 7.1.1.1-8.

Soil LUFA 5M
In soil LUFA 5M, amounts of BAS 750 F in the total extracts slightly decreased from an average of 98.2% and 98.9% TAR at day 0 to 80.8% and 81.2% TAR after 121 days of incubation for the chlorophenyl- and triazole-labeled test item.

Several metabolites were detected, none of them exceeding 0.8% TAR at any sampling time for soil samples treated with the chlorophenyl-labeled test item (sum of metabolites ≤ 1.6% TAR). Metabolites detected in extracts of soil samples treated with the triazole-labeled test item were formed in low amounts as well. Metabolites M750F001 and M750F003 were detected in maximum amounts of 0.5% TAR (121 DAT) and 0.6% TAR (14 DAT). None of the other metabolites exceeded 0.2% TAR with the triazole-label (mean of two replicates; sum of metabolites ≤ 0.6% TAR).

Soil New Jersey
In soil extracts of soil New Jersey BAS 750 F amounts declined from an average of 98.3% TAR (day 0) to an average of 63.3% TAR after 120 days for the chlorophenyl-label and from 99.2% to 67.4% TAR for the triazole-label.

Metabolites M750F001 and M750F003 were exclusively detected in soil samples treated with the triazole-labeled test item, reaching maximum amounts of 5.1% (90 DAT) and 1.4% TAR (14 DAT). A number of other metabolites were detected in the soil extracts, none of them exceeding 1.2% TAR (both labels) at any sampling time. The sum of other metabolites never exceeded 2.2% TAR (chlorophenyl-label) and 0.5% TAR (triazole-label).

Table 7.1.1.1-7: Radio-HPLC analysis of soil extracts: after treatment of soil LUFA 5M with ^{14}C-labeled BAS 750 F (sum of ACN and ACN/water extracts [% TAR], LC188, gradient 02)

chlorophenyl-^{14}C-label				triazole-^{14}C-label					
DAT	**ERR (total)**	**42.3 min BAS 750 F**	**sum others [a]**	**DAT**	**ERR (total)**	**7.2 min M750F001**	**29.9 min M750F003**	**42.3 min BAS 750 F**	**sum others [b]**
0	99.3	98.4	0.9	0	99.1	0.1	-	98.9	0.1
0	98.7	97.9	0.8	0	99.1	0.2	-	98.9	0.0
0 (mean)	**99.0**	**98.2**	**0.9**	**0 (mean)**	**99.1**	**0.1**	**-**	**98.9**	**0.0**
3	97.8	96.1	1.6	3	97.9	0.1	0.1	97.8	0.0
3	97.5	96.9	0.5	3	96.4	0.1	0.1	96.1	0.0
3 (mean)	**97.6**	**96.5**	**1.1**	**3 (mean)**	**97.2**	**0.1**	**0.1**	**96.9**	**0.0**
7	96.2	94.7	1.5	7	96.4	0.1	0.6	95.7	0.0
7	96.6	95.0	1.6	7	96.1	0.1	0.5	95.4	0.0
7 (mean)	**96.4**	**94.9**	**1.5**	**7 (mean)**	**96.3**	**0.1**	**0.6**	**95.6**	**0.0**
14	94.6	93.5	1.1	14	93.8	0.1	0.5	93.1	0.0
14	94.0	93.2	0.8	14	93.6	0.1	0.6	92.9	0.0
14 (mean)	**94.3**	**93.4**	**0.9**	**14 (mean)**	**93.7**	**0.1**	**0.6**	**93.0**	**0.0**
30	90.4	89.4	1.0	30	91.0	0.2	0.6	89.6	0.6
30	91.5	90.6	0.9	30	91.9	0.2	0.5	91.0	0.2
30 (mean)	**90.9**	**90.0**	**0.9**	**30 (mean)**	**91.5**	**0.2**	**0.5**	**90.3**	**0.4**
62	86.7	85.9	0.9	58	88.5	0.3	0.2	87.9	0.2
62	87.1	86.3	0.8	58	86.9	0.3	0.2	86.2	0.2
62 (mean)	**86.9**	**86.1**	**0.8**	**58 (mean)**	**87.7**	**0.3**	**0.2**	**87.0**	**0.2**
90	84.2	82.9	1.2	90	84.4	0.4	0.2	83.6	0.2
90	84.6	83.4	1.2	90	85.2	0.4	0.1	84.5	0.2
90 (mean)	**84.4**	**83.2**	**1.2**	**90 (mean)**	**84.8**	**0.4**	**0.2**	**84.0**	**0.2**
121	82.0	80.4	1.6	121	82.1	0.5	-	81.4	0.2
121	82.8	81.2	1.6	121	81.7	0.6	-	81.0	0.1
121 (mean)	**82.4**	**80.8**	**1.6**	**121 (mean)**	**81.9**	**0.5**	**-**	**81.2**	**0.2**

DAT = Days after treatment

TAR = Total applied radioactivity (100% = 0.418 mg kg^{-1} (chlorophenyl-label), 0.420 mg kg^{-1} (triazole-label)

ERR = Extractable radioactive residues

[a] ≤ 0.8% TAR each for single metabolite (mean of two replicates)

[b] ≤ 0.2% TAR each for single metabolite (mean of two replicates)

Table 7.1.1.1-8: Radio-HPLC analysis of soil extracts: after treatment of soil New Jersey with ^{14}C-labeled BAS 750 F (sum of ACN and ACN/water extracts [% TAR], LC188, gradient 02)

chlorophenyl-^{14}C-label				triazole-^{14}C-label					
DAT	**ERR (total)**	**42.3 min BAS 750 F**	**sum others [a]**	**DAT**	**ERR (total)**	**7.2 min M750F001**	**29.9 min M750F003**	**42.3 min BAS 750 F**	**sum others [b]**
0	98.9	98.3	0.6	0	100.5	0.2	-	100.2	0.0
0	99.5	98.3	1.2	0	98.5	0.3	-	98.2	0.0
0 (mean)	**99.2**	**98.3**	**0.9**	**0 (mean)**	**99.5**	**0.2**	**-**	**99.2**	**0.0**
3	94.8	93.7	1.1	3	97.5	0.4	0.5	96.5	0.0
3	97.1	96.1	1.0	3	98.2	0.5	0.7	97.0	0.1
3 (mean)	**96.0**	**94.9**	**1.1**	**3 (mean)**	**97.8**	**0.4**	**0.6**	**96.7**	**0.1**
7	91.5	90.6	0.9	7	96.7	1.3	1.2	93.4	0.9
7	90.7	89.6	1.1	7	94.5	1.1	1.1	92.2	0.1
7 (mean)	**91.1**	**90.1**	**1.0**	**7 (mean)**	**95.6**	**1.2**	**1.1**	**92.8**	**0.5**
14	89.6	88.1	1.4	14	93.1	2.0	1.3	89.7	0.1
14	88.9	87.8	1.1	14	96.0	1.7	1.5	92.8	0.1
14 (mean)	**89.2**	**88.0**	**1.3**	**14 (mean)**	**94.6**	**1.8**	**1.4**	**91.3**	**0.1**
30	83.1	81.7	1.3	30	86.7	2.7	1.1	82.9	0.1
30	83.4	82.1	1.3	30	87.0	2.4	1.2	83.3	0.1
30 (mean)	**83.2**	**81.9**	**1.3**	**30 (mean)**	**86.9**	**2.6**	**1.2**	**83.1**	**0.1**
58	75.3	73.1	2.3	58	81.6	4.3	0.9	76.1	0.3
58	77.7	75.6	2.1	58	79.1	4.5	1.0	73.5	0.1
58 (mean)	**76.5**	**74.3**	**2.2**	**58 (mean)**	**80.3**	**4.4**	**1.0**	**74.8**	**0.2**
90	72.4	70.8	1.6	90	75.8	5.2	0.9	69.5	0.2
90	70.8	68.4	2.4	90	74.0	4.9	0.9	67.7	0.4
90 (mean)	**71.6**	**69.6**	**2.0**	**90 (mean)**	**74.9**	**5.1**	**0.9**	**68.6**	**0.3**
120	66.8	64.5	2.3	120	73.8	4.8	0.8	67.8	0.4
120	64.5	62.0	2.5	120	72.8	4.9	0.8	66.9	0.1
120 (mean)	**65.7**	**63.3**	**2.4**	**120 (mean)**	**73.3**	**4.9**	**0.8**	**67.4**	**0.3**

DAT = Days after treatment

TAR = Total applied radioactivity (100% = 0.421 mg kg^{-1} (chlorophenyl-label), 0.416 mg kg^{-1} (triazole-label)

ERR = Extractable radioactive residues

[a] ≤ 1.2% TAR each for single metabolite (mean of two replicates)

[b] ≤ 0.4% TAR each for single metabolite (mean of two replicates)

Chiral HPLC results

Throughout the incubation period, *R*- and *S*-enantiomers of BAS 750 F were almost equally present in the pooled acetonitrile as well as acetonitrile/water extracts of soil LUFA 5M. In pooled extracts of soil New Jersey, the ratio changed from an equal distribution of both enantiomers to a very slightly higher ratio (~ 45:55) of the *S*-enantiomer of BAS 750 F at the end of the study. Detailed results and representative chromatograms of chiral HPLC are given in the final report.

E. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

Results of the non-extractable residues characterization performed by humic substance fractionation are given in Table 7.1.1.1-9 to Table 7.1.1.1-12.

Soil LUFA 5M

More than half of the amount of NER remained unextractable and was assigned to the humin fraction (3.0 to 7.4% TAR (chlorophenyl-label) and 2.7 to 6.7% TAR (triazole-label)). Smaller amounts of radioactivity were associated with the humic acid (1.0 to 2.5% and 0.9 to 2.5% TAR, respectively) and the fulvic acid fraction (0.8 to 2.1% TAR (chlorophenyl-label), the latter being higher for the triazole-labeled test item (2.2 to 8.7% TAR). About one half (chlorophenyl-label) and about one third (triazole-label) of the amount of radioactivity in the fulvic acid fraction could be partitioned from the fulvic acid fraction into ethyl acetate.

Soil New Jersey

About half of the NER remained unextractable upon extraction with NaOH and was assigned to the humin fraction (2.8 to 12.2% TAR (chlorophenyl-label) and 2.8 to 13.1% TAR (triazole-label)). Slightly lower amounts of radioactivity were associated with the humic acid (1.5 to 4.1% TAR and 0.9 to 4.2% TAR, respectively) than with the fulvic acid fraction (1.0 to 3.7% TAR and 1.0 to 9.4% TAR, respectively). About one half (chlorophenyl-label) and one-third (triazole-label) of the amount of radioactivity in the fulvic acid fraction could be partitioned from the fulvic acid fraction into ethyl acetate (0.4 to 1.3% TAR and 0.7 to 2.9% TAR, respectively).

Table 7.1.1.1-9: Characterization of the non-extractable residues (NER) in soil LUFA 5M after treatment with chlorophenyl-^{14}C-BAS 750 F [% TAR]

DAT	NER	NaOH extract (sum)	Fulvic acids			Humic acids	Humins
			Total	Aqueous phase	Ethyl acetate		
14	5.0	2.0	0.8	0.4	0.4	1.0	2.9
30	7.1	2.7	1.1	0.5	0.6	1.5	4.2
62	10.1	3.8	1.5	0.8	0.7	2.2	5.6
90	11.6	4.5	1.8	0.9	0.8	2.5	6.6
121	12.8	5.0	2.1	1.1	1.0	2.4	7.4

TAR = Total applied radioactivity (100% = 0.418 mg kg^{-1})
DAT = Days after treatment
NER = Non-extractable radioactive residues
Ethyl acetate = Organic phase after liquid-liquid extraction of fulvic acids

Table 7.1.1.1-10: Characterization of the non-extractable residues (NER) in soil LUFA 5M after treatment with triazole-^{14}C-BAS 750 F [% TAR]

DAT	NER	NaOH extract (sum)	Fulvic acids			Humic acids	Humins
			Total	Aqueous phase	Ethyl acetate		
14	5.5	3.1	2.2	1.4	0.6	0.9	2.7
30	8.1	4.7	3.1	2.4	0.9	1.2	3.7
58	12.4	7.1	4.9	3.9	1.3	1.8	5.1
90	15.0	9.3	6.6	5.3	1.5	2.5	6.0
121	18.2	10.9	8.7	6.6	1.8	2.2	6.7

TAR = Total applied radioactivity (100% = 0.420 mg kg^{-1})
DAT = Days after treatment
NER = Non-extractable radioactive residues
Ethyl acetate = Organic phase after liquid-liquid extraction of fulvic acids

Table 7.1.1.1-11: Characterization of the non-extractable residues (NER) in soil New Jersey after treatment with chlorophenyl-^{14}C-BAS 750 F [% TAR]

DAT	NER	NaOH extract (sum)	Fulvic acids			Humic acids	Humins
			Total	Aqueous phase	Ethyl acetate		
7	5.1	2.3	1.0	0.6	0.4	1.5	2.8
14	6.9	2.9	1.4	0.9	0.5	1.5	3.8
30	10.8	4.5	2.0	1.2	0.8	2.3	6.2
58	14.8	5.8	2.5	1.6	1.0	3.0	8.6
90	17.1	7.1	3.0	2.0	1.1	3.8	10.7
120	19.7	8.5	3.7	2.4	1.3	4.1	12.2

TAR = Total applied radioactivity (100% = 0.421 mg kg^{-1})
DAT = Days after treatment
NER = Non-extractable radioactive residues
Ethyl acetate = Organic phase after liquid-liquid extraction of fulvic acids

Table 7.1.1.1-12: Characterization of the non-extractable residues (NER) in soil New Jersey after treatment with triazole-^{14}C-BAS 750 F [% TAR]

DAT	NER	NaOH extract (sum)	Fulvic acids			Humic acids	Humins
			Total	Aqueous phase	Ethyl acetate		
7	5.2	2.3	1.0	0.8	0.7	0.9	2.8
14	6.9	3.2	2.2	1.3	1.0	1.1	3.7
30	12.6	6.0	3.7	2.5	1.6	2.0	6.7
58	19.1	9.8	6.4	4.3	2.3	3.1	9.9
90	24.1	12.6	8.5	6.2	2.5	3.7	11.4
120	26.7	14.2	9.4	6.8	2.9	4.2	13.1

TAR = Total applied radioactivity (100% = 0.416 mg kg^{-1})
DAT = Days after treatment
NER = Non-extractable radioactive residues
Ethyl acetate = Organic phase after liquid-liquid extraction of fulvic acids

F. KINETIC MODELING RESULTS

The estimated best-fit $DegT_{50}$ and $DegT_{90}$ values of BAS 750 F in aerobic soil are summarized in Table 7.1.1.1-13.

Table 7.1.1.1-13: Trigger endpoints for BAS 750 F (non-GLP)

Soil	Label	$DegT_{50}$ [d]	$DegT_{90}$ [d]	Kinetic model	χ^2 error
LUFA 5M	Chlorophenyl	>1000	>1000	FOMC	0.6
	Triazole	543.5	>1000	DFOP	0.2
New Jersey	Chlorophenyl	201.9	761.3	DFOP	0.8
	Triazole	474.5	>1000	FOMC	0.9

III. CONCLUSION

BAS 750 F degraded slowly in loamy sand soil LUFA 5M and loam soil New Jersey during incubation under aerobic conditions.

Depending on the label, the major sink for BAS 750 F degradation in soil under aerobic conditions was either formation of non-extractable residues and mineralization (chlorophenyl label), or formation of non-extractable residues (triazole label). Metabolites were formed only in low amounts.

Report:	CA 7.1.1.1/2 Staudenmaier H.,Dalkmann P., 2015 a Aerobic soil metabolism of Trifluoromethylphenyl-labeled BAS 750 F 2015/1003306
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The aerobic soil metabolism of trifluoromethylphenyl-^{14}C-labeled BAS 750 F was investigated in one agricultural soil, originating from the area of Frenchtown (New Jersey/United States; soil New Jersey).

The test item was applied in batch application at a nominal rate of 0.4 mg BAS 750 F per kg dry (corresponding to 150 g BAS 750 F ha^{-1}). Soil aliquots of 100 g (dry weight basis) were weighed into test vessels incubated in the dark under aerobic conditions at 40% of the maximum water holding capacity and 20 °C. A closed incubation system with continuous aeration (moistened air) was used with an attached trapping system for the determination of volatile compounds.

Samples were taken at 0, 3, 7, 14, 30, 59, 90 and 121 days after treatment (DAT). At each sampling time, two replicate soil samples were analyzed.

Soil portions of 100 g (dry weight) were extracted twice with acetonitrile, twice with acetonitrile/water (80/20, v/v), and twice with acetonitrile/water (50/50, v/v). The individual extracts were analyzed by liquid scintillation counting (LSC). The combined acetonitrile, as well as the combined acetonitrile/water extracts were concentrated and analyzed by radio-HPLC. The remaining soil after extraction was combusted in order to determine the amount of non-extractable soil bound residues (NER). The NER were further characterized by NaOH extraction and subsequent fractionation into fulvic acids, humic acids, and humins. The fulvic acid fraction was characterized by partitioning with ethyl acetate.

Amounts of extractable radioactivity (ERR) slightly decreased from an average of 99.5% TAR at day 0 to 70.1% TAR at the end of the study after 121 days of incubation. Mineralization was low and amounted to 5.7% TAR at the end of the study. The material balance ranged from 98.8 to 102.8% TAR.

The most prominent peak present in the extracts of soil New Jersey consisted of the parent compound BAS 750 F. During the course of the study, the amounts of BAS 750 F in the total extracts decreased from an average of 97.7% at day 0 down to 64.9% TAR after 121 days of incubation. A number of metabolites were detected in low amounts, none of them exceeding 1.7% TAR at any sampling time (mean of two replicates). One metabolite was identified as M750F003 by means of mass spectrometry, reaching a maximum amount of 1.6% TAR at 30 DAT. The sum of all other metabolites (mean of two replicates) never exceeded 5.5% TAR.

Non-extractable radioactive residues (NER) were formed in moderate amounts. They increased from an average of 0.5% TAR on day 0 to a maximum of 24.0% TAR. About half of the NER fraction could be attributed to the humin fraction.

Chiral analysis
During the incubation period, the ratio of *R*- and *S*-enantiomers of BAS 750 F in pooled extracts of soil New Jersey changed from an equal distribution to a slightly higher ratio of the *S*-enantiomer at the end of the study.

Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values for parent BAS 750 F was performed following the recommendations of the FOCUS Kinetics workgroup. The analysis was done by non-linear regression methods using the software package KinGUI 2. Calculated best-fit $DegT_{50}$ and $DegT_{90}$ values used as trigger endpoints were 433.7 and > 1000 days, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	(2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$
Position of radiolabel:	trifluoromethylphenyl-ring-U-^{14}C
Batch No.:	CFQ42039
Specific radioactivity of a.s.:	8.288 MBq mg^{-1}
Radiochemical purity:	98.3%
Purity:	96.3%

2. Soil

The US agricultural soil New Jersey (BASF soil No. 14/1720/01) from the area of Frenchtown (New Jersey/United States) used in this study was sampled from 0-15 cm depth. After collecting the soil from the field, the soil was kept at room temperature until sieving. The soil was passed through a 2 mm sieve, remoistened to approximately 19% soil moisture and stored at about 4 °C in the dark no longer than 3 months before use. An overview of soil parameters is given in Table 7.1.1.1-14.

Table 7.1.1.1-14: Soil characteristics

Soil designation	**New Jersey** **BASF soil No. 14/1720/01** United States (Frenchtown, New Jersey/United States)
DIN 4220 Particle size distribution *[%]* sand 0.063 – 2 mm silt 0.002 – 0.063 mm clay < 0.002 mm textural class	 31 48 21 sandy silt loam
USDA Particle size distribution *[%]* sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 33 46 21 loam
Organic C *[%]*	1.3
pH [H_2O] pH [$CaCl_2$]	6.8 6.4
cation exchange capacity *[$cmol^+ kg^{-1}$]*	8.5
Max. water holding capacity *[g /100 g dry weight]*	33.3
microbial biomass (from certificate) *[mg C/100 g dry soil]*	65.0
microbial biomass (after 63 days of incubation) *[mg C/100 g dry soil]* [a]	42.0
microbial biomass (after 123 days of incubation) *[mg C/100 g dry soil]* [a]	38.8

n.d. = Not determined
[a] Determined at BASF test facility Limburgerhof

B. STUDY DESIGN

1. Experimental conditions

The test item was applied at a nominal concentration of 0.4 mg ^{14}C-BAS 750 per kg dry soil which corresponds to a field application rate of 150 g a.s. ha^{-1} (calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}). Portions of 100 g soil (dry weight basis) were then filled into test vessels.

For incubation, all test vessels were connected in line with aeration tubes in the soil metabolism apparatus. During the study, samples were continuously aerated with a slight stream of moistened synthetic air. For removing carbon dioxide, the air was passed through a bottle with NaOH before passing the test vessels. For trapping of volatiles possibly evolving from soil during the incubation, test vessels were connected to three gas washing flasks containing 50 mL ethylene glycol, 50 mL 0.5 M H_2SO_4, and 50 mL 0.5 M NaOH. The treated soils were incubated at 40% of the maximum water holding capacity and 20 ± 2 °C in the dark.

To determine the microbial biomass at 63 and 123 days after treatment (DAT), an additional soil portion (1050 g dry equivalents of each soil, 40% maximum water holding capacity) was treated only with 758 µL acetonitrile (without test item). The samples were incubated under the same conditions (dark, 20 ± 2 °C) as the treated soil.

2. Sampling

Sampling times were 0, 3, 7, 14, 30, 59, 90 and 121 DAT.

3. Description of analytical procedures

For the determination of the extractable radioactive residues (ERR), the 100 g (dry weight basis) soil samples were consecutively extracted twice with acetonitrile (ACN), twice with ACN/water (80/20; v/v), and twice with ACN/water (50/50; v/v). Each extract was analyzed for radioactivity by liquid scintillation counting (LSC). The ACN-extracts as well as the ACN/water-extracts were pooled and each solution was concentrated (rotary evaporator T≈ 40 °C). Then the residues were re-dissolved in a well-defined volume of solvent and analyzed by radio HPLC as well as chiral analysis.

The soil residues remaining after extraction were dried at 60 °C, homogenized by means of a small mill, and aliquots were combusted in a biological oxidizer. The evolved $^{14}CO_2$ from each combusted aliquot was trapped in Oxysolve C-400 scintillator and measured by LSC to determine the amount of the non-extractable residues (NER).

From 7 DAT onwards, the non-extractable residues were further characterized by NaOH extraction. The samples were consecutively extracted with NaOH (three times) and water (once). Finally, all extracts were pooled and acidified with concentrated hydrochloric acid to pH 1.5 to precipitate the humic acid fraction. After centrifugation, the supernatant (fulvic acids) was separated from the precipitate. The precipitate (humic acid fraction) was dissolved in NaOH. The humic acid fraction and the fulvic acid fraction were measured for radioactivity. The remaining soil samples after NaOH and water extraction were dried at room temperature. Afterwards, aliquots were combusted. The released $^{14}CO_2$ was trapped and analyzed by LSC to determine the ^{14}C-residues in the humin fraction.

The fulvic acid fraction was partitioned with ethyl acetate. The organic phase was further analyzed by LSC and radio HPLC.

The microbial biomass in the soil samples treated with solvent only was determined at 63 and 123 DAT. The method was based on the determination of oxygen consumption upon addition of glucose. The microbial biomass declined over the incubation phase. However, the results demonstrate that the soil was still viable and microbially active at days 63 and 123 (end) of the study.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) as well as modeling endpoints. Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*]. The software package KinGUI (version 2.2014.224.1704) was used for parameter fitting [*SCHÄFER et al. (2007); WITT et al. (2014)*]. The error tolerance and the number of iterations of the optimization tool were set to 0.000001 and 100, respectively.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC), are already implemented in KinGUI. The Goodness-of-fit was evaluated by visual assessment, χ^2 minimum error, and type-I-error rate (t-test) [for details see Chapter 6.3 in *FOCUS (2006)*].

Replicate measurements were considered for the parameter estimation. The initial concentration of the applied test item was set to the material balance recovered at day 0. The χ^2 value was calculated for the kinetic model as recommended by FOCUS, considering the average of replicate measurements. For the individual parameters, the t-test statistics were based on number of individual measurements.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Total recoveries of radioactivity extracted from the soil are summarized in Table 7.1.1.1-15. The overall mean values for the material balance were in the range of 99.7% - 102.6% TAR.

Table 7.1.1.1-15: Recovery and distribution of radioactivity in soil New Jersey after treatment with trifluoromethylphenyl-14C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues				NER	Volatiles [a]			Material balance
	ACN	ACN/H_2O (80/20)	ACN/H_2O (50/50)	Total		CO_2	Others [b]	Total	
0	90.0	7.7	0.6	98.3	0.5	n.a.	n.a.	n.a.	98.8
0	92.2	7.9	0.6	100.7	0.5	n.a.	n.a.	n.a.	101.2
0 (mean)	**91.1**	**7.8**	**0.6**	**99.5**	**0.5**	**-**	**-**	**-**	**100.0**
3	85.4	9.0	1.5	96.0	3.9	0.1	0.0	0.1	99.9
3	85.1	9.1	1.5	95.6	3.8	0.1	0.0	0.1	99.5
3 (mean)	**85.3**	**9.1**	**1.5**	**95.8**	**3.8**	**0.1**	**0.0**	**0.1**	**99.7**
7	84.7	9.9	2.4	97.1	5.9	0.2	0.0	0.2	103.1
7	83.8	9.8	2.4	96.0	5.7	0.2	0.0	0.2	102.0
7 (mean)	**84.2**	**9.9**	**2.4**	**96.5**	**5.8**	**0.2**	**0.0**	**0.2**	**102.6**
14	78.8	10.2	2.4	91.4	8.3	0.6	0.0	0.6	100.3
14	80.8	10.5	2.4	93.8	8.1	0.6	0.0	0.6	102.4
14 (mean)	**79.8**	**10.4**	**2.4**	**92.6**	**8.2**	**0.6**	**0.0**	**0.6**	**101.3**
30	73.3	10.3	3.6	87.2	12.6	1.4	0.0	1.4	101.2
30	73.7	10.5	3.6	87.7	12.4	1.4	0.0	1.4	101.5
30 (mean)	**73.5**	**10.4**	**3.6**	**87.5**	**12.5**	**1.4**	**0.0**	**1.4**	**101.4**
59	66.3	11.0	3.5	80.8	19.1	3.0	0.0	3.0	102.8
59	63.5	10.5	3.5	77.5	18.9	3.0	0.0	3.0	99.4
59 (mean)	**64.9**	**10.7**	**3.5**	**79.2**	**19.0**	**3.0**	**0.0**	**3.0**	**101.1**
90	60.4	9.8	4.6	74.8	21.1	4.5	0.0	4.5	100.4
90	59.1	9.7	4.5	73.3	21.3	4.5	0.0	4.5	99.1
90 (mean)	**59.8**	**9.8**	**4.5**	**74.1**	**21.2**	**4.5**	**0.0**	**4.5**	**99.7**
121	55.5	10.1	4.9	70.5	24.0	5.7	0.0	5.7	100.2
121	54.8	10.1	4.9	69.8	23.9	5.7	0.0	5.7	99.5
121 (mean)	**55.1**	**10.1**	**4.9**	**70.1**	**24.0**	**5.7**	**0.0**	**5.7**	**99.9**

TAR = Total applied radioactivity (100% = 0.407 mg kg^{-1})

n.a. = Not available

[a] Cumulative values

[b] Sum of H_2SO_4 and ethylene glycol trap

B. EXTRACTABLE AND BOUND RESIDUES

In the New Jersey soil the amount of ERR decreased from an average of 99.5% TAR at day 0 to 70.1% TAR at the end of the study after 121 days of incubation.

Non-extractable radioactive residues (NER) were formed in moderate amounts. They increased from an average of 0.5% TAR on day 0 to a maximum of 24.0% TAR at the end of the study.

C. VOLATILIZATION

Low mineralization occurred during the course of the study with CO_2 amounts reaching in total 5.7% TAR after 121 days of incubation. No other volatile compounds were observed.

D. TRANSFORMATION OF PARENT COMPOUND

A summary of HPLC results are presented in Table 7.1.1.1-16.

During the course of the study, the amounts of trifluoromethylphenyl-^{14}C-labeled-BAS 750 F in the total extracts decreased from an average of 97.7% TAR at day 0 to 64.9% TAR after 121 days of incubation.

A number of metabolites formed in low amounts were detected in the soil extracts, none of them exceeding 1.8% TAR at any sampling time. The sum of metabolites (mean of two replicates) never exceeded 5.5% TAR. One metabolite, representing the metabolite M750F003, was identified by means of mass spectrometry (LC-MS/MS). M750F003 was detected in a maximum amount of 1.6% TAR at 30 DAT (mean of two replicates).

Table 7.1.1.1-16: Radio-HPLC analysis of soil extracts: after treatment of soil New Jersey with trifluoromethylphenyl-^{14}C-labeled BAS 750 F (sum of ACN and ACN/water extracts) [% TAR]

Days after treatment	ERR (total)	4.2 min	28.2 min M750F003	40.1 min BAS 750 F	Sum others [a]
0	98.3	-	-	96.5	1.8
0	100.7	-	-	99.0	1.7
0 (mean)	**99.5**	**-**	**-**	**97.7**	**1.8**
3	96.0	-	0.6	93.4	2.0
3	95.6	-	0.5	93.1	2.1
3 (mean)	**95.8**	**-**	**0.5**	**93.2**	**2.0**
7	97.1	-	0.9	93.8	2.3
7	96.0	-	1.0	92.5	2.5
7 (mean)	**96.5**	**-**	**1.0**	**93.2**	**2.4**
14	91.4	-	1.4	87.3	2.6
14	93.8	-	1.3	89.8	2.7
14 (mean)	**92.6**	**-**	**1.4**	**88.6**	**2.6**
30	87.2	0.1	1.6	82.6	2.9
30	87.7	0.1	1.7	83.3	2.6
30 (mean)	**87.5**	**0.1**	**1.6**	**83.0**	**2.8**
62	80.8	0.2	1.3	75.8	3.5
62	77.5	0.1	1.1	73.2	3.1
62 (mean)	**79.2**	**0.2**	**1.2**	**74.5**	**3.3**
90	74.8	0.2	1.4	69.1	4.2
90	73.3	0.2	1.2	68.0	3.8
90 (mean)	**74.1**	**0.2**	**1.3**	**68.6**	**4.0**
121	70.5	0.2	1.1	65.1	4.1
121	69.8	0.3	1.2	64.6	3.7
121 (mean)	**70.1**	**0.2**	**1.2**	**64.9**	**3.9**

TAR = Total applied radioactivity (100% = 0.407 mg kg^{-1})

ERR = Extractable radioactive residues

[a] ≤ 1.7% TAR for single metabolite (mean of two replicates)

Chiral HPLC results
During the incubation period, the ratio of *R*- and *S*-enantiomers of BAS 750 F in pooled extracts of soil New Jersey changed from an equal distribution to a slightly higher ratio (~ 45:55) of the S-enantiomer at the end of the study. Detailed results and representative chromatograms of chiral HPLC are given in the final report.

E. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

Results of the non-extractable residues characterization performed by humic substance fractionation are given in Table 7.1.1.1-17.

Upon extraction with NaOH, more than half of the amount of NER remained unextractable and was assigned to the humin fraction. Smaller amounts of radioactivity were associated with the humic acid and the fulvic acid fraction. About two thirds of the amount of radioactivity could be partitioned from the fulvic acid fraction into ethyl acetate, while one-third remained in the aqueous phase.

HPLC analysis showed that the low amounts of radioactivity in the ethyl acetate phase of the fulvic acid fraction were predominantly attributed to metabolite M750F003.

Table 7.1.1.1-17: Characterization of the non-extractable residues (NER) in soil New Jersey after treatment with trifluoromethylphenyl-^{14}C-BAS 750 F [% TAR]

DAT	NER	NaOH extract (sum)	Fulvic acids			Humic acids	Humins
			Total	Aqueous phase	Ethyl acetate		
7	5.9	2.0	1.0	0.3	0.7	1.1	3.9
14	8.3	3.2	1.6	0.5	1.1	1.6	5.4
30	12.6	4.9	2.5	0.9	1.7	2.4	7.7
59	19.1	7.5	3.8	1.5	2.4	3.5	11.7
90	21.3	9.1	4.6	2.0	2.8	4.2	12.0
121	24.0	10.7	5.1	2.3	2.8	5.3	13.6

TAR = Total applied radioactivity (100% = 0.407 mg kg^{-1})
DAT = Days after treatment
NER = Non-extractable radioactive residues
Ethyl acetate = Organic phase after liquid-liquid extraction of fulvic acids

F. KINETIC MODELING RESULTS

The estimated best-fit $DegT_{50}$ and $DegT_{90}$ values of BAS 750 F and metabolites in aerobic soil are summarized in Table 7.1.1.1-18.

Table 7.1.1.1-18: Trigger endpoints for BAS 750 F (non-GLP)

Label	$DegT_{50}$ *[d]*	$DegT_{90}$ *[d]*	Best-fit model	χ^2 error
trifluoromethylphenyl	433.7	>1000	FOMC	1.3

III. CONCLUSION

BAS 750 F degraded slowly in loam soil New Jersey during incubation under aerobic conditions.

No major metabolites were formed. The major degradation sink of trifluoromethylphenyl-^{14}C-labeled BAS 750 F in soil under aerobic conditions was the formation of non-extractable residues, while mineralization was of minor importance.

CA 7.1.1.2 Anaerobic degradation

Report:	CA 7.1.1.2/1 Leed M.G., 2015 a Anaerobic soil metabolism of 14C-BAS 750 F 2014/7003496
Guidelines:	EPA 835.4200, OECD 307 (2002), SETAC Procedures for assessing the environmental fate and ecotoxicity for pesticides (March 1995)
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The metabolism of BAS 750 F under anaerobic conditions was conducted in four different soils. Two of the soils used were from Germany (Li10 and LUFA 5M) and two of the soils were from locations in the United States (IN and NJ). The soils were treated with BAS 750 F using two separate radiolabeled BAS 750 F compounds which were either [chlorophenyl-U-^{14}C] or [triazole-3(5)-^{14}C]-BAS 750 F. The radiochemical purity of [chlorophenyl-U-^{14}C]-BAS 750 F was 98.9% and had a specific activity of 472,680 dpm μg^{-1} or 7.878 MBq mg^{-1}. The second radiolabeled compound, [triazole-3(5)-^{14}C]-BAS 750 F, had a radiochemical purity of 98.8% and had a specific activity of 327,600 dpm μg^{-1} or 5.46 MBq mg^{-1}. BAS 750 F was applied at a rate of 0.6 µg a.s. g^{-1} dry soil, which corresponds to the proposed maximum field application rate of 150 g a.s. ha^{-1} (assuming that the test substance is distributed into 2.5 cm depth of soil and a bulk density of soil of 1.0 g cm^{-3}). Additional samples were dosed at approximately 3.0 µg a.s. g^{-1} dry soil (5× maximum application rate) to facilitate the identification of the metabolites.
Soil equivalent to 50 g of dry weight was placed in tared test vessels and connected to a flow-through test system. Samples from all four soils were dosed with the triazole-labeled BAS 750 F and samples from one soil (NJ) was also dosed with the chlorophenyl-labeled BAS 750 F. Moisturized and CO_2-free air or nitrogen were passed over the soil in order to maintain the aerobic or anaerobic conditions respectively. Samples were analyzed during both the aerobic and anaerobic phases of the experiment. For the aerobic phase, the soil was maintained aerobically in the dark at 20 ± 2°C until 30 days had passed. The anaerobic phase was initiated by adding an aliquot of nitrogen-purged water to each sample container. The soil was maintained anaerobically in the dark at 20 ± 2°C for 90 days post-flooding. Air leaving the system was passed through an ethylene glycol trap and two aqueous solutions of NaOH (1N) in order to collect any volatiles produced.
During the aerobic phase of the experiment, duplicate samples (rep1 and rep2) were analyzed at 0, 7, 11, and 30 days after treatment (DAT). After the anaerobic phase of the experiment was initiated, duplicate samples (rep1 and rep2) were analyzed at 2, 7, 14, 30, 61, and 90 days after flooding. The soil samples were extracted six times with acetonitrile and mixtures of acetonitrile and water. Aliquots were analyzed by LSC in triplicate. The amount of CO_2 and other volatiles produced was determined at each sampling time (excluding 0 DAT). The traps were assayed directly by adding aliquots of the trapping solutions into scintillation cocktail and counting by liquid scintillation counting. The solutions in the traps were replaced at each sampling interval.

In Li10 soil, the mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 101.72–82.20% of the TAR. The extractable radioactive residue (ERR) from soil decreased from 99.73% TAR at 0 DAT to 78.02% TAR at 120 DAT. The non-extractable [^{14}C]-residues (NER) increased from 0.27% TAR at 0 DAT to 8.39% TAR at 120 DAT. The recovered volatile radioactivity reached a maximum of 0.41% TAR at 120 DAT.

In LUFA 5M soil, the mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 109.68-92.97% of the TAR. The extractable radioactive residue (ERR) from soil decreased from 98.93% TAR at 0 DAT to 93.26% TAR at 120 DAT. The non-extractable [^{14}C]-residues (NER) increased from 1.07% TAR at 0 DAT to 13.98% TAR at 120 DAT. The recovered volatile radioactivity reached a maximum of 0.38% TAR at 120 DAT.

In IN soil, the mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 108.19–90.32% of the TAR. The extractable radioactive residue (ERR) from soil decreased from 99.75% TAR at 0 DAT to 80.47% TAR at 120 DAT. The non-extractable [^{14}C]-residues (NER) increased from 0.25% TAR at 0 DAT to 9.49% TAR at 120 DAT. The recovered volatile radioactivity reached a maximum of 0.35% TAR at 120 DAT.

In NJ soil, the mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 107.49-83.86% of the TAR. The extractable radioactive residue (ERR) from soil decreased from 99.64% TAR at 0 DAT to 91.72% TAR at 120 DAT. The non-extractable [^{14}C]-residues (NER) increased from 0.36% TAR at 0 DAT to 15.51% TAR at 120 DAT. The recovered volatile radioactivity reached a maximum of 0.27% TAR at 120 DAT.

In NJ soil, the mean total radioactivity recovered (material balance) from [chlorophenyl-U-^{14}C]-BAS 750 F ranged from 102.82–96.48% of the TAR. The extractable radioactive residue (ERR) from soil decreased from 99.21% TAR at 0 DAT to 82.95% TAR at 120 DAT. The non-extractable [^{14}C]-residues (NER) increased from 0.79% TAR at 0 DAT to 16.37% TAR at 120 DAT. The recovered volatile radioactivity reached a maximum of 2.16% TAR at 120 DAT.

At all sampling points, the parent compound was the major residue detected by HPLC analysis, for all soil types and labels.

The data generated in this study was analyzed in accordance with FOCUS kinetic guidance to determine DT_{50}, DT_{75}, and DT_{90} trigger endpoints for BAS 750 F. A SFO (single first-order) model was used for the fittings. The calculated DT_{50} values in three soils ranged from 349 to 899 days. No reliable DT_{90} values could be determined. In one soil, LUFA 5M, no discernible decline was detected, and no kinetic endpoints were calculated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in two ^{14}C-labeled forms.

Internal code:	BAS 750 F
Chemical name (IUPAC):	(2*RS*)-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

1. Chlorophenyl-U-^{14}C-label

Batch No.:	CFQ41561
Specific radioactivity of a.s.:	7.878 MBq mg^{-1}
Radiochemical purity:	98.9
Purity:	99.1%

2. Triazole-3(5)-^{14}C-label

Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%
Purity:	98.9%

Unlabeled

Internal code:	BAS 750 F
Reg. No.:	5834378
Common name:	NA
Batch No.:	L84-238
Purity:	99.7%

2. Soil

The soils used (Li10, LUFA 5M, IN, NJ) were a loamy fine sand, sandy loam, loam and a loam soil respectively (according to the USDA Texture Class). After sampling from the field, the soil was allowed to dry at room temperature and then sieved though a 2 mm sieve before use. Prior to dosing, the moisture of the soil was adjusted to approximately 50% of the MWHC by adding the appropriate amount of deionized water. The soil moisture was determined as the difference between the weights of fresh soil and dried soil (after drying in an oven at ~110°C) divided by dried weights. The soil characteristics are summarized in Table 7.1.1.2-1.

Table 7.1.1.2-1: Physicochemical Characteristics of Test Soils

	Li10 Soil	LUFA 5M Soil	IN Soil	NJ Soil
USDA Texture Class	loamy fine sand	sandy loam	loam	loam
Sand (%)	83.7	62.6	41	35
Silt (%)	11.3	27.3	40	42
Clay (%)	5.0	10.2	19	23
pH (water)	6.9	8.0	5.6	6.9
pH ($CaCl_2$)	6.1	7.2	5.6	6.6
Total Organic Matter (%)	1.64[a]	1.55[a]	2.1	2.0
Total Organic Carbon (%)	0.95	0.90	1.2	1.1
Soil Biomass-0 DAT [μg g^{-1} dry soil]	244	408	190.8	246.3
Soil Biomass- 30 DAT (μg g^{-1} dry soil)	381.3	475.3	462.8	479.4
Cation Exchange Capacity [$cmol^+$ kg^{-1}] or meq/100g]	5.7	10.9	11.4	9.3
Maximum Water Holding Capacity [g 100 g^{-1} dry soil]	25.8	28.9	42.3	37.0
Bulk Density [g L^{-1}]	1353	1235	1120	1060

B. STUDY DESIGN

1. Experimental conditions

The proposed maximum single application rate for ^{14}C-BAS 750 F is 150 g a.s. ha^{-1}. Assuming that the applied ^{14}C-BAS 750 F distributes evenly into a soil depth of 0-2.5 cm and the bulk density of the soil is 1 g cm^{-3}, the concentration of BAS 750 F in soil will be 0.6 ppm. For the kinetic studies, ^{14}C-BAS 750 F was applied at a rate of 0.6 μg g^{-1} of dry soil (0.6 ppm). The application of the test solution to the soil samples was performed by applying the dosing solution (either 33 μL of App0001 or 72 μL of App0002) drop-wise to each test vessel on the soil surface using an automatic pipette. After dosing, each test vessel was gently shaken by hand to incorporate the test substance into the soil and was replaced in the flow-through system. The final concentration of BAS 750 F was approximately 0.6 ppm. High dosed vessels were treated at a higher rate (5-fold application rate). High dose vessels were treated with 157 μL of App0003 or 360 μL of App002 by drop-wise addition with an automatic pipette.

2. Sampling

The aerobic samples were sampled on 0, 7, 11, and 30 DAT. The anaerobic samples were sampled on 2, 7, 14, 30, 61, and 90 DAF.

3. Description of analytical procedures

Immediately after removing from the incubator system, the soil samples were extracted twice with 100 mL of each of the following solvents: ACN, ACN:water (8:2), and ACN:water (1:1) by shaking for 30 minutes at 300 rpm followed by centrifugation for 15 minutes at 3000 rpm. The supernatant was decanted into a graduated cylinder, and the volume was recorded and an aliquot was assayed by LSC. All of the extracts were combined, and an aliquot (~15 mL) was concentrated to ~1 mL. The pooled extracts were analyzed by LSC and by HPLC.
For the anaerobic phase of the study (post-flooding), the soil samples were first centrifuged for 15 minutes at 3000 rpm and the water was decanted into a graduated cylinder and the volume recorded before an aliquot was counted by LSC. Samples were then treated to the same extraction procedure outlined above. The water layer was pooled with the rest of the extracts.
The aqueous sodium hydroxide and ethylene glycol traps were assayed at all sampling times (except for 0 DAT) by directly adding aliquots of the trapping solution into liquid scintillation cocktail and counting by liquid scintillation counting (LSC). The traps were replaced with fresh aqueous sodium hydroxide (1N), and ethylene glycol, as appropriate, at each sampling time.

4. Calculation of the degradation rate

The data generated in this study was analyzed in accordance with FOCUS kinetic guidance to determine DT_{50}, DT_{75}, and DT_{90} trigger endpoints for BAS 750 F. A SFO (single first-order) model was used for the fittings.

II. RESULTS AND DISCUSSION

A. TEST CONDITIONS

On each sampling day after flooding, the pH, redox, and dissolved oxygen values were measured. Slight variations in pH readings were detected, but no clear trends were evident. The concentration of dissolved oxygen in the water layer over all soils was less than 1 mg L^{-1} by 7 DAF, and continued to decrease, indicating that the system was anaerobic for the majority of the study. The redox potential was negative for all soils after 30 day of flooding, and by the next sampling point (61 DAF) the water layer also had a negative redox potential.

B. MASS BALANCE

Li10 soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-2.
Average material balance ranged from 82.20% TAR at 11 DAT to 101.72% TAR at 32 DAT, displaying no clear trends between length of time after dosing and total recovery. The lower (< 90% TAR) recovery for two sampling points are not indicative of an overall trend or deficiency of the method, but only reflect small, isolated instances in the analytical procedure for the individual time points. This is not expected to have any impact on the overall study.

LUFA 5M soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-3.
The material balance of [triazole-3(5)-^{14}C]-BAS 750 F ranged from 92.97 to 109.68% of the TAR.

IN soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-4.
The mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 90.32 at 120 DAT to 108.19% at 7 DAT.

NJ soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-5.
The mean total radioactivity recovered (material balance) from [triazole-3(5)-^{14}C]-BAS 750 F ranged from 83.86 to 107.49% of the TAR. The lower (< 90% TAR) recovery for two sampling points are not indicative of an overall trend, but only reflect small, isolated mistakes in the analytical procedure for the individual time points. This is not expected to have any impact on the overall study.

NJ soil treated with [chlorophenyl-U-^{14}C]-BAS 750 F: See Table 7.1.1.2-6.
The mean total radioactivity recovered (material balance) from [chlorophenyl-U-^{14}C]-BAS 750 F ranged from 96.48 to 102.82% of the TAR.

Table 7.1.1.2-2: Material Balance of [Triazole-3(5)-^{14}C]-BAS 750 F in Li10 Soil [%TAR]

DAT	DAF	Extracts							ERR	NER	Volatiles			Material Balance
		Water	1	2	3	4	5	6			NaOH	EG	Total	
0 rep1	NA	NA	84.23	10.05	4.16	0.69	0.22	0.07	99.42	0.31	NA	NA	NA	99.73
0 rep2	NA	NA	85.10	9.62	4.24	0.75	0.24	0.09	100.04	0.24	NA	NA	NA	100.27
0 mean	**NA**	NA	**84.66**	**9.84**	**4.20**	**0.72**	**0.23**	**0.08**	**99.73**	**0.27**	**NA**	**NA**	**NA**	**100.00**
7 rep1	NA	NA	76.23	10.47	4.61	1.05	0.63	0.24	93.23	1.76	0 26	<LOQ	0 26	95.26
7 rep2	NA	NA	76.03	11.47	5.47	1.29	0.68	0.25	95.19	1.98	0 26	<LOQ	0 26	97.43
7 mean	NA	NA	**76.13**	**10.97**	**5.04**	**1.17**	**0.66**	**0.25**	**94.21**	**1.87**	**0 26**	**<LOQ**	**0 26**	**96.35**
11 rep1	NA	NA	63.07	10.81	4.25	0.96	0.37	0.24	79.71	2.25	0 30	<LOQ	0 30	82.26
11 rep2	NA	NA	65.13	9.02	4.16	0.94	0.38	0.22	79.84	2.00	0 30	<LOQ	0 30	82.15
11 mean	NA	NA	**64.10**	**9.92**	**4.20**	**0.95**	**0.38**	**0.23**	**79.78**	**2.13**	**0 30**	**<LOQ**	**0 30**	**82.20**
30 rep1	NA	NA	23.46	10.51	55.77	10.31	1.62	0.44	102.10	3.39	0 35	<LOQ	0 35	105.84
30 rep2	NA	NA	51.52	8.21	26.64	4.40	0.88	0.31	91.96	2.76	0 35	<LOQ	0 35	95.08
30 mean	NA	NA	**37.49**	**9.36**	**41.20**	**7.35**	**1.25**	**0.38**	**97.03**	**3.08**	**0 35**	**<LOQ**	**0 35**	**100.46**
32 rep1	2 rep1	3.01	79.72	10.85	2.58	0.67	0.33	0.22	97.38	3.22	0 37	<LOQ	0 37	100.96
32 rep2	2 rep2	10.85	72.59	10.91	2.55	0.71	0.35	0.26	98.21	3.90	0 37	<LOQ	0 37	102.48
32 mean	**2 mean**	**6.93**	**76.15**	**10.88**	**2.57**	**0.69**	**0.34**	**0.24**	**97.80**	**3.56**	**0 37**	**<LOQ**	**0 37**	**101.72**
37 rep1	7 rep1	4.39	75.77	10.65	2.82	0.93	0.49	0.33	95.37	4.67	0 38	<LOQ	0 38	100.42
37 rep2	7 rep2	8.06	58.56	8.82	2.36	0.74	0.43	0.28	79.24	4.38	0 38	<LOQ	0 38	84.00
37 mean	**7 mean**	**6.22**	**67.16**	**9.73**	**2.59**	**0.84**	**0.46**	**0.30**	**87.31**	**4.53**	**0 38**	**<LOQ**	**0 38**	**92.21**
44 rep1	14 rep1	5.63	73.77	11.67	3.38	1.08	0.51	0.35	96.39	5.94	0 38	<LOQ	0 38	102.71
44 rep2	14 rep2	12.01	64.35	10.60	3.14	0.95	0.53	0.34	91.91	4.46	0 38	<LOQ	0 38	96.75
44 mean	**14 mean**	**8.82**	**69.06**	**11.13**	**3.26**	**1.02**	**0.52**	**0.34**	**94.15**	**5.20**	**0 38**	**<LOQ**	**0 38**	**99.73**
60 rep1	**30 rep1**	6.26	64.32	11.16	3.39	1.07	0.56	0.38	87.14	7.05	0 39	<LOQ	0 39	94.59
60 rep2	30 rep2	10.35	65.42	11.43	3.22	0.92	0.51	0.33	92.19	4.06	0 39	<LOQ	0 39	96.64
60 mean	**30 mean**	**8.30**	**64.87**	**11.30**	**3.31**	**1.00**	**0.54**	**0.36**	**89.67**	**5.56**	**0 39**	**<LOQ**	**0 39**	**95.62**
91 rep1	61 rep1	3.22	64.37	11.31	3.70	1.27	0.67	0.55	85.09	9.87	0.40	<LOQ	0.40	95.37
91 rep2	61 rep2	6.00	66.04	11.55	3.98	1.30	0.76	0.56	90.19	9.38	0.40	<LOQ	0.40	99.97
91 mean	**61 mean**	**4.61**	**65.21**	**11.43**	**3.84**	**1.29**	**0.71**	**0.55**	**87.64**	**9.62**	**0.40**	**<LOQ**	**0.40**	**97.67**
120 rep1	90 rep1	2.82	64.16	11.34	3.61	1.13	0.67	0.45	84.18	8.81	0.41	<LOQ	0.41	93.40
120 rep2	90 rep2	4.17	52.41	9.84	3.29	1.10	0.64	0.42	71.87	7.97	0.41	<LOQ	0.41	80.25
120 mean	**90 mean**	**3.49**	**58.29**	**10.59**	**3.45**	**1.11**	**0.65**	**0.44**	**78.02**	**8.39**	**0.41**	**<LOQ**	**0.41**	**86.83**

Extract 1 = ACN
Extract 2 = ACN
Extract 3 = ACN:water (8:2)
Extract 4 = ACN:water (8:2)
Extract 5 = ACN:water (1:1)
Extract 6 = ACN:water (1:1)
EG = Ethylene glycol
ERR= Extractable Radioactive Residues
NER = Non Extractable Residues (by combustion)
NA = Not Applicable (no sample analyzed)
LOQ = 0.06% TAR

Table 7.1.1.2-3: Material Balance of [Triazole-3(5)-^{14}C]-BAS 750 F in LUFA 5M Soil [%TAR]

DAT	DAF	Extracts							ERR	NER	Volatiles			Material Balance
		Water	1	2	3	4	5	6			NaOH	EG	Total	
0 rep1	NA	NA	85.10	13.71	6.20	1.28	0.55	0.29	107.15	1.15	NA	NA	NA	108.30
0 rep2	NA	NA	70.51	12.66	5.58	1.23	0.51	0.22	90.70	0.99	NA	NA	NA	91.70
0 mean	NA	**NA**	**77.81**	**13.19**	**5.89**	**1.26**	**0.53**	**0.26**	**98.93**	**1.07**	**NA**	**NA**	**NA**	**100.00**
7 rep1	NA	NA	79.42	12.34	7.10	1.85	1.14	0.52	102.37	4.00	0.27	<LOQ	0.27	106.64
7 rep2	NA	NA	80.94	12.20	7.23	1.81	1.04	0.50	103.71	4.09	0.27	<LOQ	0.27	108.07
7 mean	NA	**NA**	**80.18**	**12.27**	**7.17**	**1.83**	**1.09**	**0.51**	**103.04**	**4.04**	**0.27**	**<LOQ**	**0.27**	**107.36**
11 rep1	NA	NA	73.20	11.07	6.59	1.69	0.72	0.48	93.75	4.27	0.30	<LOQ	0.30	98.33
11 rep2	NA	NA	65.50	10.15	6.11	1.77	0.72	0.47	84.72	4.41	0.30	<LOQ	0.30	89.43
11 mean	NA	**NA**	**69.35**	**10.61**	**6.35**	**1.73**	**0.72**	**0.47**	**89.23**	**4.34**	**0.30**	**<LOQ**	**0.30**	**93.88**
30 rep1	NA	NA	81.04	12.93	7.16	2.23	0.83	0.47	104.66	5.49	0.33	<LOQ	0.33	110.48
30 rep2	NA	NA	76.02	14.67	7.87	2.49	0.80	0.48	102.34	6.04	0.33	<LOQ	0.33	108.71
30 mean	NA	**NA**	**78.53**	**13.80**	**7.52**	**2.36**	**0.81**	**0.47**	**103.50**	**5.77**	**0.33**	**<LOQ**	**0.33**	**109.60**
32 rep1	2 rep1	4.52	78.80	13.21	4.29	1.45	0.84	0.58	103.67	6.84	0.33	<LOQ	0.33	110.85
32 rep2	2 rep2	4.80	70.48	11.80	3.87	1.27	0.71	0.47	93.40	5.89	0.33	<LOQ	0.33	99.62
32 mean	**2 mean**	**4.66**	**74.64**	**12.50**	**4.08**	**1.36**	**0.77**	**0.52**	**98.54**	**6.37**	**0.33**	**<LOQ**	**0.33**	**105.24**
37 rep1	7 rep1	5.91	73.00	11.98	3.69	1.33	0.87	0.59	97.36	7.11	0.34	<LOQ	0.34	104.81
37 rep2	7 rep2	4.79	59.94	9.95	3.22	1.16	0.72	0.52	80.30	6.07	0.34	<LOQ	0.34	86.71
37 mean	**7 mean**	**5.35**	**66.47**	**10.97**	**3.46**	**1.25**	**0.79**	**0.56**	**88.83**	**6.58**	**0.34**	**<LOQ**	**0.34**	**95.75**
44 rep1	14 rep1	4.23	60.86	11.47	3.97	1.42	0.76	0.52	83.23	6.87	0.34	<LOQ	0.34	90.44
44 rep2	14 rep2	3.80	65.12	11.92	4.28	1.51	0.82	0.59	88.04	7.13	0.34	<LOQ	0.34	95.51
44 mean	**14 mean**	**4.01**	**62.99**	**11.69**	**4.13**	**1.46**	**0.79**	**0.55**	**85.63**	**7.00**	**0.34**	**<LOQ**	**0.34**	**92.97**
60 rep1	30 rep1	4.19	68.90	14.54	5.08	1.81	1.07	0.77	96.36	9.78	0.35	<LOQ	0.35	106.49
60 rep2	30 rep2	5.37	65.92	15.65	5.02	1.71	1.05	0.78	95.50	9.55	0.35	<LOQ	0.35	105.40
60 mean	**30 mean**	**4.78**	**67.41**	**15.10**	**5.05**	**1.76**	**1.06**	**0.77**	**95.93**	**9.66**	**0.35**	**<LOQ**	**0.35**	**105.95**
91 rep1	61 rep1	3.18	69.27	14.51	5.33	1.93	1.15	0.90	96.26	13.51	0.37	<LOQ	0.37	110.15
91 rep2	61 rep2	3.49	67.14	14.63	5.34	1.99	1.20	0.94	94.73	14.11	0.37	<LOQ	0.37	109.21
91 mean	**61 mean**	**3.34**	**68.20**	**14.57**	**5.33**	**1.96**	**1.18**	**0.92**	**95.50**	**13.81**	**0.37**	**<LOQ**	**0.37**	**109.68**
120 rep1	90 rep1	4.49	66.52	13.03	5.01	1.99	1.13	0.85	93.03	13.81	0.38	<LOQ	0.38	107.22
120 rep2	90 rep2	4.73	63.78	14.48	5.87	2.22	1.45	0.98	93.50	14.15	0.38	<LOQ	0.38	108.03
120 mean	**90 mean**	**4.61**	**65.15**	**13.75**	**5.44**	**2.11**	**1.29**	**0.92**	**93.26**	**13.98**	**0.38**	**<LOQ**	**0.38**	**107.63**

Extract 1 = ACN
Extract 2 = ACN
Extract 3 = ACN:water (8:2)
Extract 4 = ACN:water (8:2)
Extract 5 = ACN:water (1:1)
Extract 6 = ACN:water (1:1)

EG = Ethylene glycol
ERR= Extractable Radioactive Residues
NER = Non Extractable Residues (by combustion)
NA = Not Applicable (no sample analyzed)
LOQ = 0.06% TAR

Table 7.1.1.2-4: Material Balance of [Triazole-3(5)-^{14}C]-BAS 750 F in IN Soil [%TAR]

DAT	DAF	Extracts							ERR	NER	Volatiles			Material Balance
		Water	1	2	3	4	5	6			NaOH	EG	Total	
0 rep1	**NA**	NA	81.70	11.74	3.51	0.66	0.21	0.09	97.92	0 23	NA	NA	NA	98.15
0 rep2	NA	NA	86.27	10.81	3.47	0.70	0.23	0.09	101 57	0 28	NA	NA	NA	101.85
0 mean	NA	**NA**	**83.99**	**11.28**	**3.49**	**0.68**	**0.22**	**0.09**	**99.75**	**0 25**	**NA**	**NA**	**NA**	**100.00**
7 rep1	NA	NA	84.84	14.48	4.86	1.52	1.27	0.54	107 51	2.78	0 23	<LOQ	0.23	110.52
7 rep2	NA	NA	80.55	13.58	5.07	1.68	1.39	0.50	102.77	2.85	0 23	<LOQ	0.23	105.86
7 mean	NA	**NA**	**82.69**	**14.03**	**4.97**	**1.60**	**1.33**	**0.52**	**105 14**	**2 82**	**0 23**	**<LOQ**	**0.23**	**108.19**
11 rep1	NA	NA	76.30	13.25	5.05	1.84	0.83	0.52	97.79	1.83	0 26	<LOQ	0.26	99.88
11 rep2	NA	NA	66.00	11.51	4.23	1.41	0.72	0.47	84.36	3.64	0 26	<LOQ	0.26	88.25
11 mean	NA	**NA**	**71.15**	**12.38**	**4.64**	**1.63**	**0.78**	**0.50**	**91.07**	**2.73**	**0 26**	**<LOQ**	**0.26**	**94.06**
30 rep1	NA	NA	75.03	12.95	4.86	2.28	0.88	0.52	96.52	5.81	0 30	<LOQ	0.30	102.63
30 rep2	NA	NA	68.13	11.44	4.34	1.99	0.76	0.47	87.12	4.86	0 30	<LOQ	0.30	92.28
30 mean	NA	**NA**	**71.58**	**12.19**	**4.60**	**2.13**	**0.82**	**0.49**	**91.82**	**5 33**	**0 30**	**<LOQ**	**0.30**	**97.45**
32 rep1	2 rep1	6.11	73.51	13.39	4.44	1.59	0.94	0.64	100.63	6.84	0 30	<LOQ	0.30	107.77
32 rep2	2 rep2	6.34	69.49	12.19	4.07	1.52	0.86	0.62	95.09	6 50	0 30	<LOQ	0.30	101.89
32 mean	**2 mean**	**6.23**	**71.50**	**12.79**	**4.26**	**1.56**	**0.90**	**0.63**	**97.86**	**6.67**	**0 30**	**<LOQ**	**0.30**	**104.83**
37 rep1	7 rep1	5.99	68.31	11.71	3.84	1.49	0.84	0.57	92.75	6.09	0 30	<LOQ	0.30	99.14
37 rep2	7 rep2	8.31	71.41	13.20	4.14	1.50	0.91	0.61	100.09	7 15	0 30	<LOQ	0.30	107.54
37 mean	**7 mean**	**7.15**	**69.86**	**12.46**	**3.99**	**1.49**	**0.88**	**0.59**	**96.42**	**6.62**	**0 30**	**<LOQ**	**0.30**	**103.34**
44 rep1	14 rep1	9.05	65.90	13.04	4.77	1.80	0.96	0.71	96.22	7.63	0 31	<LOQ	0.31	104.16
44 rep2	14 rep2	11.83	65.82	12.95	4.55	1.76	1.01	0.72	98.63	7 96	0 31	<LOQ	0.31	106.90
44 mean	**14 mean**	**10.44**	**65.86**	**12.99**	**4.66**	**1.78**	**0.98**	**0.72**	**97.42**	**7.79**	**0 31**	**<LOQ**	**0.31**	**105.53**
60 rep1	30 rep1	7.23	64.19	13.23	5.13	1.82	1.02	0.73	93.34	8 36	0 32	<LOQ	0.32	102.02
60 rep2	30 rep2	6.90	47.97	20.19	5.41	1.60	0.88	0.63	83.58	7 33	0 32	<LOQ	0.32	91.22
60 mean	**30 mean**	**7.06**	**56.08**	**16.71**	**5.27**	**1.71**	**0.95**	**0.68**	**88.46**	**7 84**	**0 32**	**<LOQ**	**0.32**	**96.62**
91 rep1	61 rep1	5.63	62.10	13.42	5.42	2.10	1.27	0.96	90.90	11.43	0 34	<LOQ	0.34	102.67
91 rep2	61 rep2	6.60	59.24	12.98	4.87	1.84	1.11	0.82	87.46	9.84	0 34	<LOQ	0.34	97.63
91 mean	**61 mean**	**6.11**	**60.67**	**13.20**	**5.14**	**1.97**	**1.19**	**0.89**	**89.18**	**10.64**	**0 34**	**<LOQ**	**0.34**	**100.15**
120 rep1	90 rep1	4.12	59.18	11.55	4.27	1.82	1.06	0.81	82.81	9.86	0 35	<LOQ	0.35	93.03
120 rep2	90 rep2	5.35	53.01	11.93	4.29	1.74	1.07	0.75	78.13	9 12	0 35	<LOQ	0.35	87.60
120 mean	**90 mean**	**4.74**	**56.09**	**11.74**	**4.28**	**1.78**	**1.07**	**0.78**	**80.47**	**9.49**	**0 35**	**<LOQ**	**0.35**	**90.32**

Extract 1 = ACN
Extract 2 = ACN
Extract 3 = ACN:water (8:2)
Extract 4 = ACN:water (8:2)
Extract 5 = ACN:water (1:1)
NA = Not Applicable (no sample analyzed)
Extract 6 = ACN:water (1:1)
EG = Ethylene glycol
ERR= Extractable Radioactive Residues
NER = Non Extractable Residues (by combustion)
LOQ = 0.06% TAR

Table 7.1.1.2-5: Material Balance of [Triazole-3(5)-^{14}C]-BAS 750 F in NJ Soil [%TAR]

DAT	DAF	Extracts							ERR	NER	Volatiles			Material Balance
		Water	1	2	3	4	5	6			NaOH	EG	Total	
0 rep1	NA	NA	88.12	13.84	3.98	0.80	0.33	0.13	107.20	0.39	NA	NA	NA	107.59
0 rep2	NA	NA	74.30	12.83	3.72	0.78	0.30	0.14	92.08	0.33	NA	NA	NA	92.41
0 mean	**NA**	**NA**	**81.21**	**13.33**	**3.85**	**0.79**	**0.32**	**0.14**	**99.64**	**0.36**	**NA**	**NA**	**NA**	**100.00**
7 rep1	NA	NA	82.57	14.60	5.13	1.55	1.51	0.52	105.89	3.60	0.13	<LOQ	0.13	109.62
7 rep2	NA	NA	69.92	12.31	4.31	1.44	1.32	0.59	89.90	3.60	0.13	<LOQ	0.13	93.63
7 mean	**NA**	**NA**	**76.25**	**13.46**	**4.72**	**1.50**	**1.42**	**0.55**	**97.90**	**3.60**	**0.13**	**<LOQ**	**0.13**	**101.63**
11 rep1	NA	NA	75.20	13.93	5.06	1.59	0.85	0.59	97.21	5.36	0.16	<LOQ	0.16	102.74
11 rep2	NA	NA	77.50	14.29	5.21	1.80	0.78	0.58	100.17	5.32	0.16	<LOQ	0.16	105.65
11 mean	**NA**	**NA**	**76.35**	**14.11**	**5.14**	**1.69**	**0.82**	**0.58**	**98.69**	**5.34**	**0.16**	**<LOQ**	**0.16**	**104.19**
30 rep1	NA	NA	69.43	13.53	5.20	2.38	0.97	0.58	92.09	7.23	0.22	<LOQ	0.22	99.55
30 rep2	NA	NA	70.11	12.96	4.61	2.20	0.88	0.57	91.31	7.30	0.22	<LOQ	0.22	98.83
30 mean	**NA**	**NA**	**69.77**	**13.24**	**4.90**	**2.29**	**0.93**	**0.57**	**91.70**	**7.27**	**0.22**	**<LOQ**	**0.22**	**99.19**
32 rep1	2 rep1	8.04	63.68	11.66	3.40	1.40	0.83	0.57	89.57	8.46	0.23	<LOQ	0.23	98.26
32 rep2	2 rep2	10.19	70.59	12.63	3.68	1.55	0.94	0.67	100.25	9.37	0.23	<LOQ	0.23	109.84
32 mean	**2 mean**	**9.11**	**67.13**	**12.14**	**3.54**	**1.48**	**0.89**	**0.62**	**94.91**	**8.91**	**0.23**	**<LOQ**	**0.23**	**104.05**
37 rep1	7 rep1	10.94	69.45	13.27	3.81	1.47	1.01	0.72	100.66	8.40	0.23	<LOQ	0.23	109.29
37 rep2	7 rep2	7.36	55.40	10.10	3.20	1.35	0.83	0.59	78.84	11.21	0.23	<LOQ	0.23	90.28
37 mean	**7 mean**	**9.15**	**62.42**	**11.68**	**3.51**	**1.41**	**0.92**	**0.65**	**89.75**	**9.81**	**0.23**	**<LOQ**	**0.23**	**99.79**
44 rep1	14 rep1	12.70	58.62	12.84	4.28	1.78	1.09	0.80	92.10	10.55	0.23	<LOQ	0.23	102.88
44 rep2	14 rep2	11.15	64.23	13.78	4.57	2.01	1.09	0.80	97.63	10.11	0.23	<LOQ	0.23	107.97
44 mean	**14 mean**	**11.92**	**61.42**	**13.31**	**4.42**	**1.90**	**1.09**	**0.80**	**94.86**	**10.33**	**0.23**	**<LOQ**	**0.23**	**105.43**
60 rep1	30 rep1	6.92	53.00	11.40	3.83	1.57	1.03	0.76	78.52	9.38	0.24	<LOQ	0.24	88.14
60 rep2	30 rep2	6.97	46.38	10.35	3.68	1.56	0.96	0.69	70.59	8.74	0.24	<LOQ	0.24	79.57
60 mean	**30 mean**	**6.94**	**49.69**	**10.87**	**3.76**	**1.57**	**0.99**	**0.73**	**74.56**	**9.06**	**0.24**	**<LOQ**	**0.24**	**83.86**
91 rep1	61 rep1	5.57	60.16	13.57	5.28	2.50	1.51	1.19	89.78	16.65	0.26	<LOQ	0.26	106.69
91 rep2	61 rep2	5.58	52.69	11.85	4.64	2.15	1.38	1.05	79.35	14.51	0.26	<LOQ	0.26	94.13
91 mean	**61 mean**	**5.58**	**56.42**	**12.71**	**4.96**	**2.32**	**1.45**	**1.12**	**84.57**	**15.58**	**0.26**	**<LOQ**	**0.26**	**100.41**
120 rep1	90 rep1	6.42	61.42	13.77	5.05	2.39	1.56	1.09	91.70	15.21	0.27	<LOQ	0.27	107.17
120 rep2	90 rep2	7.41	59.53	14.22	5.44	2.44	1.60	1.09	91.74	15.81	0.27	<LOQ	0.27	107.82
120 mean	**90 mean**	**6.91**	**60.48**	**14.00**	**5.25**	**2.42**	**1.58**	**1.09**	**91.72**	**15.51**	**0.27**	**<LOQ**	**0.27**	**107.49**

Extract 1 = ACN
Extract 2 = ACN
Extract 3 = ACN:water (8:2)
Extract 4 = ACN:water (8:2)
Extract 5 = ACN:water (1:1)
Extract 6 = ACN:water (1:1)
EG = Ethylene glycol
ERR= Extractable Radioactive Residues
NER = Non Extractable Residues (by combustion)
LOQ = 0.06% TAR
NA = Not Applicable (no sample analyzed)

Table 7.1.1.2-6: Material Balance of [Chlorophenyl-U-^{14}C]-BAS 750 F in NJ Soil [%TAR]

DAT	DAF	Extracts							ERR	NER	Volatiles			Material Balance
		Water	1	2	3	4	5	6			NaOH	EG	Total	
0 rep1	NA	NA	80.31	14.38	3.95	0.85	0.42	0.21	100.12	0.74	NA	NA	NA	100.86
0 rep2	NA	NA	79.59	13.31	3.88	0.85	0.41	0.26	98.30	0.84	NA	NA	NA	99.14
0 mean	**NA**	**NA**	**79.95**	**13.84**	**3.91**	**0.85**	**0.41**	**0.24**	**99.21**	**0.79**	**NA**	**NA**	**NA**	**100.00**
7 rep1	NA	NA	74.89	13.84	4.89	1.56	1.65	0.62	97.45	4 91	0.80	<LOQ	0.80	103.16
7 rep2	NA	NA	69.90	12.68	4.43	1.47	1.55	0.60	90.63	4.77	0.80	<LOQ	0.80	96.20
7 mean	**NA**	**NA**	**72.39**	**13.26**	**4.66**	**1.52**	**1.60**	**0.61**	**94.04**	**4 84**	**0 80**	**<LOQ**	**0.80**	**99.68**
11 rep1	NA	NA	71.55	13.37	4.86	1.77	0.78	0.60	92.93	6 18	0 97	<LOQ	0.97	100.08
11 rep2	NA	NA	69.23	13.27	4.58	1.62	0.70	0.56	89.95	5.84	0 97	<LOQ	0.97	96.77
11 mean	**NA**	**NA**	**70.39**	**13.32**	**4.72**	**1.69**	**0.74**	**0.58**	**91.44**	**6.01**	**0 97**	**<LOQ**	**0.97**	**98.42**
30 rep1	NA	NA	72.88	12.51	5.09	2.43	0.97	0.60	94.48	8 39	1 59	<LOQ	1.59	104.46
30 rep2	NA	NA	70.33	12.29	4.85	2.31	0.99	0.55	91.32	8 27	1 59	<LOQ	1.59	101.18
30 mean	**NA**	**NA**	**71.60**	**12.40**	**4.97**	**2.37**	**0.98**	**0.57**	**92.90**	**8 33**	**1 59**	**<LOQ**	**1.59**	**102.82**
32 rep1	2 rep1	7.36	59.84	10.89	3.18	1.36	0.82	0.56	84.01	9.08	1.62	<LOQ	1.62	94.71
32 rep2	2 rep2	6.67	63.97	11.04	3.33	1.41	0.84	0.58	87.83	9 27	1.62	<LOQ	1.62	98.72
32 mean	**2 mean**	**7.02**	**61.91**	**10.97**	**3.25**	**1.38**	**0.83**	**0.57**	**85.92**	**9 17**	**1.62**	**<LOQ**	**1.62**	**96.72**
37 rep1	7 rep1	9.97	55.50	10.45	3.29	1.38	0.88	0.61	82.08	8 37	1.64	<LOQ	1.64	92.09
37 rep2	7 rep2	6.98	64.96	11.66	3.45	1.41	0.89	0.63	89.97	9 26	1.64	<LOQ	1.64	100.87
37 mean	**7 mean**	**8.47**	**60.23**	**11.05**	**3.37**	**1.40**	**0.88**	**0.62**	**86.03**	**8 81**	**1.64**	**<LOQ**	**1.64**	**96.48**
44 rep1	14 rep1	9.24	57.82	11.84	4.02	1.63	0.99	0.72	86.25	9 97	1.69	<LOQ	1.69	97.91
44 rep2	14 rep2	8.45	59.79	11.93	4.16	1.77	1.07	0.78	87.96	10.60	1.69	<LOQ	1.69	100.25
44 mean	**14 mean**	**8.85**	**58.81**	**11.89**	**4.09**	**1.70**	**1.03**	**0.75**	**87.10**	**10.29**	**1.69**	**<LOQ**	**1.69**	**99.08**
60 rep1	30 rep1	6.82	59.90	13.24	4.72	1.92	1.18	0.83	88.62	11.66	1.83	<LOQ	1.83	102.11
60 rep2	30 rep2	6.72	58.82	12.94	4.58	1.98	1.25	0.85	87.14	11.63	1.83	<LOQ	1.83	100.60
60 mean	**30 mean**	**6.77**	**59.36**	**13.09**	**4.65**	**1.95**	**1.22**	**0.84**	**87.88**	**11.65**	**1 83**	**<LOQ**	**1.83**	**101.36**
91 rep1	61 rep1	4.73	53.45	12.40	4.69	2.01	1.27	0.98	79.54	14.98	1 98	<LOQ	1.98	96.51
91 rep2	61 rep2	5.24	56.81	12.51	4.97	2.25	1.40	1.09	84.28	15.34	1 98	<LOQ	1.98	101.61
91 mean	**61 mean**	**4.99**	**55.13**	**12.45**	**4.83**	**2.13**	**1.34**	**1.04**	**81.91**	**15.16**	**1 98**	**<LOQ**	**1.98**	**99.06**
120 rep1	90 rep1	4.18	57.17	12.46	4.70	2.16	1.37	1.05	83.08	16.67	2 16	<LOQ	2.16	101.91
120 rep2	90 rep2	3.47	55.96	13.43	5.09	2.38	1.43	1.08	82.83	16.07	2 16	<LOQ	2.16	101.06
120 mean	**90 mean**	**3.82**	**56.56**	**12.94**	**4.89**	**2.27**	**1.40**	**1.06**	**82.95**	**16.37**	**2 16**	**<LOQ**	**2.16**	**101.49**

Extract 1 = ACN
Extract 2 = ACN
Extract 3 = ACN:water (8:2)
Extract 4 = ACN:water (8:2)
Extract 5 = ACN:water (1:1)
NA = Not Applicable (no sample analyzed)

Extract 6 = ACN:water (1:1)
EG = Ethylene glycol
ERR= Extractable Radioactive Residues
NER = Non Extractable Residues (by combustion)
LOQ = 0.06% TAR

C. EXTRACTABLE AND BOUND RESIDUES

Li10 soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-2.
The mean extractable radioactive residues (ERR) from the soil decreased from approximately 99.73% TAR at 0 DAT to approximately 78.02% TAR at 120 DAT. The majority of the residues were obtained by replicate extraction with acetonitrile (46.85-94.5% TAR). In general, subsequent extractions with acetonitrile:water (80:20 v/v) yielded an additional 3.26-6.21% TAR (in one case 48.55% TAR was recovered, which compensated for lower recoveries in the first extractions). A final solvent system of acetonitrile:water (1:1, v/v) produced only 0.31- 1.63% TAR, to which the last extraction contributed only slightly (≤ 0.55%).
Non-extractable [^{14}C]-residues (NER) were formed in moderate amounts for this soil and label combination. The mean values increased from approximately 0.27% TAR at 0 DAT to 8.39% TAR at 120 DAT.

LUFA 5M soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-3.
The mean solvent extracted radioactive residues (ERR) from the soil decreased from 98.93% TAR at 0 DAT to 93.26% TAR at 120 DAT. Extraction with 100% acetonitrile was most effective, producing 74.68 to 92.45% TAR. Additional extractions with acetonitrile:water (80:20, v/v) generated 4.71-9.88% TAR, with lower values occurring after flooding. The following extractions with acetonitrile:water (1:1, v/v) yielded 0.79-2.21% TAR, with only minimal activity detected in the final step (≤0.92% TAR).
The mean non-extractable [^{14}C]-residues (NER) detected were in moderate levels, showing an increase from 1.07% TAR at 0 DAT to 13.98% TAR at 120 DAT.

IN soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-4.
The mean extracted radioactive residues (ERR) from the soil decreased from approximately 99.75% TAR at 0 DAT to approximately 80.47% TAR at 120 DAT. Replicate extraction by acetonitrile produced 67.83-96.72% TAR. The following two extractions with 8:2 acetonitrile:water generated 4.17-7.11% TAR, while the final two extractions combined produced only 0.31-2.08% TAR.
The mean total non-extractable [^{14}C]-residues (NER) increased from approximately 0.25% TAR at 0 DAT to 9.49% TAR at 120 DAT.

NJ soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-5.
The mean solvent extracted radioactive residues (ERR) from the soil decreased from approximately 99.64% TAR at 0 DAT to approximately 91.72% TAR at 120 DAT. Extractions with acetonitrile ranged from 60.56 to 94.54% TAR. Two subsequent extractions with 8:2 acetonitrile:water produced 4.64-7.67% TAR, while final extractions with 1:1 acetonitrile:water yielded up to 2.67% of the activity.
The mean total non-extractable [^{14}C]-residues (NER) increased from approximately 0.36% TAR at 0 DAT to 15.51% TAR at 120 DAT.

NJ soil treated with [chlorophenyl-U-^{14}C]-BAS 750 F: See Table 7.1.1.2-6.
The mean solvent extracted radioactive residues (ERR) from the soil decreased from approximately 99.21% TAR at 0 DAT to approximately 82.95% TAR at 120 DAT. Of this, pure acetonitrile extracted a sum of 67.58%-93.79% TAR. Further extraction produced 4.63%-7.16% with 8:2 acetonitrile:water. Two final portions of 1:1 acetonitrile:water extracted up to 2.46% TAR, of which no more than 1.06% was contained in the last extraction.
The mean total non-extractable [^{14}C]-residues (NER) increased from approximately 0.79% TAR at 0 DAT to 16.37% TAR at 120 DAT.

D. VOLATILISATION

Li10 soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-2.
Mineralization of BAS 750 F was minimal during the course of the study. The mean $^{14}CO_2$ recovered at 7 DAT was 0.26% TAR and reached a maximum of 0.41% TAR at 120 DAT. No other radioactive volatile compounds were detected.

LUFA 5M soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-3.
Very few volatile residues related to BAS 750 F were detected in this system. The mean total recovered $^{14}CO_2$ at 7 DAT was 0.27% TAR and reached a maximum of 0.38% TAR at 120 DAT. No volatiles were recovered in other traps.

IN soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-4.
Very little $^{14}CO_2$ was detected in this soil system. The mean total recovered volatile radioactivity at 7 DAT was 0.23% TAR and reached a maximum of 0.35% TAR at 120 DAT. Ethylene glycol traps captured no detectable radioactive residues.

NJ soil treated with [triazole-3(5)-^{14}C]-BAS 750 F: See Table 7.1.1.2-5.
The total recovered volatile radioactivity, collected in a sodium hydroxide trap, at 7 DAT was 0.13% TAR and reached a maximum of 0.27% TAR at 120 DAT.

NJ soil treated with [chlorophenyl-U-^{14}C]-BAS 750 F: See Table 7.1.1.2-6.
No volatile organic compounds were detected in an ethylene glycol trap. The total recovered volatile radioactivity at 7 DAT was 0.80% TAR and reached a maximum of 2.16% TAR at 120 DAT, and was found only in the sodium hydroxide traps.

E. TRANSFORMATION OF PARENT COMPOUND

The pooled extract for each sampling point was analyzed by HPLC to determine the speciation of detected activity. The amounts of BAS 750 F and its metabolites recovered at each time point for each soil are shown in the following tables as percentages of the total applied radioactivity.

Li10 soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
The mean amount of the parent compound (triazole-3(5)-^{14}C-BAS 750 F) decreased from 97.18% TAR at 0 DAT (See Table 7.1.1.2-7) to 75.96% TAR at 120 DAT. Two minor transformation products were observed and neither of them exceeded an average of 5% TAR at any of the retention times.

LUFA 5M soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
The mean amount of the parent compound (triazole-3(5)-^{14}C-BAS 750 F) decreased from 97.79% TAR at 0 DAT (See Table 7.1.1.2-8) to 92.62% TAR at 120 DAT. Two minor transformation products were observed and neither of them exceeded an average of 5% TAR at any of the retention times.

IN soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
The mean amount of the parent compound (triazole-3(5)-^{14}C-BAS 750 F) decreased from 97.15% TAR at 0 DAT (See Table 7.1.1.2-9) to 78.72% TAR at 120 DAT. Two minor transformation products were observed and neither of them exceeded an average of 5% TAR at any of the retention times.

NJ soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
The mean amount of the parent compound (triazole-3(5)-^{14}C-BAS 750 F) decreased from approximately 97.56% TAR at 0 DAT (See Table 7.1.1.2-10) to approximately 88.95% TAR at 120 DAT. Two minor transformation products were observed and neither of them exceeded an average of 5% TAR at any of the retention times.

NJ soil treated with [chlorophenyl-U-^{14}C]-BAS 750 F:
The mean amount of the parent compound (chlorophenyl-U-^{14}C-BAS 750 F) decreased from approximately 97.74% TAR at 0 DAT (See Table 7.1.1.2-11) to approximately 81.69% TAR at 120 DAT. Three minor transformation products were observed and none of them exceeded an average of 5% TAR at any of the retention times.

Table 7.1.1.2-7: HPLC Quantitation of [Triazole-3(5)-^{14}C]-BAS 750 F Residues in Li10 Extract [% TAR]

				BAS 750 F
	t_R (min)	**3.7-4.0**	**4.3-4.7**	**36.6-38.1**
DAT	**DAF**			
0 rep1	NA	1.81	0.69	96.91
0 rep2	NA	1.81	0.77	97.45
0 mean	**NA**	**1.81**	**0.73**	**97.18**
7 rep1	NA	1.09	< LOQ	92.14
7 rep2	NA	1.57	< LOQ	93.62
7 mean	**NA**	**1.33**	**< LOQ**	**92.88**
11 rep1	NA	2.77	< LOQ	76.95
11 rep2	NA	1.19	< LOQ	78.65
11 mean	**NA**	**1.98**	**< LOQ**	**77.80**
30 rep1	NA	2.28	< LOQ	99.40
30 rep2	NA	0.97	< LOQ	91.00
30 mean	**NA**	**1.63**	**< LOQ**	**95.20**
32 rep1	2 rep1	1.08	< LOQ	96.30
32 rep2	2 rep2	1.57	< LOQ	96.64
32 mean	**2 mean**	**1.33**	**< LOQ**	**96.47**
37 rep1	7 rep1	1.36	< LOQ	94.01
37 rep2	7 rep2	5.63	< LOQ	73.61
37 mean	**7 mean**	**3.50**	**< LOQ**	**83.81**
44 rep1	14 rep1	1.02	< LOQ	95.37
44 rep2	14 rep2	1.10	< LOQ	90.40
44 mean	**14 mean**	**1.06**	**< LOQ**	**92.89**
60 rep1	30 rep1	1.97	< LOQ	84.85
60 rep2	30 rep2	1.25	< LOQ	90.69
60 mean	**30 mean**	**1.61**	**< LOQ**	**87.77**
91 rep1	61 rep1	0.55	< LOQ	84.27
91 rep2	61 rep2	1.14	< LOQ	88.60
91 mean	**61 mean**	**0.85**	**< LOQ**	**86.44**
120 rep1	90 rep1	1.47	< LOQ	82.70
120 rep2	90 rep2	2.03	0.64	69.21
120 mean	**90 mean**	**1.75**	**0.32**	**75.96**

NA = Not Applicable
LOQ = 0.59% TAR
Arithmetic mean values may be reported as a value less than the stated LOQ value. Individual replicates reported as <LOQ have been treated as a zero value in the calculation of arithmetic mean values.

Table 7.1.1.2-8: HPLC Quantitation of [Triazole-3(5)-^{14}C]-BAS 750 F Residues in LUFA 5M Extract [% TAR]

				BAS 750 F
	t_R (min)	**3.1-4.3**	**4.3-4.7**	**36.7-38.1**
DAT	**DAF**			
0 rep1	NA	< LOQ	< LOQ	106.44
0 rep2	NA	< LOQ	1.25	89.14
0 mean	**NA**	**< LOQ**	**0.63**	**97.79**
7 rep1	NA	0.64	< LOQ	101.72
7 rep2	NA	0.60	< LOQ	103.11
7 mean	**NA**	**0.62**	**< LOQ**	**102.42**
11 rep1	NA	< LOQ	< LOQ	93.27
11 rep2	NA	< LOQ	< LOQ	84.27
11 mean	**NA**	**< LOQ**	**< LOQ**	**88.77**
30 rep1	NA	0.68	< LOQ	103.77
30 rep2	NA	< LOQ	< LOQ	101.84
30 mean	**NA**	**0.34**	**< LOQ**	**102.81**
32 rep1	2 rep1	0.66	< LOQ	103.01
32 rep2	2 rep2	< LOQ	< LOQ	93.23
32 mean	**2 mean**	**0.33**	**< LOQ**	**98.12**
37 rep1	7 rep1	0.61	< LOQ	96.75
37 rep2	7 rep2	< LOQ	< LOQ	80.05
37 mean	**7 mean**	**0.31**	**< LOQ**	**88.40**
44 rep1	14 rep1	< LOQ	< LOQ	82.71
44 rep2	14 rep2	< LOQ	< LOQ	87.56
44 mean	**14 mean**	**< LOQ**	**< LOQ**	**85.14**
60 rep1	30 rep1	< LOQ	< LOQ	96.18
60 rep2	30 rep2	< LOQ	< LOQ	94.99
60 mean	**30 mean**	**< LOQ**	**< LOQ**	**95.59**
91 rep1	61 rep1	< LOQ	< LOQ	95.89
91 rep2	61 rep2	< LOQ	< LOQ	94.46
91 mean	**61 mean**	**< LOQ**	**< LOQ**	**95.18**
120 rep1	90 rep1	< LOQ	< LOQ	92.69
120 rep2	90 rep2	0.95	< LOQ	92.54
120 mean	**90 mean**	**0.48**	**< LOQ**	**92.62**

NA = Not Applicable
LOQ = 0.59% TAR
Arithmetic mean values may be reported as a value less than the stated LOQ value. Individual replicates reported as <LOQ have been treated as a zero value in the calculation of arithmetic mean values.

Table 7.1.1.2-9: HPLC Quantitation of [Triazole-3(5)-^{14}C]-BAS 750 F Residues in IN Extract [% TAR]

				BAS 750 F
	t_R (min)	**3.7-4.3**	**4.3-4.7**	**36.7-38.1**
DAT	**DAF**			
0 rep1	NA	1.49	1.47	94.97
0 rep2	NA	1.36	0.89	99.32
0 mean	**NA**	**1.43**	**1 18**	**97.15**
7 rep1	NA	1.54	< LOQ	108.47
7 rep2	NA	0.97	< LOQ	101.81
7 mean	**NA**	**1.26**	**< LOQ**	105.14
11 rep1	NA	2.94	< LOQ	94.84
11 rep2	NA	1.25	< LOQ	82.61
11 mean	**NA**	**2.10**	**< LOQ**	**88.73**
30 rep1	NA	4.88	< LOQ	91.63
30 rep2	NA	1.32	< LOQ	85.80
30 mean	**NA**	**3.10**	**< LOQ**	**88.72**
32 rep1	2 rep1	1.90	< LOQ	98.72
32 rep2	2 rep2	2.02	< LOQ	92.90
32 mean	**2 mean**	**1.96**	**< LOQ**	**95.81**
37 rep1	7 rep1	1.29	< LOQ	91.46
37 rep2	7 rep2	2.25	< LOQ	97.38
37 mean	**7 mean**	**1.77**	**< LOQ**	**94.42**
44 rep1	14 rep1	1.03	< LOQ	95.19
44 rep2	14 rep2	1.82	< LOQ	96.43
44 mean	**14 mean**	**1.43**	**< LOQ**	**95.81**
60 rep1	30 rep1	1.64	< LOQ	91.47
60 rep2	30 rep2	2.65	< LOQ	80.62
60 mean	**30 mean**	**2.15**	**< LOQ**	**86.05**
91 rep1	61 rep1	2.14	< LOQ	88.76
91 rep2	61 rep2	2.17	< LOQ	84.92
91 mean	**61 mean**	**2.16**	**< LOQ**	**86.84**
120 rep1	90 rep1	1.54	< LOQ	81.27
120 rep2	90 rep2	1.27	0.70	76.16
120 mean	**90 mean**	**1.41**	**0 35**	**78.72**

NA = Not Applicable
LOQ = 0.59% TAR
Arithmetic mean values may be reported as a value less than the stated LOQ value. Individual replicates reported as <LOQ have been treated as a zero value in the calculation of arithmetic mean values.

Table 7.1.1.2-10: HPLC Quantitation of [Triazole-3(5)-^{14}C]-BAS 750 F Residues in NJ Extract [% TAR]

				BAS 750 F
	t_R (min)	**3.7-4.0**	**4.2-4.7**	**36.7-38.1**
DAT	**DAF**			
0 rep1	NA	1.28	1.16	104.78
0 rep2	NA	1.08	0.66	90.33
0 mean	**NA**	**1.18**	**0.91**	**97.56**
7 rep1	NA	1.39	< LOQ	104.51
7 rep2	NA	1.58	< LOQ	88.32
7 mean	**NA**	**1.49**	**< LOQ**	**96.42**
11 rep1	NA	4.32	< LOQ	92.90
11 rep2	NA	1.79	< LOQ	98.38
11 mean	**NA**	**3.06**	**< LOQ**	**95.64**
30 rep1	NA	5.78	< LOQ	86.31
30 rep2	NA	2.36	< LOQ	88.96
30 mean	**NA**	**4.07**	**< LOQ**	**87.64**
32 rep1	2 rep1	1.46	< LOQ	88.11
32 rep2	2 rep2	2.03	< LOQ	98.23
32 mean	**2 mean**	**1.75**	**< LOQ**	**93.17**
37 rep1	7 rep1	2.49	1.01	97.17
37 rep2	7 rep2	< LOQ	< LOQ	78.84
37 mean	**7 mean**	**1.25**	**0.51**	**88.01**
44 rep1	14 rep1	1.91	< LOQ	90.19
44 rep2	14 rep2	2.62	< LOQ	94.50
44 mean	**14 mean**	**2.27**	**< LOQ**	**92.35**
60 rep1	30 rep1	1.40	< LOQ	76.82
60 rep2	30 rep2	1.96	< LOQ	68.44
60 mean	**30 mean**	**1.68**	**< LOQ**	**72.63**
91 rep1	61 rep1	2.33	< LOQ	87.45
91 rep2	61 rep2	1.74	< LOQ	77.15
91 mean	**61 mean**	**2.04**	**< LOQ**	**82.30**
120 rep1	90 rep1	2.94	< LOQ	88.55
120 rep2	90 rep2	2.23	< LOQ	89.35
120 mean	**90 mean**	**2.59**	**< LOQ**	**88.95**

NA = Not Applicable
LOQ = 0.59% TAR
Arithmetic mean values may be reported as a value less than the stated LOQ value. Individual replicates reported as <LOQ have been treated as a zero value in the calculation of arithmetic mean values.

Table 7.1.1.2-11: HPLC Quantitation of [Chlorophenyl-U-^{14}C]-BAS 750 F Residues in NJ Extract [% TAR]

			BAS 750 F		
	t_R (min)	**33.92**	**36.7-38.1**	**40.0-41.7**	**42.4-42.6**
DAT	**DAF**				
0 rep1	NA	< LOQ	98.46	< LOQ	1.66
0 rep2	NA	< LOQ	97.01	< LOQ	1.29
0 mean	**NA**	**< LOQ**	**97.74**	**< LOQ**	**1.48**
7 rep1	NA	< LOQ	95.10	1.10	1.25
7 rep2	NA	< LOQ	87.84	1.64	1.15
7 mean	**NA**	**< LOQ**	**91.47**	**1.37**	**1.20**
11 rep1	NA	< LOQ	92.63	< LOQ	< LOQ
11 rep2	NA	< LOQ	89.08	0.87	< LOQ
11 mean	**NA**	**< LOQ**	**90.86**	**0.44**	**< LOQ**
30 rep1	NA	< LOQ	93.83	0.64	< LOQ
30 rep2	NA	< LOQ	90.69	0.63	< LOQ
30 mean	**NA**	**< LOQ**	**92.26**	**0.64**	**< LOQ**
32 rep1	2 rep1	< LOQ	83.54	< LOQ	< LOQ
32 rep2	2 rep2	< LOQ	87.15	0.68	< LOQ
32 mean	**2 mean**	**< LOQ**	**85.35**	**0.34**	**< LOQ**
37 rep1	7 rep1	0.70	80.34	1.04	< LOQ
37 rep2	7 rep2	< LOQ	89.42	< LOQ	< LOQ
37 mean	**7 mean**	**0.35**	**84.88**	**0.52**	**< LOQ**
44 rep1	14 rep1	< LOQ	85.28	0.97	< LOQ
44 rep2	14 rep2	< LOQ	86.31	1.64	< LOQ
44 mean	**14 mean**	**< LOQ**	**85.80**	**1.31**	**< LOQ**
60 rep1	30 rep1	< LOQ	88.18	< LOQ	< LOQ
60 rep2	30 rep2	< LOQ	86.35	0.79	< LOQ
60 mean	**30 mean**	**< LOQ**	**87.27**	**0.40**	**< LOQ**
91 rep1	61 rep1	< LOQ	78.28	1.26	< LOQ
91 rep2	61 rep2	< LOQ	83.01	1.27	< LOQ
91 mean	**61 mean**	**< LOQ**	**80.65**	**1.27**	**< LOQ**
120 rep1	90 rep1	< LOQ	82.06	1.01	< LOQ
120 rep2	90 rep2	< LOQ	81.32	1.51	< LOQ
120 mean	**90 mean**	**< LOQ**	**81.69**	**1.26**	**< LOQ**

NA = Not Applicable
LOQ = 0.59% TAR
Arithmetic mean values may be reported as a value less than the stated LOQ value. Individual replicates reported as <LOQ have been treated as a zero value in the calculation of arithmetic mean values.

F. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

The non-extractable residues in all soils were further characterized by repetitive extractions with aqueous sodium hydroxide to evaluate the activity in each fraction of the soil. Two time points (30 DAT and 120 DAT) were examined. Results for each soil are listed below and in the indicated tables. No further HPLC characterization was performed on the soil fractions due to low levels of detected activity.

Li10 soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
For the 30 DAT samples, the NER (mean 3.08%) were further characterized for their fulvic (mean 1.10%), humic (mean 0.65%), and humin (mean 1.21%) content. At 120 DAT, the NER content increased to 8.39% and the fulvic (mean 1.50%), humic (mean 1.27%), and humin (mean 5.31%) components were determined (see Table 7.1.1.2-12).

LUFA 5M soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
For the 30 DAT samples, the NER (mean 5.77%) were further characterized for their fulvic (mean 2.82%), humic (mean 0.81%), and humin (mean 2.01%) content. At 120 DAT, the NER content increased to 13.98% and the fulvic (mean 3.76%), humic (mean 1.76%), and humin (mean 8.22%) components were determined (see Table 7.1.1.2-13).

IN soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
For the 30 DAT samples, the NER (mean 5.33%) were further characterized for their fulvic (mean 1.75%), humic (mean 0.71%), and humin (mean 2.81%) content. At 120 DAT, the NER content increased to 9.49% and the fulvic (mean 2.11%), humic (mean 1.05%), and humin (mean 6.31%) components were determined (see Table 7.1.1.2-14).

NJ soil treated with [triazole-3(5)-^{14}C]-BAS 750 F:
For the 30 DAT samples, the NER (mean 7.27%) were further characterized for their fulvic (mean 2.41%), humic (mean 0.95%), and humin (mean 3.89%) content. At 120 DAT, the NER content increased to 15.51% and the fulvic (mean 3.04%), humic (mean 1.67%), and humin (mean 10.80%) components were determined (see Table 7.1.1.2-15).

NJ soil treated with [chlorophenyl-U-^{14}C]-BAS 750 F:
For the 30 DAT samples, the NER (mean 8.33%) were further characterized for their fulvic (mean 1.96%), humic (mean 1.65%), and humin (mean 4.58%) content. At 120 DAT, the NER content increased to 16.37% and the fulvic (mean 2.89%), humic (mean 2.11%), and humin (mean 11.29%) components were determined (see Table 7.1.1.2-16).

Table 7.1.1.2-12: Characterization of Bound Residues in [Triazole-3(5)-^{14}C]-BAS 750 F Treated Li10 Soil (Expressed as % TAR)

DAT	Rep	NER	Fulvic Acid	Humic Acid	Humins	% Recovery
30	1	3.39	1.19	0.67	1.33	94.26
	2	2.76	1.01	0.64	1.09	98.99
	mean	**3.08**	**1.10**	**0.65**	**1.21**	**96.62**
120 (90 DAF)	1	8.81	1.69	1.32	5.54	97.01
	2	7.97	1.31	1.23	5.07	95.54
	mean	**8.39**	**1.50**	**1.27**	**5.31**	**96.27**

NER: Non-Extractable Residues (by combustion)
% Recovery = 100 × (Fulvic Acids + Humic Acids + Humins) / NER
Values have been rounded to two decimal places. All calculations were performed using the full precision data reported in ALADIN. Calculations based on the rounded values in the tables may vary slightly from those shown above.

Table 7.1.1.2-13: Characterization of Bound Residues in [Triazole-3(5)-^{14}C]-BAS 750 F Treated LUFA 5M Soil (Expressed as % TAR)

DAT	Rep	NER	Fulvic Acid	Humic Acid	Humins	% Recovery
30	1	5.49	2.69	0.74	1.93	97.63
	2	6.04	2.96	0.88	2.10	98.29
	mean	**5.77**	**2.82**	**0.81**	**2.01**	**97.96**
120 (90 DAF)	1	13.81	3.70	1.69	8.11	97.75
	2	14.15	3.83	1.84	8.34	98.94
	mean	**13.98**	**3.76**	**1.76**	**8.22**	**98.35**

NER: Non-Extractable Residues (by combustion)
% Recovery = 100 × (Fulvic Acids + Humic Acids + Humins) / NER
Values have been rounded to two decimal places. All calculations were performed using the full precision data reported in ALADIN. Calculations based on the rounded values in the tables may vary slightly from those shown above.

Table 7.1.1.2-14: Characterization of Bound Residues in [Triazole-3(5)-^{14}C]-BAS 750 F Treated IN Soil (Expressed as % TAR)

DAT	Rep	NER	Fulvic Acid	Humic Acid	Humins	% Recovery
30	1	5.81	1.94	0.78	3.04	99.21
	2	4.86	1.56	0.63	2.58	98.21
	mean	**5.33**	**1.75**	**0.71**	**2.81**	**98.71**
120 (90 DAF)	1	9.86	2.26	1.07	6.66	101.33
	2	9.12	1.97	1.02	5.96	98.19
	mean	**9.49**	**2.11**	**1.05**	**6.31**	**99.76**

NER: Non-Extractable Residues (by combustion)
% Recovery = 100 × (Fulvic Acids + Humic Acids + Humins) / NER
Values have been rounded to two decimal places. All calculations were performed using the full precision data reported in ALADIN. Calculations based on the rounded values in the tables may vary slightly from those shown above.

Table 7.1.1.2-15: Characterization of Bound Residues in [Triazole-3(5)-^{14}C]-BAS 750 F Treated NJ Soil (Expressed as % TAR)

DAT	Rep	NER	Fulvic Acid	Humic Acid	Humins	% Recovery
30	1	7.23	2.47	0.87	3.87	99.68
	2	7.3	2.35	1.04	3.90	99.92
	mean	**7.27**	**2.41**	**0.95**	**3.89**	**99.80**
120 (90 DAF)	1	15.21	2.92	1.69	10.61	100.05
	2	15.81	3.16	1.66	10.98	99.90
	mean	**15.51**	**3.04**	**1.67**	**10.80**	**99.97**

NER: Non-Extractable Residues (by combustion)
% Recovery = 100 × (Fulvic Acids + Humic Acids + Humins) / NER
Values have been rounded to two decimal places. All calculations were performed using the full precision data reported in ALADIN. Calculations based on the rounded values in the tables may vary slightly from those shown above.

Table 7.1.1.2-16: Characterization of Bound Residues in [Chlorophenyl-U-^{14}C]-BAS 750 F Treated NJ Soil (Expressed as % TAR)

DAT	Rep	NER	Fulvic Acid	Humic Acid	Humins	% Recovery
30	1	8.39	1.97	1.70	4.63	98.94
	2	8.27	1.94	1.59	4.52	97.39
	mean	**8.33**	**1.96**	**1.65**	**4.58**	**98.17**
120 (90 DAF)	1	16.67	3.06	2.01	11.15	97.30
	2	16.07	2.72	2.21	11.42	101.68
	mean	**16.37**	**2.89**	**2.11**	**11.29**	**99.49**

NER: Non-Extractable Residues (by combustion)
% Recovery = 100 × (Fulvic Acids + Humic Acids + Humins) / NER
Values have been rounded to two decimal places. All calculations were performed using the full precision data reported in ALADIN. Calculations based on the rounded values in the tables may vary slightly from those shown above.

G. CHIRAL ANALYSIS

Chiral analysis was performed on representative samples from the beginning, middle and end of the study. The ratio showed no statistically relevant shift throughout the study (~ 52:48). Detailed results may be found in the full study report.

G. KINETIC MODELING RESULTS

The data generated in this study was analyzed in accordance with FOCUS kinetic guidance to determine DT_{50}, DT_{75}, and DT_{90} trigger endpoints for BAS 750 F. The kinetic endpoint values are presented in Table 7.1.1.2-17. A SFO (single first-order) model was used for the fittings. The calculated DT_{50} values in three soils ranged from 349 to 899 days. No reliable DT_{90} values could be determined. In one soil, LUFA 5M, no discernible decline was detected, and no kinetic endpoints were calculated.

Table 7.1.1.2-17: Kinetic Analysis of BAS 750 F Degradation in Soil Under Anaerobic Conditions

Soil	Kinetic model	Parameter estimates [a]	p-values	χ^2 error	r^2	DT_{50}	DT_{75} [b]	DT_{90}
Li10	SFO	M_o = 93.44 k = 0.0020	k: < 0.01	3.51	0.41	349	699	NR [c]
LUFA 5M	-- [d]	--	--	--	--	--	--	--
Indiana	SFO	M_o = 94.09 k = 0.0018	k: < 0.01	2.80	0.56	390	780	NR
New Jersey	SFO	M_o = 87.85 k = 0.0008	k: < 0.05	2.80	0.10	899	NR	NR

Units: M_o [% TAR]; k [d^{-1}]
[a] Reported value for rate constant, k, rounded for table presentation. Any calculations based on k used the full precision of the estimate, as found in the appropriate Figure.
[b] DT_{75} values were manually calculated as ln(4)/k, using optimized values of k for SFO
[c] NR: Not reliable, not reported. Kinetic endpoint was extrapolated > 10 times the study duration and considered unreliable. A value of 1000 days is proposed as a practical conservative approximation
[d] '--' : No discernible decline trend for BAS 750 F in LUFA 5M soil. Therefore, kinetics were not investigated

III. CONCLUSION

BAS 750 F degrades slowly under anaerobic soil conditions, as demonstrated in this laboratory study. At all sampling points, the parent compound was the major residue detected by HPLC analysis, for all soil types and labels. DT_{50} values for BAS 750 F ranged from not determined to 899 days. Formation of NER was the major end product during incubation. No new or novel metabolites were discovered under anaerobic conditions that were not seen under aerobic conditions. There is no discernible isomerization of the enantiomers, or preferred degradation of either enantiomer.

CA 7.1.1.3 Soil photolysis

Report:	CA 7.1.1.3/1 Hassink J., Delgado M., 2014 a Soil photolysis of (triazole-3(5)-C14 and chlorophenyl-U-C14) BAS 750 F 2014/1181666
Guidelines:	EPA Subdivision N, §161-3 Photodegradation Studies on Soil EPA: OPPTS 835.2410 – Photodegradation on Soil SETAC Europe: Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides Draft OECD Guideline "Phototransformation of Chemicals on Soil Surfaces", Jan. 02 Directive 91/414/EEC Annex II, amended by Commission Directive 95/36/EC
GLP:	yes (certified by Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz)

EXECUTIVE SUMMARY

A soil photolysis study was conducted with chlorophenyl-U-^{14}C- and triazole-3(5)-^{14}C-labeled BAS 750 F to investigate the behavior of BAS 750 F in soil under the influence of light.

After passing through a 2 mm sieve before use, the soil was treated with the labeled test item at a nominal rate of 1 mg per kg dry soil, corresponding to a field application rate of 150 g ha $^{-1}$. Soil aliquots of 30 g (dry weight basis) were weighed into test vessels and placed into a Suntest apparatus for continuous irradiation (light intensity 3 mW cm^{-2}) and aeration. The incubation temperature was kept at 22 °C ± 1 °C and the soil moisture was adjusted daily to approximately 60% of the maximum water holding capacity. A closed incubation system with continuous air flow was used with an attached trapping system for the determination of volatile compounds. The samples for the dark control were kept in an incubation cabinet and treated analogously but without irradiation.

Samples were taken at 0, 1, 3, 6 (7), 10 and 15 days after treatment (DAT). All soil samples were worked up in duplicate. The soil samples were extracted three times with acetonitrile (ACN) and twice with ACN/water (1:1, v/v). The combined ACN extracts were analyzed by LSC and HPLC. The ACN/water extracts were analyzed by LSC and by HPLC, only if TAR (total applied radioactivity) > 5%. The remaining soil after extraction was extracted three times with NaOH to determine the amount of alkali-soluble components. The dried soil was then combusted, in order to determine the amount of non-extractable soil bound residues.

The overall values for the material balance in the photolysis and the dark control were in the range of 100.0-104.3% TAR. Carbon dioxide was the only trapped volatile degradation product found in the trapping solutions. After 15 days of treatment, 3.5% TAR (chlorophenyl label) and 0.3% TAR (triazole label) were mineralized in the photolysis test and 1.2% and 0.1% TAR in the dark control samples.

The extractability did not significantly differ between the photolysis test and the dark control. At the end of the study about 5.2% TAR for the chlorophenyl label and 6.9% TAR for the triazole label were not extractable from the illuminated soil samples (about 4.6% and 4.7% TAR for the corresponding dark control samples). The alkali-soluble radioactivity amounted to 2.3% TAR and 4.2% TAR, for the chlorophenyl label and the triazole label respectively, in the period of 1-15 DAT in the photolysis representing the fulvic acids and the humic acids. The dark control resulted in similar values (2.0% TAR and 2.3% TAR, respectively).

The amount of extractable radioactive residues (ERR) in the study with the chlorophenyl label decreased from 98.9% TAR on day 0 to 92.1% TAR on day 15 in the photolysis test, and from 99.9% TAR on day 1 to 96.4% TAR on day 15 in the dark control. In the study with the triazole label the amount of ERR decreased from 99.0% TAR on day 0 to 97.0% TAR on day 15 in the photolysis test, and from 102.1% TAR to 99.3% TAR in the dark control. The majority of the extractable radioactive residues were always obtained by extraction with ACN.

After 15 days, the amount of chlorophenyl-labeled BAS 750 F decreased to 87.3% TAR in the photolysis experiment and to 93.1% TAR in the dark control samples. The amount of triazole-labeled BAS 750 F decreased in the same time to 93.8% TAR in the photolysis experiment and to 95.8% TAR in the dark control samples. For both labels, several degradation products were detected in the extracts, but none of them appeared in amounts higher than 1.17% TAR.

Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values (non-GLP) for BAS 750 F in soil was performed to derive trigger endpoints following the recommendations of the FOCUS Kinetics workgroup. For both labels, the results could be best described by the SFO kinetic fit approach. Derived $DegT_{50}$ and $DegT_{90}$ values for the photolysis test were 93.1 and 309.1 days and 170.0 and 564.7 days for the chlorophenyl- and the triazole-labeled test item, respectively. Corresponding $DegT_{50}$ and $DegT_{90}$ values for the dark control were 172.9 and 574.5 days and 225.0 and 747.3 days.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in two ^{14}C-labeled forms.

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	40487-42-1
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

1. Chlorophenyl-U-^{14}C-label

Batch No.:	CFQ41561
Specific radioactivity of a.s.:	7.878 MBq mg^{-1}
Radiochemical purity:	98.9%
Purity:	99.1%

2. Triazole-3(5)-^{14}C-label

Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%
Purity:	98.9%

Unlabeled

Internal code:	BAS 750 F
Batch No.:	L84-238
Purity:	99.7%

2. Soil

Two batches of German agricultural soil LUFA 5M (BASF soil No. 1651) from LUFA (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) used in this study were sampled from 0-20 cm depth. After collecting the soil from the field, the soil was kept at room temperature until sieving. The soil was passed through a 2 mm sieve, remoistened to approximately 8-12% soil moisture and stored at about 4 °C in the dark no longer than 3 months before use. An overview of soil parameters is listed in Table 7.1.1.3-1.

Table 7.1.1.3-1: Soil characteristics

Soil designation	**LUFA 5M** **BASF soil No. 13/1651/03** Germany (Origin LUFA Speyer)	**LUFA 5M** **BASF soil No. 13/1651/02** Germany (Origin LUFA Speyer)
DIN 4220 Particle size distribution *[%]* sand 0.063 – 2 mm silt 0.002 – 0.063 mm clay < 0.002 mm textural class	 54.6 33.5 11.9 loamy sand	 80.0 13.9 6.1 loamy sand
USDA Particle size distribution *[%]* sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 59.0 29.1 11.9 sandy loam	 82.8 11.1 6.1 loamy sand
Organic C *[%]*	2.08	2.03
pH [H_2O] pH [$CaCl_2$]	7.9 7.1	7.9 7.2
Cation exchange capacity *[$cmol^+ kg^{-1}$]*	11.4	11.4
Max. water holding capacity *[g per 100 g dry weight]*	25.6	25.2
Microbial biomass (start of study) *[mg C per 100 g dry soil]*	29.9	26.5

B. STUDY DESIGN

1. Experimental conditions

Ten small aluminum dishes (88 mm x 43 mm x 12 mm) were filled with soil for photolysis testing and for dark control, respectively, containing 30 g dry soil per dish. The dishes, which were later treated with the test item, were arranged in a rectangular bowl with a connected thermostat. The temperature of the dishes used for photolysis was adjusted and controlled by an external tempering unit (22 °C ± 1 °C) while the dishes for dark control were put into an incubator at 22 °C ± 1 °C. The light intensity for the photolysis test group was set to 3 mW cm^{-2} (UVA range). The bowl was closed airtight with a quartz glass covering and the whole incubation device was continuously aerated with CO_2-depleted (0.5 M NaOH) and remoistened air via an air inlet and an air outlet.

In order to trap potentially evolving volatiles (including $^{14}CO_2$), the emergent air was bubbled through three different trapping solutions located between dish and pump: 1. NaOH (0.5 M); 2. ethylene glycol; 3. H_2SO_4 (0.5 M).

The incubation bowl for photolysis was placed under a SUNTEST CPS plus (Atlas) equipped with a Xenon lamp emitting light with a spectrum similar to the intensity of sunlight, about 3 mW cm^{-2} (UVA range). This corresponds to a clear summer day in Southern Germany (about 49° N). Wavelengths < 290 nm were filtered off to simulate natural sunlight.

To maintain the temperature especially on the quartz glass surface in order to avoid a rapid drying of the soil surface, the air space between lamp and quartz glass within the SUNTEST device was cooled by an external apparatus (Yeti, Seveso). To maintain the initial water content as constant as possible, dishes were weighed at each incubation day and the evaporated water was replaced.

The amount of test item to be applied on the soil surface was calculated based on a recommended field application rate of 150 g ha^{-1}. If a soil layer of 1 cm and bulk density of 1.5 kg L^{-1} is assumed the application rate corresponds to about 1 mg test item per kg dry soil (and about 30 µg per dish).

2. Sampling

The sampling dates were 0, 1, 3, 6, 10 and 15 days after treatment (DAT) for chlorophenyl-labeled BAS 750 F and 0, 1, 3, 7, 10 and 15 DAT for triazole-labeled BAS 750 F. Two vessels were taken at each sampling time from each photolysis test system and the dark control (with exception of DAT 0, where no dark control samples were taken). At each sampling time, the respective volatile trapping solutions were removed.

3. Description of analytical procedures

Each soil sample was consecutively extracted three times with 40 mL of acetonitrile and twice with 40 mL of acetonitrile/water (1:1, v/v). For each extraction step, the suspension was shaken for 30 min. After each extraction step, solid and extract were separated by centrifugation and filtered. The three corresponding acetonitrile and two acetonitrile/water extracts were combined, respectively, and measured for radioactivity by liquid scintillation counting (LSC).

After the last extraction, the soil residues were air-dried and stored at room temperature. For determination of the amount of non-extractable residues by combustion, the residues were homogenized by milling. Aliquots of each sample were combusted in a sample oxidizer. The trapped $^{14}CO_2$ was analyzed by LSC.

The samples of day 0 and day 15 for the two labels were characterized on a chiral HPLC column to verify the enantiomeric composition of the radiolabeled test substance materials. All samples were measured for radioactivity (LSC) and were analyzed by HPLC to determine the metabolite pattern.

Therefore, prior to injection, the solvent of the extracts was completely evaporated by a rotary evaporator at 35 °C and afterwards, the residues were re-dissolved in a well-defined volume of the respective extraction solvent without any loss of significant radioactive material (recovery > 90%).

Since the samples of the photolysis and the dark control from day 15 for the chlorophenyl-labeled BAS 750 F and the samples of the photolysis from day 10 and day 15, as well as of the dark control from day 15 for the triazole-labeled BAS 750 F showed > 5% of the total applied radioactivity (TAR) of non-extractable residues, further analyses were performed before combustion to separate humic and fulvic acids from humin.

Samples were extracted three times with 0.5 M NaOH on a rotary shaker and twice washed with water. Aliquots of the NaOH and the water extracts were analyzed by LSC. NaOH extracts and water extracts were pooled, representing together the fulvic and humic acid fraction. Since the amount of radioactivity did not exceed 5% TAR, no acidic precipitation of the fulvic acids was performed.

The soil residue after the last washing was air-dried, homogenized and the weight was determined. Three aliquots were combusted and analyzed by LSC in order to determine the amount of radioactivity in the non-soluble humins.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints). Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*]. The software package KinGUII (version 2.2012.202) was used for parameter fitting [*Meyer et al. (2012)*]. The error tolerance and the number of iterations of the optimization tool were set to 0.00001 and 100, respectively.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC), are already implemented in KinGUI. The Goodness-of-fit was evaluated by visual assessment, χ^2 minimum error, and type-I-error rate (t-test) [for details see Chapter 6.3 in *FOCUS (2006)*].

Replicate measurements were considered for the parameter estimation. The initial concentration of the applied test item was set to the material balance recovered at day 0. The χ^2 value was calculated for the kinetic model as recommended by FOCUS, considering the average of replicate measurements. For the individual parameters, the t-test statistics were based on number of individual measurements.

II. RESULTS AND DISCUSSION

B. MASS BALANCE

Total recoveries of radioactivity extracted from soil are summarized in Table 7.1.1.3-2 to Table 7.1.1.3-5. The overall mean values for the material balance in the photolysis and in the dark control were in the range of 100.0-104.4% TAR.

Table 7.1.1.3-2: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with chlorophenyl-^{14}C-labeled BAS 750 F and incubation under irradiated conditions [% TAR]

DAT	ACN	ACN/water	total extractable	non-extractable	volatiles*	material balance
0/I	92.4	4.4	96.8	1.2	0.0	97.9
0/II	96.7	4.3	101.0	1.1	0.0	102.1
0 mean	**94.5**	**4.4**	**98.9**	**1.1**	**0.0**	**100.0**
1/I	96.0	2.3	98.3	1.8	0.3	100.4
1/II	95.2	2.0	97.2	2.5	0.3	99.9
1 mean	**95.6**	**2.2**	**97.7**	**2.1**	**0.3**	**100.2**
3/I	96.7	2.3	99.0	3.4	0.6	103.0
3/II	97.4	2.7	100.1	3.5	0.6	104.2
3 mean	**97.1**	**2.5**	**99.5**	**3.4**	**0.6**	**103.6**
6/I	94.8	3.0	97.9	3.6	1.1	102.6
6/II	94.4	2.9	97.4	4.5	1.1	103.0
6 mean	**94.6**	**3.0**	**97.6**	**4.1**	**1.1**	**102.8**
10/I	91.7	2.7	94.4	4.2	2.1	100.7
10/II	94.6	2.6	97.2	4.6	2.1	103.8
10 mean	**93.2**	**2.6**	**95.8**	**4.4**	**2.1**	**102.3**
15/I	89.6	2.5	92.1	5.2	3.5	100.8
15/II	89.3	2.7	92.1	5.1	3.5	100.7
15 mean	**89.5**	**2.6**	**92.1**	**5.2**	**3.5**	**100.8**

TAR = total applied radioactivity
DAT = days after treatment
ACN = acetonitrile
* no other volatiles than CO_2 were found

Table 7.1.1.3-3: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with chlorophenyl-^{14}C-labeled BAS 750 F and incubation under dark conditions [% TAR]

DAT	ACN	ACN/water	total extractable	non-extractable	volatiles*	material balance
1/I	97.3	3.0	100.3	2.4	0.3	103.0
1/II	97.8	1.8	99.6	2.9	0.3	102.7
1 mean	**97.6**	**2.4**	**99.9**	**2.6**	**0.3**	**102.8**
3/I	96.5	2.7	99.2	3.3	0.6	103.1
3/II	96.1	3.0	99.1	2.9	0.6	102.5
3 mean	**96.3**	**2.9**	**99.1**	**3.1**	**0.6**	**102.8**
6/I	94.9	2.8	97.7	3.2	0.8	101.7
6/II	95.9	2.6	98.4	3.2	0.8	102.5
6 mean	**95.4**	**2.7**	**98.0**	**3.2**	**0.8**	**102.1**
10/I	94.1	3.1	97.2	3.9	1.0	102.1
10/II	95.2	3.0	98.3	3.8	1.0	103.1
10 mean	**94.7**	**3.1**	**97.7**	**3.8**	**1.0**	**102.6**
15/I	94.5	3.3	97.8	4.5	1.2	103.4
15/II	91.7	3.3	95.0	4.7	1.2	100.8
15 mean	**93.1**	**3.3**	**96.4**	**4.6**	**1.2**	**102.1**

TAR = total applied radioactivity
DAT = days after treatment
ACN = acetonitrile
* no other volatiles than CO_2 were found

Table 7.1.1.3-4: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with triazole-^{14}C-labeled BAS 750 F and incubation under irradiated conditions [% TAR]

DAT	ACN	ACN/water	total extractable	non-extractable	volatiles*	material balance
0/I	94.7	5.0	99.7	1.1	0.0	100.8
0/II	93.0	5.2	98.2	1.0	0.0	99.2
0 mean	**93.9**	**5.1**	**99.0**	**1.0**	**0.0**	**100.0**
1/I	100.1	1.8	101.9	1.5	0.0	103.4
1/II	100.3	1.8	102.1	1.6	0.0	103.8
1 mean	**100.2**	**1.8**	**102.0**	**1.5**	**0.0**	**103.6**
3/I	97.5	2.2	99.7	2.4	0.1	102.2
3/II	97.0	1.9	98.9	2.6	0.1	101.6
3 mean	**97.3**	**2.0**	**99.3**	**2.5**	**0.1**	**101.9**
7/I	96.3	2.8	99.0	4.0	0.1	103.2
7/II	97.2	2.5	99.7	4.2	0.1	104.1
7 mean	**96.7**	**2.6**	**99.3**	**4.1**	**0.1**	**103.6**
10/I	96.9	2.5	99.4	5.1	0.2	104.7
10/II	96.1	2.5	98.6	5.3	0.2	104.0
10 mean	**96.5**	**2.5**	**99.0**	**5.2**	**0.2**	**104.4**
15/I	94.1	3.3	97.4	7.2	0.3	104.9
15/II	93.4	3.2	96.6	6.5	0.3	103.5
15 mean	**93.8**	**3.3**	**97.0**	**6.9**	**0.3**	**104.2**

TAR = total applied radioactivity
DAT = days after treatment
ACN = acetonitrile
* no other volatiles than CO_2 were found

Table 7.1.1.3-5: Recovery and distribution of radioactivity in soil LUFA 5M after treatment with triazole-^{14}C-labeled BAS 750 F and incubation under dark conditions [% TAR]

DAT	ACN	ACN/water	total extractable	non-extractable	volatiles*	material balance
1/I	99.9	2.5	102.4	1.9	0.0	104.4
1/II	99.7	2.1	101.8	1.6	0.0	103.4
1 mean	**99.8**	**2.3**	**102.1**	**1.8**	**0.0**	**103.9**
3/I	98.6	2.1	100.8	2.5	0.1	103.3
3/II	100.4	2.3	102.7	2.4	0.1	105.2
3 mean	**99.5**	**2.2**	**101.7**	**2.5**	**0.1**	**104.3**
7/I	97.0	2.5	99.5	3.7	0.1	103.3
7/II	97.4	3.1	100.4	3.9	0.1	104.4
7 mean	**97.2**	**2.8**	**100.0**	**3.8**	**0.1**	**103.9**
10/I	96.2	3.3	99.5	4.1	0.1	103.7
10/II	97.4	2.8	100.2	3.8	0.1	104.0
10 mean	**96.8**	**3.0**	**99.8**	**4.0**	**0.1**	**103.9**
15/I	95.5	3.7	99.2	4.7	0.1	104.0
15/II	96.2	3.3	99.5	4.7	0.1	104.3
15 mean	**95.8**	**3.5**	**99.3**	**4.7**	**0.1**	**104.2**

TAR = total applied radioactivity
DAT = days after treatment
ACN = acetonitrile
* no other volatiles than CO_2 were found

C. EXTRACTABLE AND BOUND RESIDUES

The amount of extractable radioactive residues in the study with the chlorophenyl-label decreased from 98.9% TAR on day 0 to 92.1% TAR on day 15 in the photolysis test, and from 99.9% TAR on day 1 to 96.4% TAR on day 15 in the dark control.

In the study with the triazole-label the amount of extractable radioactive residues decreased from 99.0% TAR on day 0 to 97.0% TAR on day 15 in the photolysis test, and from 102.1% TAR to 99.3% TAR in the dark control.

In the study with the chlorophenyl-label, the amount of non-extractable residues (NER) increased from 1.1% TAR on day 0 to 5.2% TAR in photolysis compared to 4.6% TAR in dark control after 15 days. The amount of NER in the study with the triazole-label increased from 1.0% TAR on day 0 to 6.9% TAR in photolysis compared to 4.7% TAR in dark control after 15 days.

D. VOLATILISATION

Carbon dioxide was the only trapped volatile degradation product found in the trapping solutions. After 15 days of treatment, 3.5% TAR (chlorophenyl label) and 0.3% TAR (triazole label) were mineralized in the photolysis test and 1.2% and 0.1% TAR in the dark control.

E. TRANSFORMATION OF PARENT COMPOUND

Results of radio-HPLC analyses are presented in Table 7.1.1.3-6 to Table 7.1.1.3-9.

After 15 days, the amount of [chlorophenyl-U-^{14}C]-labeled BAS 750 F decreased to 87.3% TAR in the photolysis experiment and to 93.1% TAR in the dark control samples. The amount of triazole-3(5)-labeled BAS 750 F decreased in the same time (15 days) to 93.8% TAR in the photolysis experiment and to 95.8% TAR in the dark control samples.

For both labels, several degradation products were detected in the extracts, but none of them appeared in amounts higher than 1.17% TAR.

Table 7.1.1.3-6: Radio-HPLC analysis of soil extracts after treatment of soil LUFA 5M with chlorophenyl-^{14}C-labeled BAS 750 F and incubation under irradiated conditions (sum of ACN extracts) [% TAR]

DAT	Total	BAS 750 F	Unknowns* t_R~								
		t_R~38.1'	38.8'	41.0'	41.2'	41.4'	41.6'	41.8'	42.0'	42.1'	42.2'
0 I	92.36	91.55	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.81
0 II	96.66	95.38	0.59	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.69
0 mean	**94.51**	**93.46**									
1 I	95.97	94.95	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.24	n.d.	0.78
1 II	95.17	94.72	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.45
1 mean	**95.57**	**94.83**									
3 I	96.70	96.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.54
3 II	97.42	96.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.44	0.72
3 mean	**97.06**	**96.20**									
6 I	94.84	93.67	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.17	n.d.
6 II	94.42	93.58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.39	0.25	0.20
6 mean	**94.63**	**93.63**									
10 I	91.74	90.70	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.04
10 II	94.63	93.17	0.46	n.d.	n.d.	n.d.	n.d.	0.43	0.57	n.d.	n.d.
10 mean	**93.18**	**91.94**									
15 I	89.62	87.42	n.d.	n.d.	0.93	n.d.	n.d.	0.18	n.d.	n.d.	1.09
15 II	89.34	87.14	0.33	0.24	0.20	0.27	0.10	0.21	0.37	n.d.	0.48
15 mean	**89.48**	**87.28**									

ACN = acetonitrile
TAR = total applied radioactivity
DAT = days after treatment
t_R = retention time [min]
n.d. = not detected
* all peaks are shown

Table 7.1.1.3-7: Radio-HPLC analysis of soil extracts after treatment of soil LUFA 5M with chlorophenyl-^{14}C-labeled BAS 750 F and incubation under dark conditions (sum of ACN extracts) [% TAR]

DAT	Total	BAS 750 F	Unknowns* t_R~								
		t_R~38.1'	40.9'	41.0'	41.2'	41.4'	41.6'	41.8'	42.0'	42.1'	42.2'
1 I	97.31	96.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.48
1 II	97.81	97.13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.69
1 mean	**97.56**	**96.98**									
3 I	96.46	96.26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.20
3 II	96.05	95.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.18	0.21
3 mean	**96.25**	**95.96**									
6 I	94.87	94.87	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6 II	95.86	95.86	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6 mean	**95.37**	**95.37**									
10 I	94.14	92.69	0.09	0.25	0.28	0.25	n.d.	0.41	0.18	n.d.	n.d.
10 II	95.20	95.20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10 mean	**94.67**	**93.95**									
15 I	94.47	94.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15 II	91.69	91.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15 mean	**93.08**	**93.08**									

ACN = acetonitrile
TAR = total applied radioactivity
DAT = days after treatment
t_R = retention time [min]
n.d. = not detected
* all peaks are shown

Table 7.1.1.3-8: Radio-HPLC analysis of soil extracts after treatment of soil LUFA 5M with triazole-^{14}C-labeled BAS 750 F and incubation under irradiated conditions (sum of ACN extracts) [% TAR]

DAT	Total	BAS 750 F	Unknown*
		t_R~38.6'	t_R~39.0'
0 I	94.69	94.69	n.d.
0 II	98.21**	93.04	n.d.
0 mean	**96.45**	**93.86**	
1 I	100.15	100.15	n.d.
1 II	100.32	100.32	n.d.
1 mean	**100.23**	**100.23**	
3 I	97.51	97.51	n.d.
3 II	97.02	96.87	0.15
3 mean	**97.27**	**97.26**	
7 I	96.25	96.25	n.d.
7 II	97.18	97.06	0.12
7 mean	**96.72**	**96.71**	
10 I	96.95	96.95	n.d.
10 II	96.07	96.07	n.d.
10 mean	**96.51**	**96.51**	
15 I	94.10	94.10	n.d.
15 II	93.40	93.40	n.d.
15 mean	**93.75**	**93.75**	

ACN = acetonitrile
TAR = total applied radioactivity
DAT = days after treatment
t_R = retention time [min]
n.d. = not detected
* only one unknown peak detected
** sum of ACN and ACN/H_2O extracts

Table 7.1.1.3-9: Radio-HPLC analysis of soil extracts after treatment of soil LUFA 5M with triazole-^{14}C-labeled BAS 750 F and incubation under dark conditions (sum of ACN extracts) [% TAR]

DAT	Total	BAS 750 F	Unknown
		t_R~38.6'	t_R~39.0'
1 I	99.92	99.92	n.d.
1 II	99.70	99.70	n.d.
1 mean	**99.81**	**99.81**	
3 I	98.61	98.61	n.d.
3 II	100.42	100.42	n.d.
3 mean	**99.52**	**99.52**	
7 I	97.02	97.02	n.d.
7 II	97.39	97.39	n.d.
7 mean	**97.21**	**97.21**	
10 I	96.25	96.25	n.d.
10 II	97.43	97.43	n.d.
10 mean	**96.84**	**96.84**	
15 I	95.45	95.45	n.d.
15 II	96.21	96.21	n.d.
15 mean	**95.83**	**95.83**	

ACN = acetonitrile
TAR = total applied radioactivity
DAT = days after treatment
t_R = retention time [min]
n.d. = not detected

The chiral-HPLC analysis of samples of day 0 and day 15 for the two labeled test items revealed that the enantiomeric composition of both substances did not have any relevant differences in this time period. In chlorophenyl-labeled BAS 750 F, an unknown peak was observed, with TAR values not exceeding 1.7%. Results of chiral HPLC analyses are shown in Table 7.1.1.3-10.

Table 7.1.1.3-10: Chiral HPLC analysis of selected soil extracts after treatment of soil LUFA 5M with chlorophenyl-^{14}C- and triazole-^{14}C-labeled BAS 750 F [% TAR]

DAT	Chlorophenyl-U-^{14}C-BAS 750 F						Triazole-3(5)-^{14}C-BAS 750 F			
	Unknown		*R*		*S*		*R*		*S*	
	t_R~	% TAR	t_R~	% TAR	t_R~	% TAR	t_R~	% TAR	t_R~	% TAR
0d I (PA)	6.30'	1.74	13.30'	45.64	15.53'	44.98	12.06'	48.19	14.13'	46.50
15d I (DC)	6.58'	1.40	13.38'	46.38	15.60'	46.68	12.23'	48.15	14.25'	47.30
15d I (PA)	6.37'	1.26	13.38'	44.89	15.58'	43.47	12.24'	47.29	14.27'	46.81

TAR = total applied radioactivity
DAT = days after treatment
t_R = retention time [min]
PA = photolysis sample
DC = dark control sample

E. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

Results of the non-extractable residue characterization performed by humic substance fractionation are given in Table 7.1.1.3-11.

The alkali-soluble radioactivity amounted to 2.3 and 2.0% TAR at day 15 in the photolysis and dark control with the [chlorophenyl-U-^{14}C]-label, and to 4.2% TAR and 2.3% TAR at day 15 in the photolysis and dark control with the [triazole-3(5)-^{14}C]-label, respectively. Since the alkali-soluble radioactivity (humic acids) did not exceed 5% TAR, no further separation of fulvic acids was performed.

Table 7.1.1.3-11: Characterization of non-extractable residues (NER) in soil LUFA 5M after treatment with ^{14}C-BAS 750 F under irradiated conditions [% TAR]

DAT	Position of radiolabel	NER Initial	NaOH extraction	Soil residues after extraction	Sum*
15d I	chlorophenyl-^{14}C	5.2	2.3	2.3	4.6
10d I	triazole-^{14}C	5.1	2.9	1.6	4.5
15d II		7.2	4.2	2.0	6.1

TAR = total applied radioactivity
DAT = days after treatment
* slight deviations from initial NER values have to be attributed to differing LSC results

F. KINETIC MODELING RESULTS

Degradation rates of BAS 750 F were estimated using the software package KinGUI 2 following the recommendations of the FOCUS Kinetics workgroup. The soil residues for the irradiated and the dark control experiment could both be best described by the SFO kinetic fit approach. The $DegT_{50}$/$DegT_{90}$ values obtained with the selected best fit model are presented in Table 7.1.1.3-12 and Table 7.1.1.3-13.

Table 7.1.1.3-12: Trigger endpoints for chlorophenyl-U-^{14}C-BAS 750 F (non-GLP)

Test system	$DegT_{50}$ *[d]*	$DegT_{90}$ *[d]*	Best-fit model	χ^2 error
Photolysis	93.05	309.09	SFO	1.15
Dark control	172.93	574.45	SFO	0.82

Table 7.1.1.3-13: Trigger endpoints for triazole-3(5)-^{14}C-BAS 750 F (non-GLP)

Test system	$DegT_{50}$ *[d]*	$DegT_{90}$ *[d]*	Best-fit model	χ^2 error
Photolysis	169.99	564.69	SFO	0.607
Dark control	224.96	747.31	SFO	0.29

III. CONCLUSION

Irradiation in the soil photolysis experiments with chlorophenyl-U-^{14}C-labeled BAS 750 F and triazole-3(5)-^{14}C-labeled BAS 750 F did not show an influence of light on the degradation behavior and metabolite formation in soil.

There was no relevant degradation during the study period, indicating that BAS 750 F is stable in sunlight. In addition, no metabolites of relevant amounts (> 5%) could be found.

Overall Summary for the Route of Degradation in Soil

The route of degradation of BAS 750 F was investigated using all three available labels (chlorophenyl, triazole and trifluoromethylphenyl). Aerobic soil metabolism demonstrated a simple metabolic profile of BAS 750 F in soil. Two minor metabolites of BAS 750 F were observed and characterized in the soil metabolism studies of BAS 750 F. The metabolite M750F001 (1,2,4-triazole) was observed during the aerobic soil metabolism studies in very low amounts. Only in a single soil did the levels reach above 5% TAR at a single measureable time point (5.2% TAR single rep at 90 DAT; 5.1% avg of 2 reps) and then subsequent measurements showed decline of the levels of M750F001 at the end of the study. M750F003 was also observed in low amounts, never reaching higher than 2.2% TAR in any test system. The degradation of BAS 750 F in aerobic soil systems shows moderate mineralization (approximately 9.7% TAR within 121 days, chlorophenyl label).

The greatest sink observed for BAS 750 F in these systems was bound residues reaching levels of 12.6-26.7% TAR after 121 days.

The anaerobic soil metabolism also showed little overall degradation with bound residues being the main sink, giving 8.39-16.37% TAR of non-extractable residues after 120 days.

The results from a soil photolysis study showed no degradation. It can be concluded that photolysis on soil surfaces is not expected to be a major degradation pathway for BAS 750 F.

BAS 750 F is applied as a racemic mixture (1:1) of enantiomers. Therefore the enantiomeric ratio was monitored throughout all relevant studies in which enantiospecific selective degradation and/or enrichment could be envisioned. This resulted in a slight shift of enantiomeric ratio from 50:50 to 45:55 in two soils of the aerobic soil metabolism study. This is not deemed significant as it is almost within the experimental variance of the analytical method. Even so, an insignificant shift such as this would not change the overall exposure profile. In all other studies the measured ratio saw an even more insignificant shift staying almost at 50:50. A more detailed discussion of the fate of the enantiomers of BAS 750 F is provided in Document N5.

The collected data leads to the proposed metabolic scheme found below (Figure 7.1.1-1).

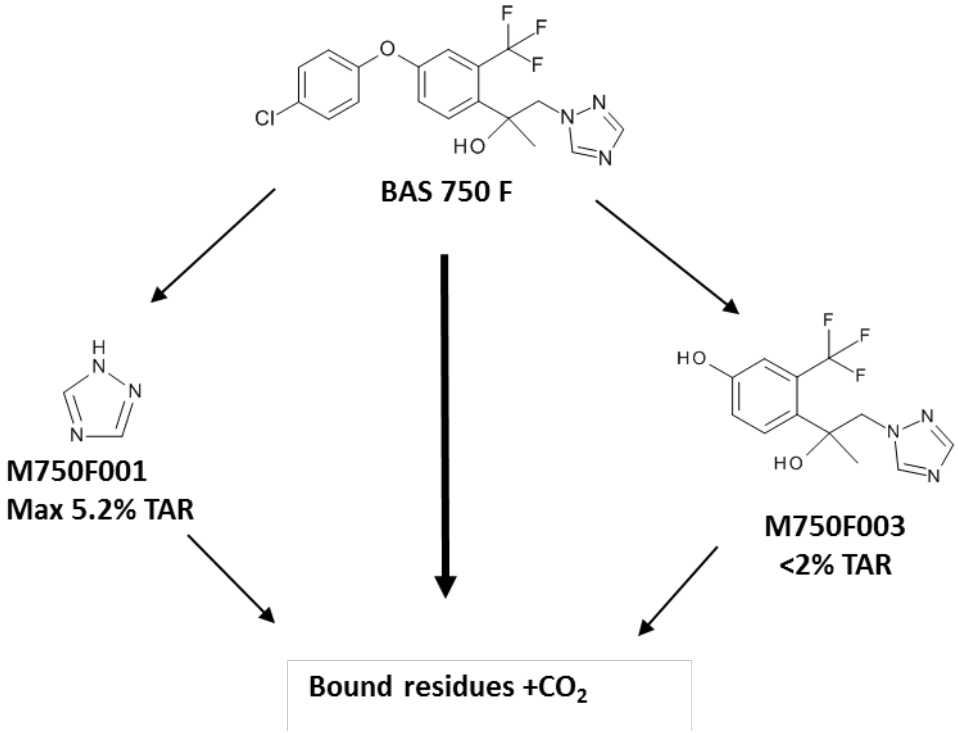


Figure 7.1.1-1: Overall Soil Metabolism Scheme of BAS 750 F

CA 7.1.2 Rate of degradation in soil

The rate of degradation of BAS 750 F was investigated in four soils using radiolabels at 3 different positions. All results from the studies are summarized at the end of the section.

CA 7.1.2.1 Laboratory studies

CA 7.1.2.1.1 Aerobic degradation of the active substance

Report:	CA 7.1.2.1.1/1 Staudenmaier H., Dalkmann, P., 2015c Degradation of BAS 750 F in soil under aerobic conditions 2014/1275178
Guidelines:	OECD 307, EPA 835.4100
GLP:	yes (certified by Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz)

EXECUTIVE SUMMARY

In the present study, the rate of degradation of ^{14}C-BAS 750 F was investigated in two soils incubated under aerobic conditions at 20 °C. The soils originated from Germany (Limburgerhof) and the USA (Indiana).

The soils were freshly collected from the field and passed through a 2 mm sieve before use. The soils were treated in a batch application at a nominal concentration of 0.4 mg test item per kg dry soil. The soils were adjusted to 40% of the maximum water holding capacity. Soil portions of 100 g (dry weight basis) were weighed into test vessels and incubated in the dark at a temperature of 20 ± 2°C. The test vessels were continuously ventilated with moistened air and the exiting air was passed through a trapping system consisting of flasks containing ethylene glycol, aqueous sulfuric acid, and aqueous sodium hydroxide for trapping organic volatiles and $^{14}CO_2$, respectively. Prior to the treatment, in the middle and at the end of the study, the microbial biomass was determined. The results showed that the soils were viable during the study.

Samples were taken at 0, 3, 7, 14, 30, 58, 91 and 120 days after treatment (DAT). At all sampling dates, soil samples were worked up in duplicate, an additional soil sample was stored in a freezer as a reserve.

Soil aliquots were consecutively extracted twice with acetonitrile, twice with acetonitrile/water (80/20, v/v), and twice with acetonitrile/water (50/50, v/v). The extracts were measured for radioactivity by liquid scintillation counting (LSC). Both acetonitrile, as well as all acetonitrile/water extracts were pooled, concentrated and subjected to radio-HPLC analysis. Non-extractable residues (NER) were determined by combustion and LSC analysis. A total balance of radioactivity in soil was established for each sampling interval.

The material balance throughout the incubation period ranged from 99.8 to 103.7% of the total applied radioactivity (TAR).

The amount of extractable radioactive residues decreased slightly from 99.5% TAR (both soils) at 0 DAT to 86.0% TAR (soil Li10) and 89.6% TAR (soil Indiana) at 120 DAT. The portion of NER increased during the course of the study from 0.5% TAR (both soils) at 0 DAT to 12.6% TAR (soil Li10) and 12.7% TAR (Indiana) at 120 DAT. Mineralization of the test item was negligible with total amounts not exceeding 0.5% TAR at the end of the study. No other volatile products were detected.

The unchanged test item ^{14}C-BAS 750 F represented the major radioactive component in the extracts. In both soils metabolites M750F001 and M750F003 were detected. M750F001 reached a maximum of 1.5% TAR (soil Li10) and 1.2% TAR (soil Indiana), respectively. M750F003 was detected in maximum amounts of 1.8% TAR (soil Li10) and 0.6% TAR (soil Indiana). Several other minor metabolites were detected, in sum never exceeding 0.8% and 1.2% TAR in soil Li10 and soil Indiana, respectively.

Throughout the incubation period, *R*- and *S*-enantiomers of BAS 750 F were almost equally distributed in the pooled acetonitrile, as well as in the pooled acetonitrile/water extracts of soil Indiana. In pooled extracts of soil Li10 the ratio changed from an equal distribution of both enantiomers to a slightly higher ratio of the *S*-enantiomer of BAS 750 F at the end of the study.

Kinetic analysis and calculations of $DegT_{50}$ and $DegT_{90}$ values for BAS 750 F in soil were performed following the recommendations of the FOCUS Kinetics workgroup. The analysis was conducted by non-linear regression methods employing the software tool KinGUI. For both soils, calculated best-fit $DegT_{50}$ and $DegT_{90}$ values used as trigger endpoints were > 1000 days, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$
Position of radiolabel:	triazole-3(5)-^{14}C
Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%, see certificate of analysis in the final report
Purity:	98.9%

2. Soil

Two different agricultural soils from Germany (Li10) and the US (Indiana) were used in this study sampled from 0-20 cm (soil Li10) and from 0-15 cm (soil Indiana) depth. After collecting the soil from the field, the soil was kept at room temperature until sieving. The soil was passed through a 2 mm sieve, remoistened to approximately 7-15% soil moisture and stored at about 4 °C in the dark no longer than 3 months before use. An overview of soil parameters is given in Table 7.1.2.1.1-1.

Table 7.1.2.1.1-1: Soil characteristics

Soil designation	**Li10** **BASF soil No. 13/1680/04** Germany (Limburgerhof)	**Indiana** **BASF soil No. 13/1806/01** Unites States (Indiana)
DIN 4220 Particle size distribution *[%]* sand 0.063 – 2 mm silt 0.002 – 0.063 mm clay < 0.002 mm textural class	 81.8 13.3 5.0 loamy sand (Sl2)	 n.d. n.d. n.d. n.d.
USDA Particle size distribution *[%]* sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 84.0 11.0 5.0 loamy sand	 35 46 19 loam
Organic C *[%]*	0.93	n.d.
Organic matter *[%]* [a]	1.60	2.0
pH [H_2O]	6.6	6.3
pH [$CaCl_2$]	6.1	5.8
cation exchange capacity *[$cmol^+ kg^{-1}$]*	3.7	10.3
Max. water holding capacity *[g /100 g dry weight]*	26.9	33.3
microbial biomass (after 0 days of incubation) *[mg C/100 g dry soil]*	25.2	42.3
microbial biomass (after 58 days of incubation) *[mg C/100 g dry soil]* [b]	18.9	29.5
microbial biomass (after 122 days of incubation) *[mg C/100 g dry soil]* [b]	10.5	24.0

n.d. = Not determined
[a] Organic matter = organic carbon x 1.724
[b] Determined at BASF test facility Limburgerhof

B. STUDY DESIGN

1. Experimental conditions

The test item was applied at a nominal concentration of 0.4 mg ^{14}C-BAS 750 F per kg dry soil, which corresponds to a field application rate of 150 g a.s. ha^{-1} (calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}). Portions of 100 g soil (dry weight basis) were then filled into test vessels.

For incubation, all test vessels were connected in line with aeration tubes in the soil metabolism apparatus. During the study, samples were continuously aerated with a slight stream of moistened synthetic air. For removing carbon dioxide, the air was passed through a bottle with NaOH before passing the test vessels. For trapping of volatiles possibly evolving from soil during the incubation, test vessels were connected to three gas washing flasks containing ethylene glycol, 0.5 M H_2SO_4, and 0.5 M NaOH. The treated soils were incubated at 40% of the maximum water holding capacity and 20 ± 2 °C in the dark.

To determine the microbial biomass at 0, 58 and 122 days after treatment (DAT), extra test vessels were treated with acetonitrile in a volume corresponding to the application solution but without test item. The samples were incubated under the same conditions (dark, 20 ± 2 °C) as the treated soils.

2. Sampling

Sampling intervals for the samples treated at the normal rate were 0, 3, 7, 14, 30, 58, 91 and 120 DAT.

3. Description of analytical procedures

For the determination of the extractable radioactive residues (ERR), soil samples were consecutively extracted twice with acetonitrile (ACN), twice with ACN/water (80/20; v/v), and twice with ACN/water (50/50; v/v). Each extract was analyzed for radioactivity by liquid scintillation counting (LSC). The ACN-extracts as well as the ACN/water-extracts were pooled and each solution was concentrated. Then the residues were re-dissolved in a well-defined volume of solvent and analyzed by radio HPLC.

The soil residues remaining after extraction were dried, homogenized by means of an analytical mill, and aliquots were combusted in a biological oxidizer. The evolved $^{14}CO_2$ from each combusted aliquot was trapped and measured by LSC to determine the amount of the non-extractable residues (NER). The NER fraction was not further characterized within this study.

The method to measure the microbial biomass was based on the determination of oxygen consumption upon addition of glucose. Results revealed, that the soils were still viable and microbially active from the beginning (0 DAT) until the end of the study (122 DAT).

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) as well as modeling endpoints. Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*].

The software package KinGUI (version 2.2014.224.1704) was used for parameter fitting [*SCHÄFER et al. (2007); WITT et al. (2014)*]. The error tolerance and the number of iterations of the optimization tool were set to 0.000001 and 100, respectively.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested in order to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO), the Gustafson-Holden model (FOMC), and bi-exponential (DFOP) kinetics are already implemented in KinGUI. The Goodness-of-fit was evaluated by visual assessment, χ^2 minimum error, and type-I-error rate (t-test) [for details see Chapter 6.3 in *FOCUS (2006)*].

Replicate measurements were considered for the parameter estimation. The initial concentration of the applied test item was set to the material balance recovered at day 0. The χ^2 value was calculated for the kinetic model as recommended by FOCUS, considering the average of replicate measurements. For the individual parameters, the t-test statistics were based on number of individual measurements.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Total recoveries of radioactivity extracted from the soils are summarized in Table 7.1.2.1.1-2 and Table 7.1.2.1.1-3. The overall material balace throughout the incubation period ranged from 98.5 to 105.4% of the total applied radioactivity (TAR).

Table 7.1.2.1.1-2: Recovery and distribution of radioactivity in soil Li10 after treatment with triazole-3(5)-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues							NER	Volatiles [a]		Material balance
	Acetonitrile		ACN/H_2O (80/20)		ACN/H_2O (50/50)		Total		NaOH (CO_2)	Others [b]	
	1	2	1	2	1	2					
0	74.5	17.2	5.4	1.4	0.4	0.1	99.0	0.5	n.a.	n.a.	99.5
0	74.9	17.8	5.4	1.4	0.4	0.1	100.0	0.5	n.a.	n.a.	100.5
0 (mean)	**74.7**	**17.5**	**5.4**	**1.4**	**0.4**	**0.1**	**99.5**	**0.5**	-	-	**100.0**
3	71.9	17.8	5.8	1.6	0.7	0.3	98.0	1.7	0.1	0.0	99.8
3	72.6	17.8	5.8	1.6	0.7	0.3	98.8	1.7	0.1	0.0	100.6
3 (mean)	**72.3**	**17.8**	**5.8**	**1.6**	**0.7**	**0.3**	**98.4**	**1.7**	**0.1**	**0.0**	**100.2**
7	71.3	17.2	6.0	1.7	0.8	0.3	97.2	2.6	0.1	0.0	100.0
7	70.7	17.7	6.0	1.7	0.8	0.4	97.3	2.6	0.1	0.0	100.0
7 (mean)	**71.0**	**17.5**	**6.0**	**1.7**	**0.8**	**0.3**	**97.3**	**2.6**	**0.1**	**0.0**	**100.0**
14	68.6	17.9	6.3	1.8	0.8	0.5	95.9	3.7	0.1	0.0	99.7
14	68.7	18.3	6.3	1.9	0.8	0.5	96.5	3.8	0.1	0.0	100.4
14 (mean)	**68.7**	**18.1**	**6.3**	**1.9**	**0.8**	**0.5**	**96.2**	**3.8**	**0.1**	**0.0**	**100.1**
30	65.4	17.3	6.6	2.1	1.1	0.5	93.0	6.1	0.2	0.0	99.3
30	65.9	17.1	6.7	2.2	1.1	0.5	93.5	5.9	0.2	0.0	99.6
30 (mean)	**65.6**	**17.2**	**6.7**	**2.2**	**1.1**	**0.5**	**93.3**	**6.0**	**0.2**	**0.0**	**99.5**
58	62.8	16.6	6.6	2.3	1.3	0.7	90.2	8.6	0.3	0.0	99.1
58	63.1	16.7	6.7	2.3	1.3	0.6	90.7	8.6	0.3	0.0	99.6
58 (mean)	**63.0**	**16.7**	**6.6**	**2.3**	**1.3**	**0.6**	**90.5**	**8.6**	**0.3**	**0.0**	**99.4**
91	60.3	16.5	6.7	2.4	1.6	0.7	88.1	10.5	0.4	0.0	99.0
91	59.7	16.5	6.7	2.3	1.6	0.7	87.5	10.6	0.4	0.0	98.5
91 (mean)	**60.0**	**16.5**	**6.7**	**2.3**	**1.6**	**0.7**	**87.8**	**10.6**	**0.4**	**0.0**	**98.8**
120	58.9	15.7	6.6	2.5	1.6	0.8	86.0	12.6	0.5	0.0	99.1
120	58.3	16.2	6.8	2.5	1.6	0.7	86.1	12.5	0.5	0.0	99.1
120 (mean)	**58.6**	**16.0**	**6.7**	**2.5**	**1.6**	**0.8**	**86.0**	**12.6**	**0.5**	**0.0**	**99.1**

TAR = Total applied radioactivity (100% = 0.425 mg kg^{-1})

ACN = Acetonitrile

NER = Non-extractable residues

n.a. = Not analyzed

[a] Values for volatile radioactive residues were calculated cumulatively

[b] Sum of volatile radioactive residues in H_2SO_4 and ethylene glycol traps

Table 7.1.2.1.1-3: Recovery and distribution of radioactivity in soil Indiana after treatment with triazole-3(5)-^{14}C-labeled BAS 750 F [% TAR]

Days after treatment	Extractable residues							NER	Volatiles [a]		Material balance
	Acetonitrile		ACN/H_2O (80/20)		ACN/H_2O (50/50)		Total		NaOH (CO_2)	Others [b]	
	1	2	1	2	1	2					
0	79.0	13.7	4.9	1.1	0.4	0.2	99.3	0.5	n.a.	n.a.	99.9
0	79.1	13.7	5.0	1.2	0.4	0.2	99.6	0.5	n.a.	n.a.	100.1
0 (mean)	**79.0**	**13.7**	**5.0**	**1.2**	**0.4**	**0.2**	**99.5**	**0.5**	-	-	**100.0**
3	77.0	14.8	5.6	1.8	0.9	0.4	100.3	2.2	0.0	0.0	102.6
3	78.9	14.8	5.8	1.9	0.9	0.4	102.6	2.2	0.0	0.0	104.9
3 (mean)	**77.9**	**14.8**	**5.7**	**1.8**	**0.9**	**0.4**	**101.5**	**2.2**	**0.0**	**0.0**	**103.7**
7	73.9	14.6	6.4	2.0	1.2	0.5	98.7	3.3	0.1	0.0	102.1
7	73.5	14.5	6.3	2.0	1.2	0.5	98.1	3.2	0.1	0.0	101.4
7 (mean)	**73.7**	**14.6**	**6.4**	**2.0**	**1.2**	**0.5**	**98.4**	**3.3**	**0.1**	**0.0**	**101.7**
14	72.2	14.5	6.3	2.3	1.1	0.8	97.0	4.8	0.1	0.0	101.9
14	72.8	14.7	6.1	2.2	1.1	0.8	97.6	4.9	0.1	0.0	102.7
14 (mean)	**72.5**	**14.6**	**6.2**	**2.2**	**1.1**	**0.8**	**97.3**	**4.9**	**0.1**	**0.0**	**102.3**
30	71.9	14.7	6.9	2.7	1.6	0.7	98.5	6.8	0.1	0.0	105.4
30	67.7	14.6	6.9	2.7	1.6	0.7	94.3	6.5	0.1	0.0	101.0
30 (mean)	**69.8**	**14.7**	**6.9**	**2.7**	**1.6**	**0.7**	**96.4**	**6.6**	**0.1**	**0.0**	**103.2**
58	67.9	15.1	7.3	2.9	2.0	0.9	96.0	9.2	0.2	0.0	105.4
58	64.1	14.0	6.9	2.7	1.9	0.9	90.6	8.8	0.2	0.0	99.6
58 (mean)	**66.0**	**14.5**	**7.1**	**2.8**	**2.0**	**0.9**	**93.3**	**9.0**	**0.2**	**0.0**	**102.5**
91	61.7	14.1	7.1	2.9	2.3	0.9	89.1	10.8	0.2	0.0	100.1
91	62.4	14.4	7.3	3.0	2.4	1.0	90.5	10.7	0.2	0.0	101.5
91 (mean)	**62.1**	**14.3**	**7.2**	**3.0**	**2.3**	**1.0**	**89.8**	**10.8**	**0.2**	**0.0**	**100.8**
120	61.6	14.1	7.2	3.2	2.3	1.0	89.4	12.7	0.3	0.0	102.4
120	62.0	13.9	7.2	3.3	2.3	1.1	89.7	12.7	0.3	0.0	102.7
120 (mean)	**61.8**	**14.0**	**7.2**	**3.2**	**2.3**	**1.0**	**89.6**	**12.7**	**0.3**	**0.0**	**102.6**

TAR = Total applied radioactivity (100% = 0.417 mg kg^{-1})
ACN = Acetonitrile
NER = Non-extractable residues
n.a. = Not analyzed
[a] values for volatile radioactive residues were calculated cumulatively
[b] sum of volatile radioactive residues in H_2SO_4 and ethylene glycol traps

B. EXTRACTABLE AND BOUND RESIDUES

The amount of extractable radioactive residues (ERR) in soil decreased slowly from 99.5% TAR (both soils) at 0 DAT to 86.0% TAR (soil Li10) and 89.6% TAR (soil Indiana) after 120 DAT, respectively.

The amounts of non-extractable radioactive residues (NER) increased during the incubation period in both soils reaching 12.6% TAR and 12.7% TAR after 120 days of incubation in soil Li10 and soil Indiana, respectively.

C. VOLATILIZATION

The formation of volatiles (exclusively related to the mineralization to $^{14}CO_2$) amounted to a total of only 0.5% TAR in soil Li10 and only 0.3% TAR in soil Indiana after 120 days of incubation. In ethylene glycol, as well as in sulfuric acid traps no significant amounts of radioactive residues were detected.

D. TRANSFORMATION OF PARENT COMPOUND

A summary of HPLC results are presented in Table 7.1.2.1.1-4 and Table 7.1.2.1.1-5.

The test item ^{14}C-BAS 750 F represented the only major radioactive fraction in the extracts of soil Li10 and soil Indiana. The degradation of BAS 750 F was rather slow in both tested soils. Amounts of ^{14}C-BAS 750 F decreased from an average value of 98.9% TAR at 0 DAT to 83.5% TAR at 120 DAT and from 98.6% TAR at 0 DAT to and 87.1% TAR at 120 DAT in soils Li10 and soil Indiana, respectively.

Considering both soils, the known metabolite M750F001 was detected, reaching a maximum of 1.5% TAR (soil Li10) and 1.3% TAR (soil Indiana) in single replicates. Metabolite M750F003 was measured in soil extracts from 3 DAT (soil Li10) and 7 DAT (soil Indiana) on, reaching maximum amounts of 1.8% TAR and 0.6% TAR in the respective soils.

In addition, up to seven other metabolites were detected in both soils. However, none of them exceeded 0.7% TAR (soil Li10) and 0.6% TAR (soil Indiana) in a single replicate at any sampling time. The sum of all other metabolites never exceeded 1.2% TAR.

Table 7.1.2.1.1-4: Radio-HPLC analysis of soil extracts: after treatment of soil Li10 with triazole-3(5)-^{14}C-labeled BAS 750 F (sum of ACN and ACN/water extracts) [% TAR]

Days after treatment	ERR (total)	7.2 min M750F001	28.4 min M750F003	42.3 min BAS 750 F	sum others [a]
0	99.0	0.4	-	98.6	0.0
0	100.0	0.8	-	99.2	0.0
0 (mean)	**99.5**	**0.6**	-	**98.9**	**0.0**
3	98.0	0.4	0.4	97.0	0.2
3	98.8	0.8	0.3	97.6	0.1
3 (mean)	**98.4**	**0.6**	**0.4**	**97.3**	**0.1**
7	97.2	0.5	0.6	96.0	0.2
7	97.3	0.5	0.8	95.9	0.1
7 (mean)	**97.3**	**0.5**	**0.7**	**95.9**	**0.1**
14	95.9	0.7	0.9	93.7	0.6
14	96.5	0.8	0.9	94.1	0.7
14 (mean)	**96.2**	**0.8**	**0.9**	**93.9**	**0.6**
30	93.0	0.6	1.7	90.3	0.4
30	93.5	1.0	1.8	89.8	1.0
30 (mean)	**93.3**	**0.8**	**1.8**	**90.0**	**0.7**
58	90.2	0.8	1.7	87.2	0.5
58	90.7	1.3	1.2	87.8	0.4
58 (mean)	**90.5**	**1.1**	**1.4**	**87.5**	**0.5**
90	88.1	1.4	1.1	84.7	0.8
90	87.5	1.5	0.9	84.3	0.7
91 (mean)	**87.8**	**1.5**	**1.0**	**84.5**	**0.8**
120	86.0	1.4	0.8	83.4	0.4
120	86.1	1.1	0.9	83.7	0.4
120 (mean)	**86.0**	**1.3**	**0.9**	**83.5**	**0.4**

TAR = total applied radioactivity (100% = 0.425 mg kg^{-1})
ERR = extractable radioactive residues
[a] ≤ 0.4% TAR each

Table 7.1.2.1.1-5: Radio-HPLC analysis of soil extracts: after treatment of soil Indiana with triazole-3(5)-^{14}C-labeled BAS 750 F (sum of ACN and ACN/water extracts) [% TAR]

Days after treatment	ERR (total)	7.2 min M750F001	28.4 min M750F003	42.3 min BAS 750 F	sum others [a]
0	99.3	0.7	-	98.5	0.1
0	99.6	0.9	-	98.8	0.0
0 (mean)	**99.5**	**0.8**	**-**	**98.6**	**0.1**
3	100.3	0.8	-	99.0	0.5
3	102.6	0.6	-	101.9	0.2
3 (mean)	**101.5**	**0.7**	**-**	**100.5**	**0.3**
7	98.7	0.5	0.0	97.9	0.2
7	98.1	0.6	-	97.3	0.2
7 (mean)	**98.4**	**0.6**	**0.0**	**97.6**	**0.2**
14	97.0	0.8	0.3	94.7	1.3
14	97.6	0.8	0.3	95.4	1.1
14 (mean)	**97.3**	**0.8**	**0.3**	**95.0**	**1.2**
30	98.5	0.6	0.5	97.2	0.3
30	94.3	0.8	0.4	92.8	0.3
30 (mean)	**96.4**	**0.7**	**0.4**	**95.0**	**0.3**
58	96.0	1.0	0.7	93.9	0.4
58	90.6	0.9	0.6	88.2	0.9
58 (mean)	**93.3**	**0.9**	**0.6**	**91.1**	**0.6**
90	89.1	0.9	0.6	86.8	0.8
90	90.5	1.1	0.4	88.0	1.1
91 (mean)	**89.8**	**1.0**	**0.5**	**87.4**	**0.9**
120	89.4	1.1	0.5	86.9	1.0
120	89.7	1.3	0.5	87.3	0.7
120 (mean)	**89.6**	**1.2**	**0.5**	**87.1**	**0.8**

TAR = total applied radioactivity (100% = 0.417 mg kg^{-1})
ERR = extractable radioactive residues
[a] ≤ 0.6% TAR each

Chiral HPLC results

The ratio of *R*- and *S*-enantiomers of BAS 750 F in soil extracts in the course of the incubation was determined by chiral HPLC. Throughout the incubation period, the BAS 750 F enantiomers were almost equally distributed in the pooled soil extracts of soil Indiana. In soil Li10, the ratio of the *S*-enantiomer of BAS 750 F slightly increased from about 50% region of interest (ROI) at day 0 to 52.9% at the end of the incubation period in the pooled acetonitrile extracts. In the pooled acetonitrile/water extracts, the ratio changed from an equal distribution to 52.0% for the *S*-enantiomer.

F. KINETIC MODELING RESULTS

The estimated best-fit $DegT_{50}$ and $DegT_{90}$ values of BAS 750 F in aerobic soil are summarized in Table 7.1.2.1.1-6.

Table 7.1.2.1.1-6: Trigger endpoints for BAS 750 F (non-GLP)

Soil	Kinetic model	χ^2 error	Visual fit	$DegT_{50}$ [d]	$DegT_{90}$ [d]
Li10	SFO	1.6	Poor	477.1	>1000
	FOMC	**0.3**	**Good**	**>1000**	**>1000**
	DFOP	0.4	Good	857.8	>1000
Indiana	SFO	1.2	Poor	569.8	>1000
	FOMC	**0.8**	**Good**	**>1000**	**>1000**
	DFOP	0.9	Good	733.6	>1000

Bold = Selected as best-fit model to derive trigger endpoints.

III. CONCLUSION

BAS 750 F degraded slowly in soil during incubation under aerobic conditions. Dissipation mainly occurred by a formation of non-extractable residues whereas mineralization was negligible.

Report: CA 7.1.2.1.1/2
Platz K., 2015 a
Normalized modelling DegT50 endpoints of BAS 750 F derived from laboratory soil degradation experiments
2015/1239053
Guidelines: FOCUS Kinetics (2006) SANCO/10058/2005 version 1.1 of Dec 2014
GLP: no

EXECUTIVE SUMMARY

The degradation of the fungicide BAS 750 F in aerobic soil has been investigated in different laboratory metabolism studies. The purpose of this evaluation was to derive modeling $DegT_{50}$ endpoints of BAS 750 F following the recommendation of the FOCUS Kinetics workgroup on the basis of the kinetic evaluations that were already included in the experimental study reports. Subsequently, the modeling endpoints were normalized to moisture reference conditions at field capacity at pF2. Normalization to standard temperature conditions was not necessary as the experiments were already conducted at 20° C.

The geometric mean value of the modeling $DegT_{50}$ values at reference conditions (20°C, pF2) is 438.6 days.

I. MATERIAL AND METHODS

The degradation of BAS 750 F in aerobic soil was examined in different metabolism studies conducted in the laboratory [*CA 7.1.1.1/1, BASF DocID 2014/1275177; CA 7.1.1.1/2, BASF DocID 2015/1003306; CA 7.1.2.1.1/1, BASF DocID 2014/1275178*]. The purpose of the study was to derive normalized modeling $DegT_{50}$ endpoints for BAS 750 F following the recommendation of the FOCUS Kinetics workgroup [*FOCUS (2006*].

The basic kinetic analysis of the BAS 750 F laboratory soil degradation experiments that is necessary to derive suitable $DegT_{50}$ endpoints for modeling purposes is already provided in the respective experimental study reports. The kinetic evaluation was originally performed to derive trigger endpoints but can also be used to derive suitable modeling endpoints.

Kinetic models included in the evaluations

For each data set, the kinetic models proposed by FOCUS Kinetics [*FOCUS (2006)*] were tested, i.e. single first order (SFO) kinetics, the Gustafson-Holden model (FOMC) and the bi-exponential (DFOP) kinetics.

The derivation of the modeling endpoints is based on the following simplified decision scheme that is mentioned in the FOCUS kinetic report [*FOCUS (2006)*]:

Modeling endpoints:
->Run SFO as a first step
->Check visual fit and calculate error percentage at which $\chi 2$ test passed
->If error % < 15% and visual fit acceptable, use SFO DT_{50}

else
->If 10% of initial reached in study period: Calculate DT_{50} as DT_{90} FOMC / 3.32
->If 10% of initial **not** reached in study period: Use longer DT_{50} (slow phase) of HS or DFOP

As in the study period of the different degradation experiments the BAS 750 F soil residues did not reach 10% of the initial amount the DFOP kinetic model was considered as appropriate bi-phasic model for kinetic analysis.

Derivation of modeling endpoints

The goodness-of-fit of the kinetic models as recommended by the FOCUS Kinetics guidance [*FOCUS (2006)*] was already assessed by visual inspection and statistical measures, as provided in the respective study reports. For derivation of modeling endpoints, the respective SFO fits were compared with the results from the bi-phasic kinetic DFOP kinetic fit approaches for each soil.

Normalization to reference conditions

According to FOCUS *(2006),* $DegT_{50}$ values for modeling purposes obtained from laboratory studies should be normalized to reference conditions at a temperature of 20°C and soil moisture at pF2 to account for the different soil temperature and moisture conditions during incubation. The temperature and soil moisture normalization was performed according to FOCUS *(2006)*.

The actual soil moisture (40% MWHC) and the reference moisture at field capacity at pF2 were taken from the experimental study reports. In case that the moisture content of the soils at pF2 was not available, the default value for each soil type given in FOCUS [*FOCUS (2000): "FOCUS groundwater scenarios in the EU review of active substances" Report of the FOCUS Groundwater Scenarios Workgroup, EC Document Reference Sanco/321/2000 rev. 2, 202 pp*.] was used.

No temperature correction had to be performed as the study was already conducted at reference conditions.

Experimental data

The statistical indices of the different kinetic modeling approaches for each soil degradation experiment taken from the experimental study reports are summarized in Table 7.1.2.1.1-7. The kinetic fit approaches that were finally considered to derive $DegT_{50}$ modeling endpoints are marked bold.

Table 7.1.2.1.1-7: Derivation of modeling endpoints

Reference	Soil/Label	Kinetic model	χ^2 error	Visual fit	p (t-test)
BASF DocID 2014/1275177	LUFA 5 M/ Chlorophenyl	SFO	1.4	Poor	k: <0.01
		DFOP	**0.4**	**Good**	**k2: <0.01**
	LUFA 5 M/ Triazole	SFO	1.4	Poor	k: <0.01
		DFOP	**0.2**	**Good**	**k2: <0.01**
	New Jersey/ Chlorophenyl	SFO	2.6	Poor	k: <0.01
		DFOP	**0.8**	**Good**	**k2: <0.01**
	New Jersey/ Triazole	SFO	2.7	Poor	k: <0.01
		DFOP	**0.9**	**Good**	**k2: not sign.**
BASF DocID 2014/1275178	Li10/ Triazole	SFO	1.6	Poor	k: <0.01
		DFOP	**0.4**	**Good**	**k2: <0.01**
	Indiana/ Triazole	**SFO**	**1.2**	**Acceptable**	**k: <0.01**
		DFOP	0.9	Good	k2: <0.01
BASF DocID 2015/1003306	New Jersey/ trifluoromethylphenyl	SFO	2.4	Poor	k: <0.01
		DFOP	**1.3**	**Good**	**k2: <0.01**

II. RESULTS AND DISCUSSION

The actual $DegT_{50}$ at study conditions ($DegT_{50,act}$), the moisture correction factors and the normalized endpoints at reference conditions (20°C, pF 2) are provided in Table 7.1.2.1.1-8.
In case that the endpoint was derived from a DFOP kinetic model, the $DegT_{50}$ of the slow phase was taken into account (conservative approach). For one soil (New Jersey, triazole label), no reliable degradation rate constants were estimated. Hence, according to FOCUS a conservative default $DegT_{50}$ value of 1000 d was taken into account for this experiment.

According to good modeling practice, the geometric mean of $DegT_{50}$ endpoints derived from identical soils were considered as representative value for this soil.

Table 7.1.2.1.1-8: Modeling $DegT_{50}$ endpoints at reference conditions

Reference	Soil/Label	Kinetic model	χ^2 error [%]	Modeling endpoints $DegT50_{act}$ [1] [d]	f_{moist}	Modeling endpoints $DegT50_{norm}$ [2] [d]
BASF DocID 2014/1275177	LUFA 5 M/ Chlorophenyl	DFOP	0.4	557.2	0.59	329.0
	LUFA 5 M/ Triazole	DFOP	0.2	602.2		355.5
Geometric mean LUFA 5 M						**342.0**
BASF DocID 2014/1275177	New Jersey/ Chlorophenyl	DFOP	0.8	240.9	0.69	166.9
	New Jersey/ Triazole	DFOP	0.9	1000 [3]		1000 [3]
BASF DocID 2015/1003306	New Jersey/ trifluoromethylphenyl	DFOP	1.3	290.1	0.54	156.4
Geometric mean New Jersey						**296.7**
BASF DocID 2014/1275178	Li10/ Triazole	DFOP	0.4	994.3	1.00	994.3
	Indiana/ Triazole	SFO	1.2	569.8	0.64	366.7
Geometric mean of different soils						**438.6**

[1] $DegT_{50}$ at actual incubation conditions
[2] Normalized $DegT_{50}$ at reference conditions
[3] Conservative default $DegT_{50}$ as k is not estimated significant different from zero

III. CONCLUSION

Modeling $DegT_{50}$ endpoints of BAS 750 F were derived following the recommendation of the FOCUS Kinetics workgroup on the basis of the kinetic evaluations that were already included in the experimental study reports. Subsequently, the modeling endpoints were normalized to reference conditions (20°C, pF 2).

The geometric mean value of the modeling $DegT_{50}$ values at reference conditions (20°C, pF2) is 438.6 days.

CA 7.1.2.1.2 Aerobic degradation of metabolites, breakdown and reaction products

Report: CA 7.1.2.1.2/1
Szegedi K., 2015 a
Estimation of the formation fraction of 1,2,4-triazole from BAS 750 F
2015/1260802
Guidelines: <none>
GLP: no

EXECUTIVE SUMMARY

The purpose of this evaluation was the estimation of a formation fraction in soil for 1,2,4-triazole (M750F001) from BAS 750 F for use in environmental fate models.

The evaluation was based on the results of aerobic soil degradation studies with BAS 750 F where 1,2,4-triazole occurred at low amounts and EU agreed degradation parameters for 1,2,4-triazole. A "shell" approach was followed in which the formation fractions of 1,2,4-triazole were adjusted manually so that the simulated residue curve covers all measured residues in the different soils (like in a shell). For this purpose, a compartment model including BAS 750 F and 1,2,4-triazole was implemented in the software package ModelMaker.

The estimated formation fractions of 1-2-4-triazole ranged from 0.11 to 0.50, with an arithmetic mean value of 0.32.

I. MATERIAL AND METHODS

The aim of the study was to estimate the formation fraction of 1,2,4-triazole from BAS 750 F (M750F001) in a conservative manner. The evaluation was based on the findings of aerobic soil degradation studies with BAS 750 F where 1,2,4-triazole was detected at low amounts of 0.6 to 5.2% TAR (one single measurement) [*CA 7.1.1.1/1, BASF DocID 2014/1275177; CA 7.1.2.1.1/1, BASF DocID 2014/1275178*].

Degradation parameters for BAS 750 F were taken from the best kinetic fits obtained in the kinetic evaluations of the soil degradation experiments in which 1,2,4-triazole was detected (Table 7.1.2.1.2-1). For 1,2,4-triazole, EU agreed endpoints recommended for use in environmental fate models were considered as degradation parameters [*CRD (2014): Triazole Derived Metabolite: 1,2,4-Triazole. Proposed revision to DT_{50} Summary, Scientific Evaluation and Assessment July 2011, revised September 2011 (after comments from MS and EFSA) and further revised January 2013 (minor clarifications added post-commenting)*].

Table 7.1.2.1.2-1: Best fit kinetic models and their parameters for BAS 750 F in four soils [a]

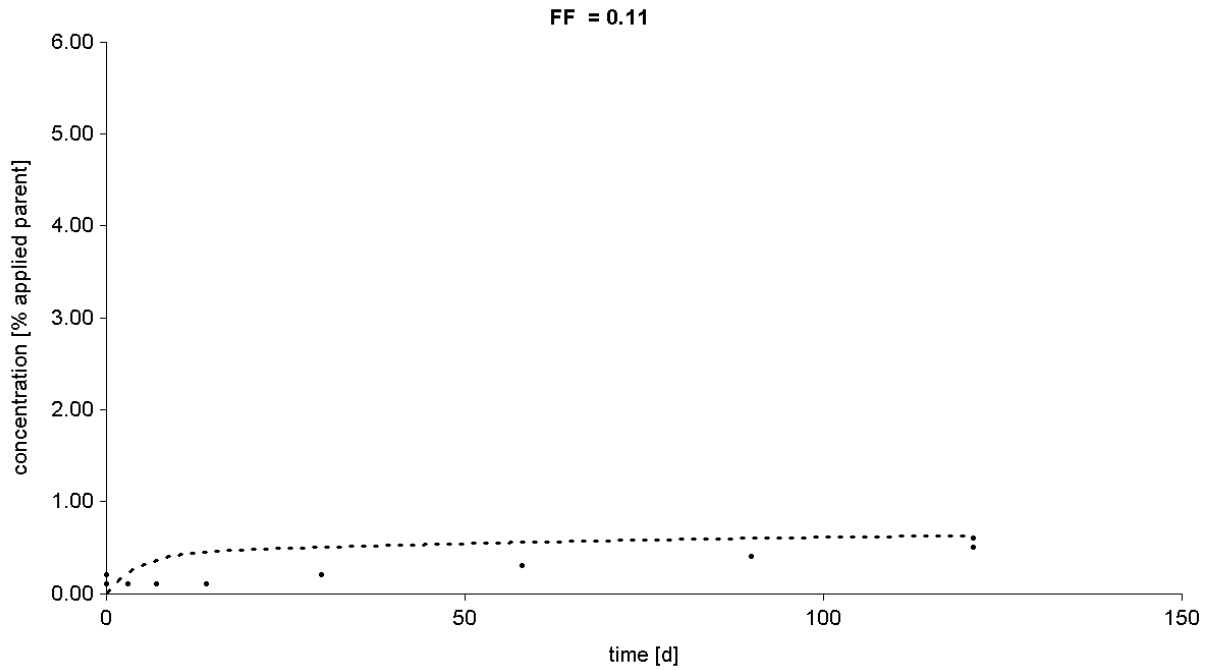
Soil	Kinetic model	Visual assessment	χ^2 error	Parameters
LUFA 5M	DFOP	Good	0.2	$k_{1\ parent} = 0.1225$ $k_{2\ parent} = 0.001151$ $g_{parent} = 0.06558$
New Jersey	FOMC	Good	0.9	$\alpha = 0.22908$ $\beta = 24.1983$
Li 10	FOMC	Good	0.3	$\alpha = 0.0656$ $\beta = 8.4325$
Indiana	FOMC	Good	0.8	$\alpha = 0.0762$ $\beta = 21.1284$

[a] Treated with triazole-labeled BAS 750 F

A compartment model including both BAS 750 F and 1,2,4-triazole was implemented in the software package ModelMaker 3.0.3. The underlying differential equations were solved without performing any optimization. Starting from 1.0, the formation fraction of 1,2,4-triazole was successively reduced to fit the predicted maximum occurrence of the metabolite to its observed maximum occurrence (shell approach). This procedure was repeated for each soil respectively.

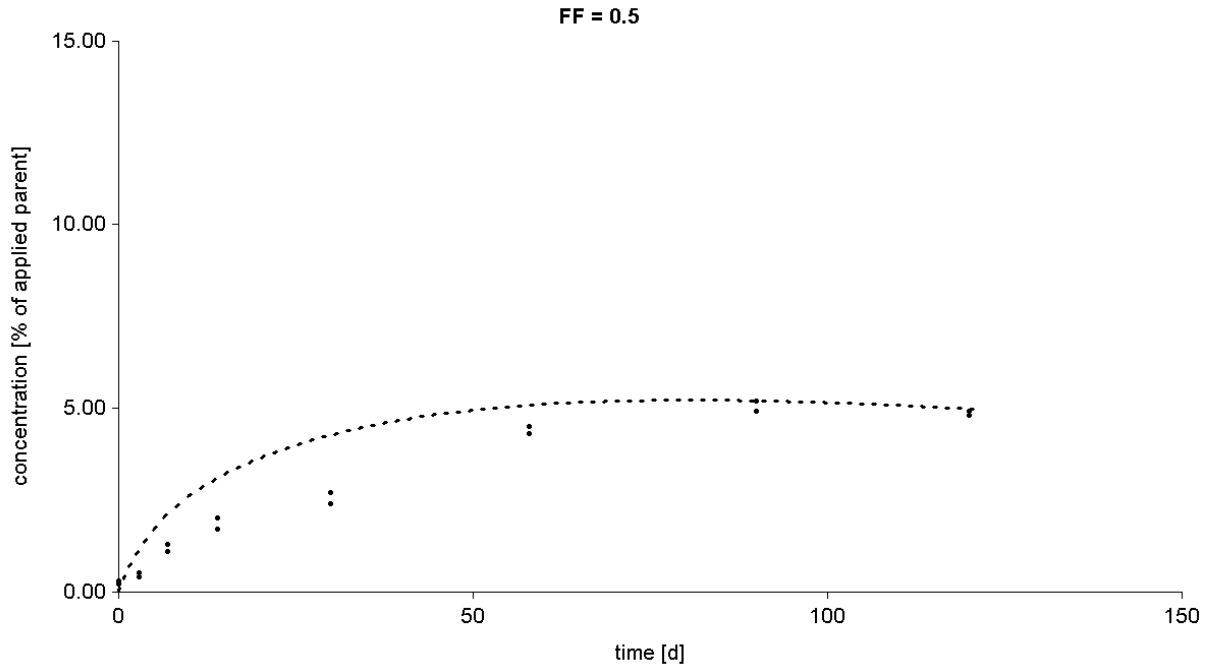
II. RESULTS AND DISCUSSION

The resulting graphs from the fitting procedure are shown below and the estimated formation fractions for 1,2,4-triazole are given in Table 7.1.2.1.2-2.



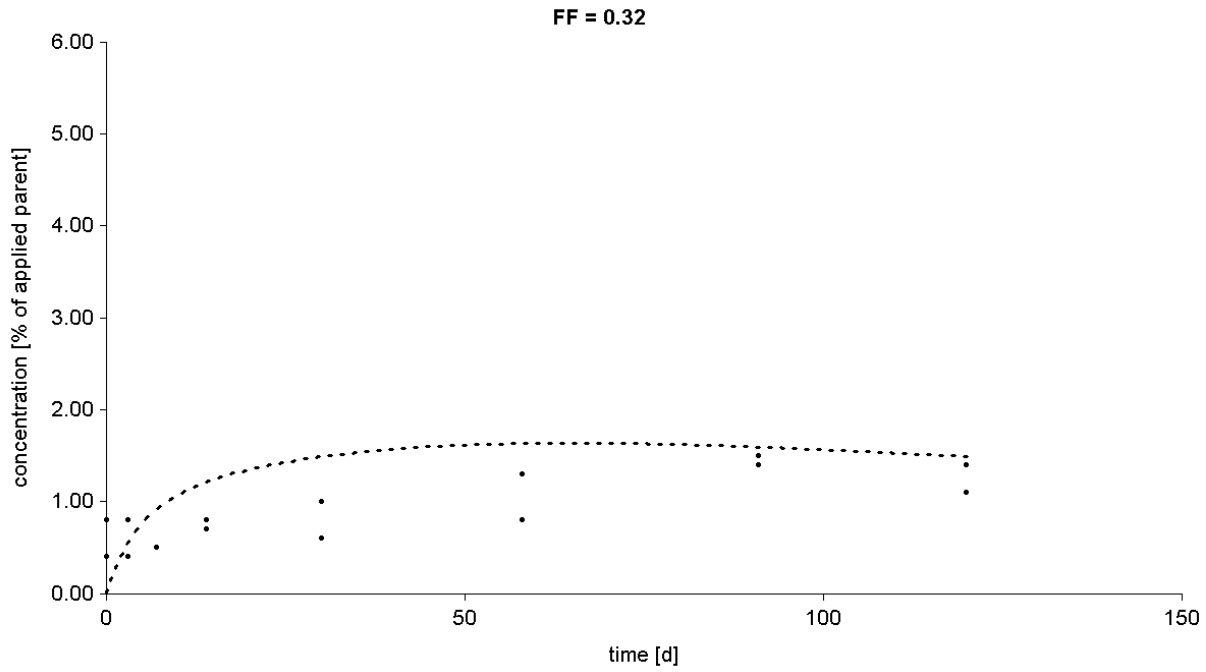
Dashed line: predicted values; dots: measured values

Figure 7.1.2-1: Predicted compared to measured concentration of 1,2,4-triazole in soil LUFA 5M using the estimated formation fraction



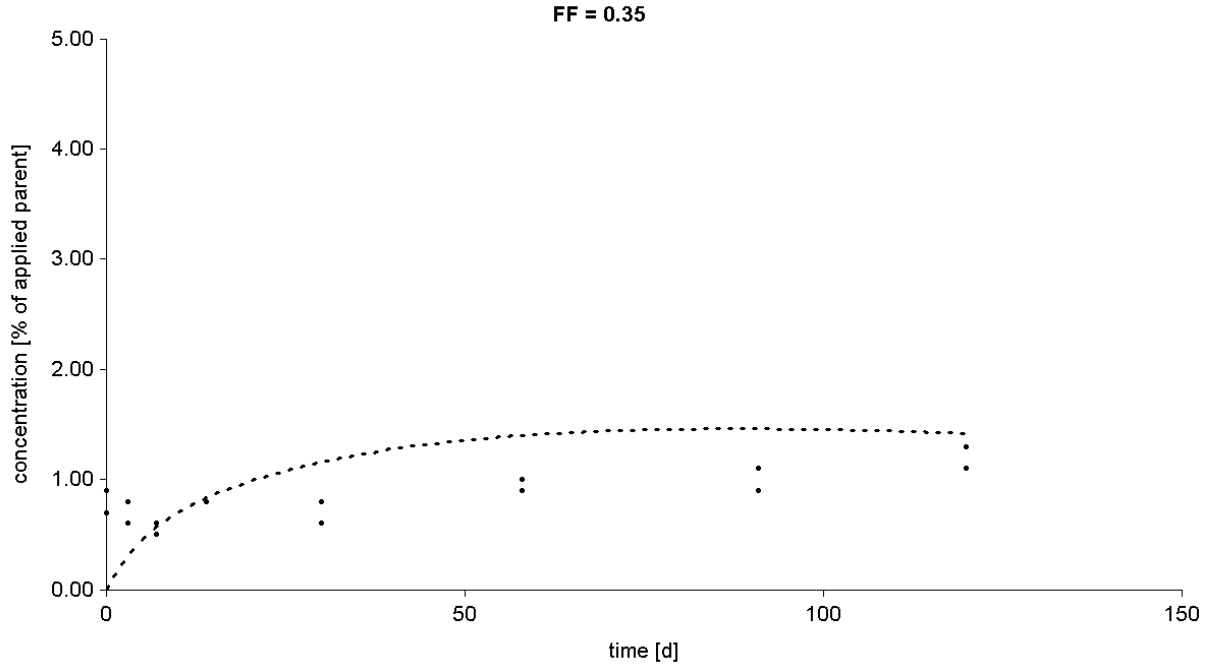
Dashed line: predicted values; dots: measured values

Figure 7.1.2-2: Predicted compared to measured concentration of 1,2,4-triazole in soil New Jersey using the estimated formation fraction



Dashed line: predicted values; dots: measured values

Figure 7.1.2-3: Predicted compared to measured concentration of 1,2,4-triazole in soil Li 10 using the estimated formation fraction



Dashed line: predicted values; dots: measured values

Figure 7.1.2-4: Predicted compared to measured concentration of 1,2,4-triazole in soil Indiana using the estimated formation fraction

Table 7.1.2.1.2-2: Estimated formation fractions of 1,2,4-triazole

Soil	Formation fraction of 1,2,4-triazole
LUFA 5M	0.11
New Jersey	0.50
Li 10	0.32
Indiana	0.35
Arithmetic mean	**0.32**

III. CONCLUSION

Formation fractions for 1,2,4-triazole from BAS 750 F for use in environmental fate models were estimated for four different soils with a shell approach. The estimated formation fractions of 1-2-4-triazole ranged from 0.11 to 0.50, with an arithmetic mean value of 0.32.

CA 7.1.2.1.3 Anaerobic degradation of the active substance

Please see the above section for anaerobic degradation of the active substance CA 7.1.1.2

Overall Summary of the Rate of Degradation in Soil

BAS 750 F degraded moderately under the given laboratory conditions. A summary of the overall trigger and modeling endpoints from this section are provided below in Table 7.1.2.1.3-1 and in Table 7.1.2.1.3-2, respectively.

No degradation half-lives were derived for the metabolites of BAS 750 F from the performed studies. Endpoints for 1,2,4-triazole (M750F001) were assessed in a separate process from the current submission. EU agreed endpoints for the exposure assessment will be taken from the evaluation provided by CRD and will be summarized in the respective CP chapters [*CRD (2014): Triazole Derived Metabolite: 1,2,4-Triazole. Proposed revision to DT50 Summary, Scientific Evaluation and Assessment July 2011, revised September 2011 (after comments from MS and EFSA) and further revised January 2013 (minor clarifications added post-commenting).*]

Formation fractions for M750F001 (1,2,4-triazole) from BAS 750 F for use in environmental fate models were estimated for four different soils. The estimated formation fractions of 1-2-4-triazole ranged from 0.11 to 0.50, with an arithmetic mean value of 0.32.

Table 7.1.2.1.3-1: Summary of Trigger Value Results from Laboratory Soil Studies

Reference	Soil/Label	$DegT_{50}$ [d]	$DegT_{90}$ [d]	Kinetic model	χ^2 error
BASF DocID 2014/1275177	LUFA 5M /Chlorophenyl	>1000	>1000	FOMC	0.6
	LUFA 5M /Triazole	543.5	>1000	DFOP	0.2
BASF DocID 2014/1275177	New Jersey /Chlorophenyl	201.9	761.3	DFOP	0.8
	New Jersey /Triazole	474.5	>1000	FOMC	0.9
BASF DocID 2015/1003306	New Jersey /Triflouromethylphenyl	433.7	>1000	FOMC	1.3
BASF DocID 2014/1275178	Li10/Triazole	>1000	>1000	FOMC	0.3
	Indiana /Triazole	>1000	>1000	FOMC	0.8

Table 7.1.2.1.3-2: Modeling $DegT_{50}$ endpoints at reference conditions

Reference	Soil/Label	Kinetic model	χ^2 error [%]	Modeling endpoints $DegT50_{act}$ [1] [d]	Modeling endpoints $DegT50_{norm}$ [2] [d]
BASF DocID 2014/1275177	LUFA 5 M/ Chlorophenyl	DFOP	0.4	557.2	329.0
	LUFA 5 M/ Triazole	DFOP	0.2	602.2	355.5
Geometric mean LUFA 5 M					**342.0**
BASF DocID 2014/1275177	New Jersey/ Chlorophenyl	DFOP	0.8	240.9	166.9
	New Jersey/ Triazole	DFOP	0.9	1000 [3]	1000 [3]
BASF DocID 2015/1003306	New Jersey/ trifluoromethylphenyl	DFOP	1.3	290.1	156.4
Geometric mean New Jersey					**296.7**
BASF DocID 2014/1275178	Li10/ Triazole	DFOP	0.4	994.3	994.3
	Indiana/ Triazole	SFO	1.2	569.8	366.7
Geometric mean of different soils					**438.6**

[1] $DegT_{50}$ at actual incubation conditions
[2] Normalized $DegT_{50}$ at reference conditions
[3] Conservative default $DegT_{50}$ as k is not estimated significant different from zero

CA 7.1.2.1.4 Anaerobic degradation of metabolites, breakdown and reaction products

No studies were required for specific degradation products under anaerobic conditions. Please see CA 7.1.1.2 for degradation of the active substance under anaerobic conditions.

CA 7.1.2.2 Field studies

The degradation of BAS 750 F was investigated under field conditions. Field plots were set up in representative growing regions of Europe. Plots were covered by a layer of sand to exclude surface processes and to enable a straightforward generation of modeling endpoints used as input for calculation of predicted environmental concentrations. A kinetic evaluation was performed in order to derive best-fit field degradation parameters for BAS 750 F according to the FOCUS kinetics guidance (2006,2014). An additional kinetic evaluation was performed to derive degradation parameters that can be used as input for modelling according to the EFSA (2014) guidance [*EFSA Guidance Document for evaluating laboratory and field dissipation studies to obtain $DegT_{50}$ values of active substances of plant protection products and transformation products of these active substances in soil. EFSA Journal 2014:12(5):3662*].

As the laboratory DT_{50} of BAS 750 F exceeds 60 days, an additional terrestrial field dissipation study was run in the US at 6 different sites without covering the plots according to NAFTA guidelines. Plot locations corresponded closely to the growing regions for the intended GAP though the test sites were not cropped. The application was also done according to intended GAP for the intended crops, although the studies themselves were not grown with actual crops (bare field plots). At the time of the writing of this dossier the study was incomplete. A comprehensive interim reporting of the data at hand has been presented in this dossier. The dissipation observed from these studies, being of higher tier and including surface processes, is in line with the overall scientific environmental picture of BAS 750 F. A final report from these studies will be provided upon completion.

An extractability study was performed to demonstrate equivalent extractability between the methods used in the metabolism studies and the residue analytical methods used for the field samples.

The DT_{50} of BAS 750 F in the field was high enough that it also triggered a field accumulation studies. At the time of compiling this dossier, it was not completed and an interim report was generated.

CA 7.1.2.2.1 Soil dissipation studies

Report: CA 7.1.2.2.1/1
Schaeufele M., 2015 d
Field soil dissipation study of Reg.No. 5834378 in the formulation EXP 5834378 F-AV on bare soil at six sites in Europe, 2013
2015/1046920

Guidelines: NAFTA Guidance Document for Conducting Terrestrial Field Dissipation Studies Regulatory Directive DIR2006-01 (March 2006), EPA 835.6100, SETAC Procedures for assessing the environmental fate and behaviour and ecotoxicity of pesticides (March 1995), EFSA Guidance to obtain DegT50 values in soil (2014), SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

Report: CA 7.1.2.2.1/2
Schaeufele M., 2015 e
Final report amendment No. 1: Field soil dissipation study of Reg.No. 5834378 in the formulation EXP 5834378 F-AV on bare soil at six sites in Europe, 2013
2015/1242234

Guidelines: NAFTA Guidance Document for Conducting Terrestrial Field Dissipation Studies Regulatory Directive DIR2006-01 (March 2006), EPA 835.6100, SETAC Procedures for assessing the environmental fate and behaviour and ecotoxicity of pesticides (March 1995), EFSA Guidance to obtain DegT50 values in soil (2014), SANCO/3029/99 rev. 4 (11 July 2000), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

EXECUTIVE SUMMARY

The dissipation of BAS 750 F and its metabolites M750F003 and 1,2,4-triazole (M750F001) under field conditions was investigated at six sites in Europe representative of Northern and Southern EU conditions. Two trials were performed in Germany (one in western and one in eastern Germany) and one trial each was performed in Denmark, Northern France, Italy, and Spain. All sites represent typical regions of agricultural practice representative for growing cereals, which are among the most important crops for the use of BAS 750 F. The trial sites consisted of an untreated and a treated plot, the latter being subdivided into 3 subplots that were assigned for replicates.

The product EXP 5834378 F-AV, containing the active ingredient BAS 750 F formulated as an emulsifiable concentrate (EC), was broadcast applied to bare soil in a single application at a nominal rate of 150 g a.s. ha^{-1} using a target water volume of 300 L ha^{-1}. Applications were conducted between early May and mid-June 2013 using a calibrated boom sprayer. The actual application rates for each trial determined by quantifying the amount of spray solution discharged ranged from 152 to 166 g a.s. ha^{-1}, with an average of 160 g a.s. ha^{-1}. The average of the results from spray broth analysis for the individual trial sites revealed concentrations between 92 and 98% of the nominal value with an average of 94% across all sites. Dose verification conducted via application monitors yielded recovery values for the individual sites ranging from 90 to 112% of the target rate and an average recovery of 103% across all sites.

Immediately after application of the test item, the plots were covered with a layer of sand of approximately 5 mm thickness to protect the applied product from surface processes like photolysis or volatilization, and to exclude any potential impact on the degradation of the test item caused by any of these processes. The application of sand was conducted until complete coverage of the soil surface. The layer of sand was controlled regularly up to 30 days after the application and was renewed when needed, ensuring that the sand cover remained intact until at least 30 days. Within this time period of 30 days, the individual fields received a total precipitation (rain and irrigation) of 86 mm (Denmark), 120 mm (Germany- East), 94 mm (Germany-West), 65 mm (France North), 63 mm (Italy) and 39 mm (Spain), respectively.

No tillage or fertilization was performed during the course of the study and no crops were grown throughout any of the trials. The plots were kept free of vegetation via the application of glyphosate, 2,4-D, MCPA, dicamba, glufosinate ammonium, quinoclamin, or flumioxazin.

Rainfall was supplemented with irrigation at sites in Denmark (217 mm), Germany-East (188 mm), Germany-West (565 mm), Northern France (273 mm), Italy (178 mm) and Spain (506 mm) and the total water input was at least 102% of the historical average rainfall during the recorded period at the test sites.

Soil specimens were taken at intervals up to 720 days after application and down to a maximum soil depth of 50 cm. Soil cores were cut into 10 cm sections. Soil segments of the same depth and subplot from a defined sampling event were pooled and homogenized and a representative sub-sample of each depth was taken for residue analysis. All soil specimens were stored at about -18°C within a maximum of 5 hours and 45 minutes after sampling and remained frozen until analysis.

In order to demonstrate stability of the residues in soil during storage and shipment, shipment verification specimens were prepared at selected sampling occasions by fortifying untreated soil from the field sites with known amounts of BAS 750 F. These specimens were stored and shipped under the same conditions as the actual residue specimens. Analysis of the shipping verification specimens on BAS 750 F yielded average recovery values, corrected for procedural recovery, of 123-136% across all sites confirming residue stability during all storage and shipment procedures.

Soil specimens were analyzed for BAS 750 F and metabolites M750F003 and 1,2,4-triazole (M750F001) according to validated BASF method L0214/01. The analytical method involved extraction of the soil with acetonitrile/water 70/30 (v/v) and final determination of the analytes by LC-MS/MS. The limit of quantification (LOQ) was 0.002 mg kg^{-1} for each individual analyte. The limit of detection (LOD) was set at 0.0004 mg kg^{-1} (20% of LOQ). Field soil specimens from the treated plot were analyzed to a depth until at least one soil layer was free of detectable residues (< LOD of 0.0004 mg kg^{-1}). Analysis was performed until a maximum of 720 days after treatment (DAT). Application monitors (Petri dish specimen) and shipping verification specimens were analyzed for parent only using the same analytical method L0214/01.

No residues above the LOD of any analyte were detected in any of the untreated control samples proving that there were no interferences of the untreated soil material with the analytical procedure used except in trial L130557 (Germany-East), where a contamination of untreated control samples with 1,2,4-triazole was apparent. 1,2,4-triazole residues detected within these untreated control samples ranged from <LOD to 0.012 mg kg^{-1}. Procedural recovery values were corrected for any corresponding control amounts for this analyte in fortified soil samples from this trial. Procedural recovery experiments performed with untreated soils spiked with the three analytes at concentrations of 0.002 and 0.02 mg kg^{-1} yielded mean recovery rates per trial ranging from 90 to 97% (BAS 750 F), 96 to 100% (M750F003) and 80 to 90% (1,2,4-triazole), confirming the validity of the analytical method used in this study.

Residue values of BAS 750 F and metabolites M750F003 and 1,2,4-triazole in mg kg^{-1} dry soil were converted to residue rates in g ha^{-1} taking into account the actual dry soil density of the individual field samples, and were summed up for all depths between 0 and 50 cm analyzed. Residue values were not corrected for procedural recoveries except for results obtained from petri dish and shipment verification analysis.

BAS 750 F residues degraded under field conditions in soil at all six European field sites. The total amount of BAS 750 F residues detected in the soil profiles decreased from an average of 122 g ha^{-1} at day 0 to an average of 30 g ha^{-1} after 24 months. DT_{50} values are presented in a separate modeling report.

Quantifiable residues of BAS 750 F were detected only in the first 20 cm of the soils. No residues above the LOQ were detected below 20 cm in any sample at any site. Altogether, it can be concluded that BAS 750 F does not show any significant tendency to move into deeper soil layers indicating low potential to leach to groundwater.

Metabolite M750F003 was also monitored during the study. No residues of M750F003 above the LOQ were detected in any sample.

Metabolite 1,2,4-triazole was also monitored during the study. No residues of 1,2,4-triazole above the LOQ were detected in any sample at sites in Denmark, Germany (West), France (North), Italy and Spain except for one single finding close to the LOQ (at 3 µg kg^{-1}) in the 0-10 cm layer at the 7 DAT sampling in France.

A different picture was seen at the trial in Germany (East), where residues of 1,2,4-triazole were detected in significant amounts in all replicates and all soil depths. These findings, however, are considered the result of a substantial background contamination of the trial site with 1,2,4-triazole, as high amounts of 1,2,4-triazole (41 g ha^{-1} within 0-50 cm soil profile) were also detected in the untreated control samples taken two days before the application at a depth of 0 - 50 cm. Therefore the analytical results for 1,2,4-triazole from the trial in Germany (East) are considered as not useful for further exposure assessments. The observed background concentration of the soil in Germany (East) might be explained by the potential application of 1,2,4-triazole containing nitrogen fertilizers since no triazole containing fungicides had been applied in the past 3 years.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test item (formulation):	EXP 5834378 F-AV
Active substance (a.s.):	BAS 750 F (Reg. No. 5834378)
Type of formulation:	EC
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Batch No.:	370168 (certificate of analysis: 432061_1)
Content of a.s.:	104.7 g L^{-1} (nominal 100 g L^{-1})
Expiration date:	January 31, 2015

2. Test sites

The dissipation of BAS 750 F under field conditions was investigated at six sites in Europe representative of Northern and Southern EU conditions. Two trials was performed in Germany (one trial each in Western and Eastern Germany) and one trial each was performed in Denmark, Northern France, Italy, and Spain. The site characteristics are presented in Table 7.1.2.2.1-1 and Table 7.1.2.2.1-2. Soil parameters were determined from soil samples taken before application from the boundaries of the treated plot following segmentation according to the soil horizons.

Table 7.1.2.2.1-1: Soil characteristics of the trial sites L130556, L130557, L130558 used to investigate the field dissipation of BAS 750 F

Trial	**L130556**		**L130557**		**L130558**	
Location	**Bogense, Denmark**		**Lentzke, Germany**		**Goch-Nierswalde, Germany**	
Soil properties	0 – 30 cm	30 – 50 cm	0 – 35 cm	35 – 50 cm	0 – 30 cm	30 – 50 cm
Soil class (DIN 4220)	Sandy loam (Sl3)	Sandy loam (Sl3)	Strong loamy sand (Sl2)	Strong loamy sand (Su3)	Loamy silt (Ut2)	Loamy silt (Uls)
sand [%]	61.1	58.6			16.1	34.2
silt [%]	29.7	30.6	68.2	66.9	75.3	56.3
clay [%]	9.2	10.8	24.6	25.7	8.5	9.8
			7.1	7.3		
Soil class (USDA)	Sandy loam	Sandy loam	Loamy sand	Sandy loam	Silt loam	Loam
sand [%]	73.3	71.5	80.0	76.3	39.0	49.5
silt [%]	15.3	16.0	12.0	12.3	51.5	41.2
clay [%]	11.4	12.5	7.9	11.5	9.6	9.3
Total organic C [%]	1.1	0.5	0.7	0.2	1.6	0.3
Organic matter [%] [a]	1.8	0.9	1.2	0.4	2.8	0.5
pH [$CaCl_2$]	6.4	7.4	5.4	4.5	6.5	6.0
pH [H_2O]	6.9	7.9	5.9	5.4	7.1	6.7
CEC [$cmol^+ kg^{-1}$]	7.2	7.0	3.8	2.6	10.2	3.8
MWHC [g $100g^{-1}$ dry weight]	32.8	28.8	22.6	19.7	39.0	26.2
pF 2.0 [g $100g^{-1}$ dry weight] [b]	18.3	16.9	15.9	14.5	34.6	23.2
pF 2.5 [g $100g^{-1}$ dry weight] [b]	11.3	11.7	10.4	9.9	21.0	16.6
Dry bulk density [g cm^{-3}] [c]	1.39 (10-20 cm)	-	1.55 (10-20 cm)	-	1.29 (10-20 cm)	-
Soil taxonomy [d]	Haplic Luvisols		Podzoluvisols		Gleyic Cambisols and Stagnic Luvisols	

CEC = Cation exchange capacity
MWHC = Maximum water holding capacity
[a] Organic matter = Organic carbon x 1.724
[b] Water retention characteristics, soil moisture at 0.1 or 0.33 bar
[c] Mean of three replicates
[d] According to WRB classification scheme

Table 7.1.2.2.1-2: Soil characteristics of the trial sites L130559, L130560, L130561 used to investigate the field dissipation of BAS 750 F

Trial	**L130559**		**L130560**		**L130561**		
Location	**Stotzheim, France**		**Poggio Renatico, Italy**		**Utrera, Spain**		
Soil properties	0 – 30 cm	30 – 50 cm	0 – 30 cm	30 – 50 cm	0 – 20 cm	20 – 40 cm	40 – 50 cm
Soil class (DIN 4220)	Silt loam (Ut4)	Silt loam (Tu4)	Silt loam (Ut4)	Silt loam (Ut4)	Weak loamy sand (St2)	Strong loamy sand (Sl3)	Sandy clay (Ts4)
sand [%]	8.1	6.7	7.8	6.3	82.9	78.2	52.5
silt [%]	70.3	67.6	67.9	68.8	8.8	11.3	13.0
clay [%]	21.6	25.5	24.3	24.8	8.3	10.4	34.5
Soil class (USDA)	Silty clay loam	Silty clay loam	Silty clay loam	Silty clay loam	Loamy sand	Loamy sand	Sandy clay
sand [%]	13.3	13.2	15.6	14.5	87.9	83.1	58.5
silt [%]	57.6	51.5	49.8	49.2	3.8	4.9	5.0
clay [%]	29.2	35.2	34.6	36.2	8.3	12.1	36.5
Total organic C [%]	0.8	0.7	1.1	1.1	0.4	0.2	0.4
Organic matter [%] [a]	1.4	1.2	1.8	1.9	0.7	0.4	0.6
pH [$CaCl_2$]	7.4	7.6	7.6	7.6	7.4	7.0	6.7
pH [H_2O]	8.0	8.3	8.3	8.2	7.9	7.6	7.1
CEC [$cmol^+ kg^{-1}$]	14.4	16.4	17.0	17.3	3.5	4.0	21.0
MWHC [g $100g^{-1}$ dry weight]	41.7	41.0	44.4	47.3	28.8	34.3	46.2
pF 2.0 [g $100g^{-1}$ dry weight] [b]	31.9	31.5	35.7	37.2	19.1	17.6	32.7
pF 2.5 [g $100g^{-1}$ dry weight] [b]	25.9	26.0	28.4	30.9	7.4	8.5	25.0
Dry bulk density [g cm^{-3}] [c]	1.43 (10-20 cm)	-	1.17 (10-20 cm)	-	1.62 (10-20 cm)	-	-
Soil taxonomy [d]	Haplic Calcisols		Calcaric Endostagnic Fluvisols		Eutric Planosol, Gleyic Luvisols, and Plinthic Luvisols		

CEC = Cation exchange capacity
MWHC = Maximum water holding capacity
[a] Organic matter = Organic carbon x 1.724
[b] Water retention characteristics, soil moisture at 0.1 or 0.33 bar
[c] Mean of three replicates
[d] According to WRB classification scheme

The selected fields represented typical regions of agricultural practice with soils representative for growing cereals that had been under cultivation for many years. The sites were flat without any significant slope. Before commencement of the study, the soil at each trial site was prepared as for sowing and was rolled if considered necessary, but then was left fallow. No product containing the test item active substance or azole fungicides had been used on the test plots in the last three years.

B. STUDY DESIGN

1. Experimental treatments

The trial area at each site was divided into two plots, one untreated control plot (size: 30 - 96 m^2) and one treated plot (size: 288 – 364.5 m^2). The treated plot consisted of three equal sized subplots A, B, and C that were assigned for replicates.

The product, formulated as emulsifiable concentrate (EC), was broadcast applied to bare soil in a single application at a nominal rate of 150 g a.s. ha^{-1} using a target water volume of 300 L ha^{-1}. Applications were conducted between early May and mid-June 2013 using calibrated boom sprayers. Treated subplots were three-fold replicated with subplot sizes ranging from 96 to 121.5 m^2. For each treated replicate, a separate spray mixture was prepared and the test item was applied to each subplot individually. Each spray mixture was visually checked for homogeneity and small aliquots of the spray mixture were taken before and after application of each individual subplot for analysis.

The actual application rates determined by quantifying the amount of spray discharged ranged from 152 to 166 g a.s. ha^{-1} averaged over the three replicates of each treated plot. In addition, the dose was verified by means of sampling Petri dishes filled with untreated soil from the trial site (approximately 40 g per dish, sieved to 2 mm). The Petri dishes with an inner diameter of nominal 9.0 cm were placed on the treated plot (ten in each subplot) before application. On completion of the application, the Petri dishes were closed with a lid, sealed with adhesive tape, stored chilled after collection, and placed on dry ice or frozen within a maximum of 62 minutes. Further details on the application are presented in Table 7.1.2.2.1-3.

Table 7.1.2.2.1-3: Application rates of field trial sites treated with EXP 5834378 F-AV (EC)

Trial Country	Application Method	No. of applications	Subplot (m^2)	Application rate per treatment				Application date
				nominal [g a.s. ha^{-1}]	actual [a] [g a.s. ha^{-1}]	dose verification [b] [g a.s ha^{-1}]	dose verification [b] % of nominal	
L130556 Denmark	broadcast spray to bare soil	1	A (96)	150	168	176	117	06 June 2013
			B (96)	150	167	164	110	
			C (96)	150	155	161 [c]	107 [c]	
			Average	**150**	**164**	**167 [d]**	**112 [d]**	
L130557 Germany (East)	broadcast spray to bare soil	1	A (108)	150	153	151	101	05 June 2013
			B (108)	150	151	149	100	
			C (108)	150	152	143	96	
			Average	**150**	**152**	**148**	**99**	
L130558 Germany (West)	broadcast spray to bare soil	1	A (96)	150	162	137	91	14 June 2013
			B (96)	150	156	129	86	
			C (96)	150	160	138	92	
			Average	**150**	**159**	**134**	**90**	
L130559 France (North)	broadcast spray to bare soil	1	A (121.5)	150	170	162	108	11 June 2013
			B (121.5)	150	160	145	97	
			C (121.5)	150	168	168	112	
			Average	**150**	**166**	**158**	**106**	
L130560 Italy	broadcast spray to bare soil	1	A (96)	150	158	152	102	05 June 2013
			B (96)	150	156	145	96	
			C (96)	150	157	164	109	
			Average	**150**	**157**	**154**	**102**	
L130561 Spain	broadcast spray to bare soil	1	A (96)	150	159	157	105	07 May 2013
			B (96)	150	162	174	116	
			C (96)	150	163	164	109	
			Average	**150**	**161**	**165**	**110**	

[a] Determined by calculation of spray liquid applied
[b] Determined by means of petri dishes filled with soil (recovery corrected)

Immediately after application of the test item and before subsequent soil sampling, the control plot and the treated replicates were covered with a thin layer of sand to protect the applied product from surface processes like photolysis or volatilization, and to exclude any potential impact on the degradation of the test item caused by any of these processes. The application of sand was conducted manually or using a fertilizer spreader, a box- or drop-spreader until complete coverage of the soil surface. The thickness of the sand layer necessary for complete coverage of the soil was between 3 and 8 mm.

The layer of sand was monitored and controlled for up to around 30 days after application and was renewed when needed, ensuring that the sand cover remained intact until at least 30 days after application. Within this time period of 30 days, the individual fields received a total precipitation (rain and irrigation) of 86 mm (Denmark), 120 mm (Germany East), 94 mm (Germany West), 65 mm (France North), 63 mm (Italy), and 39 mm (Spain), respectively.
No tillage or fertilization was performed during the course of the study from first to last sampling and no crops were grown throughout any of the trials. The plots were kept free of vegetation via the application of glyphosate, 2,4-D, MCPA, dicamba, glufosinate ammonium, quinoclamin, or flumioxacin.

Rainfall was supplemented with irrigation at sites in Denmark (217 mm), Eastern Germany (188 mm), Western Germany (565 mm), Northern France (273 mm), Italy (178 mm), and Spain (506 mm).

Actual weather data are based on records of appropriate weather stations located on-site. Monthly summary results on temperature, precipitation, and irrigation are presented in Table 7.1.2.2.1-4 and Table 7.1.2.2.1-5.

Table 7.1.2.2.1-4: Summary of climatic conditions at field trial sites used to investigate the dissipation of BAS 750 F (trial sites L130556, L130557, and L130558)

Trial	L130556 [a]			L130557 [a]			L130558 [a]		
Location	Bogense			Lentzke			Goch-Nierswalde		
	Denmark			Germany (East)			Germany (West)		
Climatic conditions	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]
Month		∑	∑		∑	∑		∑	∑
May 13	-	-	-	-	-	-	-	-	-
Jun 13	14.5	83.3	0.0	16.7	99.2	0.0	16.1	60.4	15.0
Jul 13	18.3	12.9	49.5	20.0	47.4	31.0	19.3	66.2	40.0
Aug 13	17.5	49.4	18.5	18.7	58.6	30.6	18.1	18.6	60.0
Sep 13	13.5	108.9	24.2	13.0	74.8	0.0	14.2	59.4	45.0
Oct 13	11.2	112.6	33.3	11.0	95.8	0.0	12.0	39.6	35.0
Nov 13	6.3	80.9	0.0	5.2	75.8	0.0	6.3	39.8	10.0
Dec 13	5.5	125.8	0.0	4.0	50.6	0.0	5.6	22.6	10.0
Jan 14	2.1	76.3	0.0	0.3	35.0	0.0	5.2	47.6	0.0
Feb 14	4.7	44.6	0.0	4.6	26.2	0.0	6.3	46.2	0.0
Mar 14	6.1	30.1	14.0	6.7	18.8	21.5	8.5	26.6	45.0
Apr 14	9.0	36.5	8.2	10.8	41.0	6.2	12.2	16.6	30.0
May 14	12.1	68.8	0.0	12.8	90.8	0.0	13.0	65.6	10.0
Jun 14	15.4	62.5	11.0	16.0	109.4	10.3	16.2	33.4	55.0
Jul 14	19.5	33.3	21.9	20.7	69.2	13.2	19.5	25.6	40.0
Aug 14	15.9	132.0	24.5	16.8	50.2	8.1	15.9	100.8	0.0
Sep 14	15.0	49.4	12.3	15.7	27.4	29.0	15.7	23.4	50.0
Oct 14	12.5	77.6	0.0	12.1	47.8	5.3	13.1	39.4	15.0
Nov 14	7.8	30.4	0.0	6.4	11.4	0.0	8.1	19.0	10.0
Dec 14	3.8	116.0	0.0	2.3	57.4	0.0	4.1	44.4	0.0
Jan 15	3.3	91.2	0.0	2.7	71.4	0.0	3.4	88.6	0.0
Feb 15	2.3	26.2	0.0	1.2	18.8	0.0	2.8	38.4	0.0
Mar 15	4.9	57.4	0.0	5.3	67.4	12.3	5.7	28.8	25.0
Apr 15	7.6	29.2	0.0	8.3	31.4	13.5	8.5	12.6	40.0
May 15	10.0	64.0	0.0	11.8	16.8	7.2	13.0	21.2	30.0

[a] Actual weather data refer to time period from start of trial (day of application) until end of trial (day of last sampling)

Table 7.1.2.2.1-5: Summary of climatic conditions at field trial sites used to investigate the dissipation of BAS 750 F (trial sites L130559, L130560, and L130561)

Trial	L130559 [a]			L130560 [a]			L130561 [a]		
Location	Stotzheim			Poggio Renatico			Utrera		
	France (North)			Italy			Spain		
Climatic conditions	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]	T_{mean} Air [°C]	Prec. [mm]	Irrigation [mm]
Month		∑	∑		∑	∑		∑	∑
May 13	-	-	-	-	-	-	20.3	8.0	18.9
Jun 13	18.8	38.0	6.6	22.0	29.0	33.8	25.0	0.0	41.0
Jul 13	22.2	21.0	44.4	25.0	4.6	22.3	28.2	0.0	58.6
Aug 13	19.3	57.6	25.6	23.8	63.2	19.3	28.7	0.0	74.0
Sep 13	15.7	86.6	0.0	19.7	29.7	24.7	25.5	21.5	30.0
Oct 13	12.1	140.8	9.3	14.9	98.0	0.0	21.5	88.5	0.0
Nov 13	5.6	85.8	0.0	9.4	69.2	0.0	14.2	3.0	9.3
Dec 13	3.5	35.6	0.0	3.7	10.8	0.0	12.0	59.0	24.7
Jan 14	4.6	33.8	0.0	5.8	94.4	0.0	12.9	85.0	0.0
Feb 14	5.7	47.6	0.0	7.7	87.6	0.0	13.0	85.3	0.0
Mar 14	8.9	8.0	22.2	10.4	52.0	0.0	15.1	32.8	21.3
Apr 14	12.5	25.8	35.6	13.9	68.8	10.2	19.5	93.0	0.0
May 14	14.8	55.4	13.5	17.1	48.8	19.5	22.6	10.0	23.0
Jun 14	19.8	24.4	64.0	22.3	25.1	21.0	24.8	8.0	38.9
Jul 14	19.8	210.8	0.0	22.6	67.6	15.6	26.3	0.0	55.7
Aug 14	17.6	81.4	0.0	22.4	84.9	0.0	27.0	0.0	59.7
Sept 14	16.3	12.4	33.8	18.8	64.5	0.0	24.5	61.0	28.6
Oct 14	13.2	85.2	0.0	15.8	36.8	0.0	22.7	58.0	0.0
Nov 14	7.5	61.8	0.0	11.4	53.2	0.0	16.8	212.0	0.0
Dec 14	4.4	32.0	0.0	5.6	47.6	0.0	10.9	29.5	0.0
Jan 15	3.3	75.8	0.0	3.6	13.9	0.0	10.5	56.0	0.0
Feb 15	1.9	20.8	0.0	5.1	111.7	0.0	11.6	11.0	0.0
Mar 15	7.3	26.2	18.4	9.0	71.3	0.0	14.5	55.5	10.3
Apr 15	11.2	59.4	0.0	13.0	59.1	11.4	18.4	6.5	11.7
May 15	15.4	89.0	0.0	19.1	29.2	0.0	-	-	-
Jun 15	16.3	0.0	0.0	-	-	-	-	-	-

[a] Actual weather data refer to time period from start of trial (day of application) until end of trial (day of last sampling)

Due to additional irrigation, the total water input at the test sites during the study was at least 102% of the historical average rainfall, which is considered sufficient to allow the cultivation of cereal crops. Details on the historical (long-term) weather data on precipitation and average air temperature are given in the study report.

2. Sampling

Replicate soil specimens (8 per treated subplot and 10 or 15 per control plot) were taken at intervals up to 720 days after treatment (DAT) and down to a maximum soil depth of 50 cm. At day 0, immediately after application, the treated plots were sampled down to 10 cm only. The detailed sampling intervals are presented in Table 7.1.2.2.1-6.

Table 7.1.2.2.1-6: Summary of sampling intervals of residue soil samples at each field trial site

Trial	Country	Sampling intervals [days after treatment]
L130556	Denmark	-1, 0, 6, 13, 29, 61, 92, 124, 174, 245, 363, 487, 615, 713
L130557	Germany (East)	-2, 0, 6, 14, 33, 56, 85, 118, 176, 272, 355, 476, 590, 715
L130558	Germany (West)	-3, 0, 7, 13, 27, 59, 95, 125, 185, 248, 361, 474, 613, 710
L130559	France (North)	-1, 0, 7, 14, 30, 62, 91, 120, 175, 238, 366, 471, 591, 720
L130560	Italy	-1, 0, 7, 13, 29, 56, 90, 120, 183, 285, 351, 475, 600, (712 [a]), 714 [a]
L130561	Spain	-1, 0, 6, 13, 29, 58, 92, 127, 183, 230, 353, 478, 591, 713

[a] Untreated soil samples (Sampling No. 16) were collected at 712 DAT, and the treated soil samples (Sampling No. 17) were collected at 714 DAT.

Untreated soil specimens were collected from the control plot on three occasions, between one and three days before application down to a depth of 50 cm, and after about one year and again after about two years to a depth of 10 cm. The specimens were taken randomly from the untreated plot each time and pooled according to soil depth. The 15 cores collected at the first sampling interval were taken using a common soil probe equipped with a plastic liner of 4.4 to 5.0 cm diameter. As an exception in trial L130560 (Italy), soil cores taken from 30-50 cm depth were done with plastic liner of diameter 2.5 cm. The 10 cores taken after about one and two years were collected with a metal tube of minimum 7.2 and maximum 9.8 cm diameter.

Treated soil specimens were taken randomly from eight points of each of the three treated subplots A – C and pooled according to subplot and depth. All soil specimens from 0-10 cm depth collected from the treated plots were taken separately using a metal tube of minimum 7.2 and to maximum 9.8 cm diameter which left a hole contained by a guard collar. Alternatively, samples were taken by pressing the metal tube described above into the ground and collecting the soil with a spoon or similar device. Soil specimens deeper than 10 cm were collected through the center of the excavation hole contained by the guard collar, using a common soil corer fitted with a plastic liner of diameter 4.4 to 5.0 cm. Sampling of these cores was conducted in one run or in up to two consecutive steps. As an exception in trial L130560 (Italy), soil cores taken from 30-50 cm depth at all sampling events were done with plastic liner of diameter 2.5 cm.

All main soil cores collected with the soil probe were sectioned into 10 cm segments and pooled by depth. The segmentation was done before freezing or in frozen stage. If soil cores were segmented in frozen stage, the specimens did not defrost during the segmentation process.

In addition to the main sampling described above, a second complete sampling (double sampling) was carried out. The reserve samples were not sectioned into 10 cm segments but directly put into freezers at the field test sites.

All soil specimens intended for residue analysis were stored at about -18°C within a maximum of 5.75 h after sampling and remained frozen through storage, shipment, and processing until final analysis except for short term temperature rise in trial L130559, where temperature raised to -12°C for 15 hours. Since samples were always deep-frozen, any negative impact can be excluded.

Shipment verification specimens were prepared to demonstrate stability of the residues in soil during storage and through any shipment processes. The samples were prepared at three occasions by fortification of soil with 0.1 mg kg^{-1} BAS 750 F and were subsequently handled in the same manner as the actual residue samples. The analytical results demonstrated no significant losses from the shipment verification samples. The recovery of BAS 750 F was in the range of 80 - 99% (mean of each trial and fortification level).

3. Analytical procedure

Field soil specimens, were analyzed for BAS 750 F, M750F003, and 1,2,4-triazole (M750F001) according to the analytical method L0214/01 validated in a separate study [see *KCA 4.1.2/1 2015/1039006]* provided by BASF. Petri dish and shipment verification specimens were analyzed for BAS 750 F according to the same method with minor adaptations to account for the larger quantity of soil to be extracted.

The analytical method involved extraction of the soil with acetonitrile/water 70/30 (v/v) and final determination of the analytes by LC-MS/MS. Spray broth specimens were diluted with acetonitrile to the appropriate concentration and analyzed for BAS 750 F using HPLC-MS/MS.

The limit of quantification (LOQ) was 0.002 mg kg^{-1} for each individual analyte. The limit of detection (LOD) was set at 0.0004 mg kg^{-1} (20% of LOQ).

Analysis of field soil specimens originating from the treated plots was conducted down to a depth until at least one soil layer was free of detectable residues (< LOD of 0.0004 mg kg^{-1}). Analysis was performed up to a maximum of 720 DAT.

The validity of the analytical method was proven within the present study by analysis of untreated control and fortified samples within each analytical sample set.

4. Storage stability experiments

Storage stability of BAS 750 F and its metabolite M750F003 in frozen soil was investigated in a separate study *[see KCA 7.1.2.2.1/6 2015/1050221]*. The storage stability of M750F001 was investigated in a report supporting another triazole containing a.s. in representative field sites [*see KCA 7.1.2.2.1/8 2015/1204922*]. The storage stability of M750F003 was initiated in support of this field study before the final results from the Aerobic Soil Metabolism were collected. Once the results of the metabolism study were seen in their final form, it was realized that M750F003 never reached the official trigger values to initiate a storage stability study (never observed > 2.2% TAR).

5. Calculation of degradation times

No calculation of degradation times is provided in the study report. A detailed kinetic evaluation of the degradation behavior of BAS 750 F in the six European field soils is presented in a separate modeling report [*see KCA 7.1.2.2.1/4 2015/1249176*]

II. RESULTS AND DISCUSSION

1. Spray broth concentration and application verification

Analyzed concentrations of BAS 750 F averaged for the individual trial sites were in the range of 479.6 to 512.2 mg L^{-1} corresponding to 92 - 98% of the target concentration of 523.5 mg L^{-1}. The analytical results confirm the integrity of the test item used in the trials.

Application verification was conducted by means of petri dishes filled with fine untreated soil from the trial site. As a result, the obtained application rates for the individual trials ranged from 134 to 167 g a.s. ha^{-1} representing 90 - 112% of the target application rate. The applied amount determined via the application monitors in these trials is in agreement with the nominal value of 150 g ha^{-1}, and the results from spray broth analysis.

2. Residues in field soil samples

Untreated soil specimens (control samples) of the respective soil depths from each trial were analyzed for residues of BAS 750 F, M750F001 (1,2,4-triazole), and M750F003.

No residues above the LOD of any analytes were detected in any of the control samples, except in trial L130557 (Eastern Germany). The soil obtained from this trial before the application showed a substantial background contamination with M750F001. Residues of M750F001 in these samples were detected in a range between <LOQ (1.4 µg kg^{-1}) and 12.0 µg kg^{-1} (70 to 600% LOQ, respectively). Procedural recovery values were corrected for any corresponding control amounts for this analyte in fortified soil samples from this trial. Except the soil from trial L130557 regarding M750F001, no interferences of the untreated soil material with the analytical procedures occurred.

Procedural recovery experiments performed with untreated field soil specimens spiked with all three analytes at concentration levels of 0.002 and 0.02 mg kg^{-1} yielded overall mean recovery rates for field soil samples of the individual trials between 90-97% (BAS 750 F), 80-90% (M750F001), and 96-100% (M750F003), confirming the validity of the analytical method used in this study. Detailed results are summarized in Table 7.1.2.2.1-7.

Table 7.1.2.2.1-7: Procedural recoveries of soil residue method

Analyte	Fortification level [mg kg^{-1}]	n [a]	Mean recovery ± RSD [a] [%]
BAS 750 F	0.002	7 - 15	85 - 93 ± 8.2 – 13.8
	0.02	11 - 20	93 - 102 ± 3.8 – 7.5
	All fortification levels	**18 - 35**	**90 - 97 ± 7.5 – 11.0**
M750F001 (1,2,4-triazole)	0.002	7 - 15	77 - 85 ± 6.4 – 13.3
	0.02	11 - 19	82 - 93 ± 12.7 – 20.2
	All fortification levels	**18 - 33**	**80 - 90 ± 11.4– 16.9**
M750F003	0.002	7 - 15	91 - 100 ± 3.5 – 8.9
	0.02	11 - 18	99 - 100 ± 2.5 – 5.6
	All fortification levels	**18 - 33**	**96 - 100 ± 3.2 – 7.2**

RSD = Relative standard deviation
[a] Range given for the six individual field trial sites.

These data demonstrate that the analytical method applied was able to determine residues of BAS 750 F and its metabolites M750F001 and M750F003 in soil samples accurately down to a concentration of 0.002 mg kg^{-1} for each analyte.

Field soil specimens from the treated plots were analyzed down to a depth until one soil layer was free of detectable residues (< LOD of 0.0004 mg kg^{-1}) except for soil obtained from trial L130557, as the soil showed a background contamination with M750F001, hence, all available soil layers from this trial were analyzed for M750F001 (0-50 cm depth). Obtained residue values were related to moist soil were then converted to soil dry weight values. If samples were analyzed in duplicate, the individual numbers (related to dry weight) were averaged to produce a mean for the respective soil sample. For all trials, the 0 DAT double samples of the 0-10 cm soil layer were analysed as well, in order to account for the importance of the day 0 value, and the final data were obtained by averaging the mean values of the respective main and double samples.

Residue values for BAS 750 F, M750F001, and M750F003 are presented in Table 7.1.2.2.1-7 to Table 7.1.2.2.1-10 in µg kg^{-1} and in g ha^{-1} for BAS 750 F and M750F001 (trial L130557 only) in Table 7.1.2.2.1-8 and Table 7.1.2.2.1-11. All residue values presented in these tables are related to the dry weight of the soil and are not corrected for procedural recoveries. Residue levels of the analytes in µg kg^{-1} dry soil were converted to residue rates in g ha^{-1} taking into account the actual dry soil density of the field samples, and were summed up for all depths between 0 and 50 cm analyzed.

Table 7.1.2.2.1-8: Total residues of BAS 750 F under field conditions in soil calculated to g ha-1 and summed up for all depths analyzed

Trial Country	L130556 Bogense, Denmark			L130557 Lentzke, Germany (East)			
DAT	Subplot A [g ha^{-1}]	Subplot B [g ha^{-1}]	Subplot C [g ha^{-1}]	DAT	Subplot A [g ha^{-1}]	Subplot B [g ha^{-1}]	Subplot C [g ha^{-1}]
0	109	123	91	**0**	140	101	121
6	136	135	100	**6**	118	138	137
13	161	98	107	**14**	126	113	105
29	117	125	124	**33**	118	98	105
61	94	56	92	**56**	119	120	85
92	90	89	80	**85**	113	87	75
124 [a]	69	69	74	**118** [a]	102	107	70
174	68	65	73	**176**	92	80	86
245	53	59	39	**272**	84	87	85
363 [a]	42	21	42	**355** [a]	42	42	33
487	16	14	8.7	**476**	45	43	37
615	17	18	7.8	**590**	33	57	54
713	10	7.5	5.4	**715**	42	36	25
Trial Country	**L130558 Goch-Nierswalde, Germany (West)**			**L130559 Stotzheim, France (North)**			
DAT	**Subplot A [g ha^{-1}]**	**Subplot B [g ha^{-1}]**	**Subplot C [g ha^{-1}]**	**DAT**	**Subplot A [g ha^{-1}]**	**Subplot B [g ha^{-1}]**	**Subplot C [g ha^{-1}]**
0	97	140	125	**0**	127	136	135
7	165	154	92	**7**	130	112	103
13	108	133	122	**14**	126	116	125
27	146	77	23	**30**	119	110	113
59	132	107	121	**62**	122	71	85
95	132	94	78	**91**	75	57	75
125 [a]	89	84	101	**120** [a]	61	69	60
185	106	112	63	**175**	67	59	61
248	106	104	68	**238**	61	51	58
361 [a]	37	33	24	**366** [a]	53	55	52
474	22	18	14	**471**	23	21	31
613	14	5.3	30	**591**	26	20	12
710	31	23	29	**720**	15	12	21
Trial Country	**L130560 Poggio Renatico, Italy**			**L130561 Utrera, Spain**			
DAT	**Subplot A [g ha^{-1}]**	**Subplot B [g ha^{-1}]**	**Subplot C [g ha^{-1}]**	**DAT**	**Subplot A [g ha^{-1}]**	**Subplot B [g ha^{-1}]**	**Subplot C [g ha^{-1}]**
0	112	130	107	0	141	123	133
7	124	139	149	6	116	110	126
13	108	111	96	13	120	90	112
29	118	119	127	29	101	96	96
56	126	117	99	58	69	73	95
90	111	104	92	92	94	106	84
120 [a]	87	96	102	127 [a]	78	73	72
183	95	106	90	183	78	64	54
285	131	118	111	230	72	73	52
351 [a]	94	83	63	353 [a]	46	50	59
475	70	78	68	478	40	47	40
600	82	58	63	591	20	24	21
714	63	80	80	713	21	22	18

DAT = Days after treatment
Residue values < LOQ (limit of quantification) or < LOD (limit of quantification) were reported and treated as zero.
a Samples analyzed in duplicate.

Table 7.1.2.2.1-9: Residues of M750F001 under field conditions in soil calculated in µg kg^{-1} and summed up for all depths analyzed

Trial Country	L130556 Bogense, Denmark			L130557 Lentzke, Germany (East)[c]			
DAT	Subplot A [µg kg^{-1}]	Subplot B [µg kg^{-1}]	Subplot C [µg kg^{-1}]	DAT	Subplot A [µg kg^{-1}]	Subplot B [µg kg^{-1}]	Subplot C [µg kg^{-1}]
0	< LOD	< LOD	< LOD	**0**	2.7	2.7	2.1
6	< LOD	< LOD	< LOD	**6**	32	37	37
13	< LOD	< LOD	< LOD	**14**	38	47	41
29	< LOD	< LOD	< LOD	**33**	38	39	26
61	< LOD	< LOD	< LOQ	**56**	31	27	29
92	< LOD	< LOD	< LOQ	**85**	19	27	21
124 [a]	< LOQ	< LOD	< LOD	**118** [a]	26	22	16
174	< LOD	< LOD	< LOQ	**176**	7.6	14	13
245	< LOD	< LOD	< LOD	**272**	6.1	3.0	2.5
363 [a]	< LOD	< LOQ	< LOD	**355** [a]	15	6.6	6.6
487	< LOD	< LOD	< LOD	**476**	< LOQ	2.2	2.1
615	< LOD	< LOD	< LOD	**590**	< LOQ	< LOQ	< LOQ
713	< LOD	< LOD	< LOD	**715**	< LOQ	< LOQ	< LOQ
Trial Country	**L130558 Goch-Nierswalde, Germany (West)**			**L130559 Stotzheim, France (North)**			
DAT	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**	**DAT**	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**
0	< LOD	< LOD	< LOD	**0**	< LOD	< LOD	< LOD
7	< LOD	< LOD	< LOD	**7**	< LOQ	3.1 [b]	< LOQ
13	< LOD	< LOD	< LOD	**14**	< LOQ	< LOQ	< LOQ
27	< LOD	< LOD	< LOD	**30**	< LOQ	< LOD	< LOQ
59	< LOD	< LOD	< LOD	**62**	< LOQ	< LOD	< LOQ
95	< LOD	< LOD	< LOD	**91**	< LOD	< LOD	< LOD
125 [a]	< LOD	< LOD	< LOD	**120** [a]	< LOD	< LOD	< LOD
185	< LOD	< LOD	< LOD	**175**	< LOD	< LOD	< LOD
248	< LOD	< LOD	< LOD	**238**	< LOD	< LOD	< LOD
361 [a]	< LOD	< LOD	< LOD	**366** [a]	< LOD	< LOD	< LOD
474	< LOD	< LOD	< LOD	**471**	< LOD	< LOD	< LOD
613	< LOD	< LOD	< LOD	**591**	< LOD	< LOD	< LOD
710	< LOQ	< LOD	< LOD	**720**	< LOD	< LOD	< LOD
Trial Country	**L130560 Poggio Renatico, Italy**			**L130561 Utrera, Spain**			
DAT	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**	**DAT**	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**
0	< LOQ	< LOQ	< LOQ	0	< LOD	< LOD	< LOD
7	< LOQ	< LOQ	< LOD	6	< LOD	< LOD	< LOD
13	< LOQ	< LOQ	< LOQ	13	< LOD	< LOD	< LOD
29	< LOQ	< LOQ	< LOQ	29	< LOQ	< LOQ	< LOD
56	< LOQ	< LOD	< LOQ	58	< LOD	< LOD	< LOD
90	< LOQ	< LOQ	< LOQ	92	< LOD	< LOD	< LOD
120 [a]	< LOQ	< LOQ	< LOQ	127 [a]	< LOD	< LOD	< LOD
183	< LOQ	< LOQ	< LOQ	183	< LOD	< LOD	< LOD
285	< LOQ	< LOD	< LOD	230	< LOD	< LOD	< LOD
351 [a]	< LOQ	< LOQ	< LOQ	353 [a]	< LOD	< LOD	< LOD
475	< LOD	< LOD	< LOD	478	< LOD	< LOD	< LOD
600	< LOD	< LOD	< LOD	591	< LOD	< LOD	< LOD
714	< LOD	< LOD	< LOD	713	< LOD	< LOD	< LOD

DAT = Days after treatment
LOQ (limit of quantification): 2 µg kg^{-1}; LOD (limit of detection): 0.4 µg kg^{-1}
[a] Samples analyzed in duplicate.
[b] Converting this value to g ha^{-1} = 3.3 g ha^{-1} (based on actual dry soil density)
[c] See table Table 7.1.2.2.1-11 for a more relevant conversion to g ha^{-1}

Table 7.1.2.2.1-10: Cumulative Concentration of M750F003 under field conditions in soil calculated to µg kg^{-1} and summed up for all depths analyzed

Trial Country	L130556 Bogense, Denmark			L130557 Lentzke, Germany (East)			
DAT	Subplot A [µg kg^{-1}]	Subplot B [µg kg^{-1}]	Subplot C [µg kg^{-1}]	DAT	Subplot A [µg kg^{-1}]	Subplot B [µg kg^{-1}]	Subplot C [µg kg^{-1}]
0	< LOD	< LOD	< LOD	**0**	< LOD	< LOD	< LOD
6	< LOQ	< LOQ	< LOQ	**6**	< LOD	< LOD	< LOD
13	< LOQ	< LOQ	< LOQ	**14**	< LOD	< LOD	< LOD
29	< LOQ	< LOQ	< LOQ	**33**	< LOD	< LOD	< LOD
61	< LOQ	< LOQ	< LOQ	**56**	< LOD	< LOD	< LOD
92	< LOD	< LOQ	< LOD	**85**	< LOD	< LOD	< LOD
124 [a]	< LOQ	< LOQ	< LOQ	**118** [a]	< LOD	< LOD	< LOD
174	< LOQ	< LOQ	< LOQ	**176**	< LOD	< LOD	< LOD
245	< LOQ	< LOD	< LOQ	**272**	< LOD	< LOD	< LOD
363 [a]	< LOD	< LOD	< LOD	**355** [a]	< LOD	< LOD	< LOD
487	< LOD	< LOD	< LOD	**476**	< LOD	< LOD	< LOD
615	< LOD	< LOD	< LOD	**590**	< LOD	< LOD	< LOD
713	< LOD	< LOD	< LOD	**715**	< LOD	< LOD	< LOD
Trial Country	**L130558 Goch-Nierswalde, Germany (West)**			**L130559 Stotzheim, France (North)**			
DAT	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**	**DAT**	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**
0	< LOD	< LOD	< LOD	**0**	< LOD	< LOD	< LOD
7	< LOD	< LOD	< LOD	**7**	< LOD	< LOD	< LOD
13	< LOD	< LOD	< LOD	**14**	< LOD	< LOD	< LOD
27	< LOD	< LOD	< LOD	**30**	< LOD	< LOD	< LOD
59	< LOQ	< LOD	< LOD	**62**	< LOD	< LOD	< LOD
95	< LOQ	< LOD	< LOD	**91**	< LOD	< LOD	< LOD
125 [a]	< LOQ	< LOD	< LOQ	**120** [a]	< LOD	< LOD	< LOD
185	< LOQ	< LOQ	< LOD	**175**	< LOD	< LOD	< LOD
248	< LOQ	< LOQ	< LOD	**238**	< LOD	< LOD	< LOD
361 [a]	< LOQ	< LOD	< LOD	**366** [a]	< LOD	< LOD	< LOD
474	< LOQ	< LOD	< LOD	**471**	< LOD	< LOD	< LOD
613	< LOD	< LOD	< LOQ	**591**	< LOD	< LOD	< LOD
710	< LOQ	< LOQ	< LOQ	**720**	< LOD	< LOD	< LOD
Trial Country	**L130560 Poggio Renatico, Italy**			**L130561 Utrera, Spain**			
DAT	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**	**DAT**	**Subplot A [µg kg^{-1}]**	**Subplot B [µg kg^{-1}]**	**Subplot C [µg kg^{-1}]**
0	< LOD	< LOD	< LOD	0	< LOQ	< LOQ	< LOQ
7	< LOQ	< LOD	< LOQ	6	< LOQ	< LOQ	< LOQ
13	< LOQ	< LOD	< LOD	13	< LOQ	< LOQ	< LOQ
29	< LOD	< LOD	< LOD	29	< LOQ	< LOQ	< LOQ
56	< LOQ	< LOD	< LOQ	58	< LOD	< LOQ	< LOQ
90	< LOQ	< LOD	< LOQ	92	< LOQ	< LOQ	< LOD
120 [a]	< LOD	< LOD	< LOD	127 [a]	< LOQ	< LOQ	< LOD
183	< LOD	< LOD	< LOQ	183	< LOD	< LOD	< LOD
285	< LOD	< LOD	< LOQ	230	< LOD	< LOD	< LOD
351 [a]	< LOD	< LOD	< LOD	353 [a]	< LOD	< LOD	< LOD
475	< LOD	< LOD	< LOD	478	< LOD	< LOD	< LOD
600	< LOD	< LOD	< LOD	591	< LOD	< LOD	< LOD
714	< LOQ	< LOD	< LOD	713	< LOD	< LOD	< LOD

DAT = Days after treatment
LOQ (limit of quantification): 2 µg kg^{-1}; LOD (limit of detection): 0.4 µg kg^{-1}
[a] Samples analyzed in duplicate.

Table 7.1.2.2.1-11: Total residues of M705F001 (1,2,4-triazole) under field conditions in soil calculated to g ha^{-1} and summed up for all depths analyzed (trial L130557)

Trial Country	L130557 Lentzke, Germany		
DAT	Subplot A	Subplot B	Subplot C
	[g ha^{-1}]	[g ha^{-1}]	[g ha^{-1}]
0	3.5	4.0	3.0
6	53	62	61
14	63	79	70
33	63	67	44
56	52	45	49
85	31	46	35
118 [a]	42	35	27
176	13	24	22
272	10	5.1	4.2
355 [a]	25	11	10
476	0	3.7	3.5
590	0	0	0
715	0	0	0

DAT = Days after treatment
Residue values < LOQ (limit of quantification) or < LOD (limit of quantification) were reported and treated as zero.
[a] Samples analyzed in duplicate.

Analytical data clearly shows that BAS 750 F degraded at all six European field sites. The total amount of BAS 750 F residues detected in the soil profiles decreased from an average of 122 g ha^{-1} at day 0 to an average of 30 g ha^{-1} (range 8 to 75 g ha^{-1}) after 24 months.

Considering the distribution of BAS 750 F residues in the soil profiles, residues were exclusively found in the top 0-20 cm layer of the soils. No residues above the LOQ were detected below 20 cm in any sample at any site. Altogether, it can be concluded that BAS 750 F does not show any significant tendency to move into deeper soil layers indicating low potential to leach to groundwater.

Metabolite M750F001 (1,2,4-triazole) was also monitored during the study. No residues of M750F001 above the LOQ were detected in any sample at sites in Denmark, Germany (West), France (North), Italy and Spain, except in one sample of trial L130559 in France (North), where 0.003 mg kg^{-1} (equivalent to 3.3 g ha^{-1}) were measured in one 0-10 cm sample at 7 DAT sampling.

Metabolite M750F003 was also monitored during the study. No residues of M750F003 above the LOQ were detected in any sample.

A different picture was seen at the trial in Germany (East), where residues of M750F001 were detected in significant amounts in all replicates, all soil depths and all time points. These findings, however, are considered the result of a substantial background contamination of the trial site with M750F001, as high amounts of M750F001 (41 g ha^{-1} within 0-50 cm soil profile) were also detected in the untreated control samples taken two days before the application at a depth of 0-50 cm. Therefore, the analytical results for M750F001 from the trial in Germany (East) are considered meaningless and not to be useful for further exposure assessments. The observed background concentration of the soil in Germany (East) might be explained by the potential application of M750F001 containing nitrogen fertilizers since no triazole containing fungicides had been applied in the past three years.

3. Shipment verification specimens

Shipment verification specimens were prepared to demonstrate stability of the residues in soil during storage and through any shipping process. The samples were prepared at nominal 0, 30 and 90 DAT by fortification of soil aliquots with 0.1 mg kg^{-1} BAS 750 F and were subsequently handled in the same manner as the actual residue samples. Concentrations of BAS 750 F analyzed were corrected for the mean recovery of the respective analytical set. The analyzed concentrations averaged across the individual trial sites were in the range of 0.123 to 0.136 mg kg^{-1} corresponding to 123 to 136% of the target concentration of 0.100 mg kg^{-1}.

4. Time of storage

The predominant part of the samples was analyzed within less than 11 months. Some individual samples typically foreseen for re-analysis or double sample analysis were stored for a longer time period prior to analysis. The maximum period any soil sample from the present field soil dissipation study was stored from the time of sampling to extraction was 646 days. To confirm residue stability over the maximal storage period, a storage stability study was set up and it is still running [*see KCA 7.1.2.2.1/6 2015/1050221*]. In addition, the storage stability of metabolite M750F001 was confirmed for over 2 years in a separate study [*see KCA 7.1.2.2.1/8 2015/1204922*] conducted as part of the EU renewal of BAS 555 F (metconazole).

III. CONCLUSION

BAS 750 F residues degraded under field conditions in soil at all six European field sites. The total amount of BAS 750 F residues detected in the soil profiles decreased from an average of 122 g ha^{-1} at day 0 to an average of 30 g ha^{-1} after 24 months. DT_{50} values are presented in a separate modeling report.

Quantifiable residues of BAS 750 F residues were detected only in the first 20 cm of the soils. No residues above the LOQ were detected below 20 cm in any sample at any site. Altogether, it can be concluded that BAS 750 F does not show any significant tendency to move into deeper soil layers indicating low potential to leach to groundwater.

Metabolite M750F001 (1,2,4-triazole) was also monitored during the study. No residues of 1,2,4-triazole above the LOQ were detected in any sample at sites in Denmark, Germany (West), France (North), Italy, and Spain, except for one single finding close to the LOQ (at 3 µg kg^{-1}) in the 0-10 cm layer at the 7 DAT sampling in France.

Metabolite M750F003 was also monitored during the study. No residues of M750F003 above the LOQ were detected in any sample.

A different picture was seen at the trial in Germany (East), where residues of M750F001 were detected in significant amounts in all replicates and all soil depths. These findings, however, are considered the result of a substantial background contamination of the trial site with M750F001, as high amounts of M750F001 (41 g ha^{-1} within 0-50 cm soil profile) were also detected in the untreated control samples taken two days before the application at a depth of 0-50 cm. Therefore, the analytical results for M750F001 from the trial in Germany (East) are considered to be meaningless and not useful in the environmental exposure assessment. The observed background concentration of the soil in Germany (East) might be explained by the potential application of M750F001 containing nitrogen fertilizers since no triazole containing fungicides had been applied in the past 3 years.

Report:	CA 7.1.2.2.1/3 Jacobson B. et al., 2016 a Terrestrial field dissipation of the fungicide BAS 750 F following broadcast applications of BAS 750 01 F (EC) or BAS 750 UA F (SC) 2015/7006396
Guidelines:	EPA 835.6100
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The dissipation of BAS 750 F and its metabolites M750F001 (1,2,4-triazole) and M750F003 was investigated at six sites in the U.S.A. Sampling and analysis are still ongoing, interim results are reported, therefore no final conclusions may be drawn on the overall $DisT_{50}$ until the completed final report is reviewed.

The selected fields represented typical regions of agricultural practice and had been under cultivation for many years. The sampling sites were fallow prior to the study start.

The formulated product was broadcast applied to bare soil in two to three applications at target application rates of 150 g a.s. ha^{-1} or 200 g a.s. ha^{-1}, which resulted in the maximum proposed label use rate for the crop represented at each test site. While the sites were not actually cropped, the application and practice were followed as for a cropped situation as per the label under GAP (Good Agricultural Practice).

Seasonal weather data were collected at each test site for the duration of the study period, beginning just prior to application. Sprinkler irrigation was provided to supplement normal precipitation so that the plots would receive 110% of historical average rainfall for the duration of the study period.

Depending on the plot, there were 16 or 18 scheduled sampling intervals in the treated plots: Prior to and immediately after each test substance application and then at 3, 7, 15, 30, 60, 90, 180, 270, 390, 510, 630, and 750 days after last application (DALA). Interim results are reported for sampling and analysis up to 390 DALA.

BAS 750 F dissipated slowly in all trials with preliminary $DisT_{50}$ values from 101 to 292 days. As these are preliminary data–and can be expected to change once the full data package are considered, no further consideration should be given these interim results.

The metabolites M750F001 and M750F003 were detected at all trial sites. Quantifiable amounts (>0.002 mg kg^{-1}) were found at four of the six the trial sites. M750F001 was observed in some sites prior to or at application.

An ecoregion crosswalk for the six trial sites showed that five (California, Illinois, New York, Oklahoma and Washington) out of the six terrestrial field dissipation trial sites have similar ecoregions in Europe. Therefore, results for those five trial sites of US terrestrial field dissipation study of BAS 750 F are applicable to Europe.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test item (formulation):	BAS 750 01 F
Active ingredient:	BAS 750 F (Reg. No. 5834378)
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molar mass:	397.8 g mol^{-1}
Batch No.:	FD-140113-0006 (containing 98.9 g BAS 750 F L^{-1})
Type of formulation:	EC

Test item (formulation):	BAS 750 UA F
Active ingredient:	BAS 750 F (Reg. No. 5834378)
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molar mass:	397.8 g mol^{-1}
Batch No.:	FD-140121-0040 (containing 403.5 g BAS 750 F L^{-1})
Type of formulation:	SC

2. Test sites

The dissipation of BAS 750 F and its metabolites M750F001 (1,2,4-triazole) and M750F003 was investigated at six sites in the U.S.. The selected fields represented typical regions of agricultural practice in the US and had been under cultivation for many years. The sampling sites were fallow prior to the study. The site characteristics including the basic soil parameters of the corresponding soil horizons are presented in Table 7.1.2.2.1-12 and Table 7.1.2.2.1-14.

Table 7.1.2.2.1-12: Characteristics of the trial sites used in the field dissipation study (New York and North Dakota sites)

Trial	**R140591**							
Location	**New York (NY), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Silt Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam
sand [%]	27	23	23	21	17	13	17	21
silt [%]	58	62	68	72	72	76	72	62
clay [%]	15	15	9	7	11	11	11	17
Organic matter [%]	4.3	2.7	1.09	0.31	0.31	0.22	0.17	0.22
Total organic C [%]	2.5	1.6	0.63	0.18	0.18	0.13	0.10	0.13
pH [a]	5.0	4.9	5.0	5.1	5.1	4.9	5.0	5.7
CEC [meq $100g^{-1}$]	8.4	7.4	5.2	4.2	5.3	6	5.6	7.3
Moisture (gravimetric) at 1/3 bar [%]	32.1	35.7	32.4	27.1	27.5	28.9	23.7	23.9
Trial	**R140592**							
Location	**North Dakota (ND), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Clay	Clay	Clay	Clay	Sandy Clay Loam	Clay	Clay	Clay
sand [%]	30	34	42	40	56	38	32	44
silt [%]	29	19	17	13	9	15	17	9
clay [%]	41	47	41	47	35	47	51	47
Organic matter [%]	3.2	1.9	1.6	1.4	1.4	1.2	0.95	0.95
Total organic C [%]	1.9	1.1	0.90	0.83	0.80	0.68	0.55	0.55
pH [a]	7.5	7.6	7.7	7.8	7.8	8.0	7.9	8.0
CEC [meq $100g^{-1}$]	33.9	36.5	34.9	33.3	33.0	31.1	30.2	34.1
Moisture (gravimetric) at 1/3 bar [%]	41.3	45.3	43.1	46.4	43.8	44.2	43.7	41.4

CEC = Cation exchange capacity
[a] Measured in saturated paste

Table 7.1.2.2.1-13: Characteristics of the trial sites used in the field dissipation study (Washington and California sites)

Trial	**R140593**							
Location	**Washington (WA), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Loamy Sand	Sand	Sand	Loamy Sand	Loamy Sand	Sandy Loam	Sandy Loam	Sandy Loam
sand [%]	86	88	90	84	76	70	64	68
silt [%]	11	11	9	15	21	29	33	29
clay [%]	3	1	1	1	3	1	3	3
Organic matter [%]	0.39	0.22	0.22	0.18	0.18	0.13	0.18	0.09
Total organic C [%]	0.23	0.13	0.13	0.10	0.10	0.08	0.10	0.05
pH [a]	8.3	8.2	8.2	8.2	8.1	8.1	8.4	8.4
CEC [meq $100g^{-1}$]	7.6	8.0	8.1	8.9	9.3	10.7	13.7	14.6
Moisture (gravimetric) at 1/3 bar [%]	8.1	7.4	7.0	9.4	12.0	15.3	19.3	20.1
Trial	**R140594**							
Location	**California (CA), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Loamy Sand	Loamy Sand	Loamy Sand	Loamy Sand	Loamy Sand	Loamy Sand	Loamy Sand	Loamy Sand
sand [%]	74	76	76	74	76	80	80	82
silt [%]	22	20	20	22	22	18	18	16
clay [%]	4	4	4	4	2	2	2	2
Organic matter [%]	0.70	0.48	0.31	0.13	0.13	0.09	0.26	0.31
Total organic C [%]	0.41	0.28	0.18	0.08	0.08	0.05	0.15	0.18
pH [a]	7.6	8.0	8.3	8.3	8.3	8.3	8.5	8.4
CEC [meq $100g^{-1}$]	9.5	10.0	9.9	10.1	10.1	10.3	10.0	9.9
Moisture (gravimetric) at 1/3 bar [%]	11.2	10.6	11.7	11.9	11.1	11.3	9.4	10.0

CEC = Cation exchange capacity
[a] Measured in saturated paste

Table 7.1.2.2.1-14: Characteristics of the trial sites used in the field dissipation study (Oklahoma and Illinois sites)

Trial	**R140595**							
Location	**Oklahoma (OK), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Sandy Loam	Sandy Loam	Sandy Loam	Loam	Loam	Loam	Loam	Sandy Clay Loam
sand [%]	59	59	53	47	43	39	47	55
silt [%]	26	24	28	32	34	34	30	24
clay [%]	15	17	19	21	23	27	23	21
Organic matter [%]	0.67	0.97	0.76	0.71	0.63	0.63	0.50	0.42
Total organic C [%]	0.39	0.56	0.44	0.42	0.37	0.37	0.29	0.24
pH [a]	7.1	5.8	5.8	6.3	6.4	6.7	6.8	7.0
CEC [meq $100g^{-1}$]	7.8	8.2	9.2	9.6	11.0	13.2	12.1	11.8
Moisture (gravimetric) at 1/3 bar [%]	10.8	12.8	14.7	17.0	20.4	23.1	20.0	18.9
Trial	**R140596**							
Location	**Illinois (IL), USA**							
Soil properties	**Depth [inches] ([cm])**							
	0-6 (0-15)	**6-12 (15-30)**	**12-18 (30-46)**	**18-24 (46-61)**	**24-30 (61-76)**	**30-36 (76-91)**	**36-42 (91-107)**	**42-48 (107-123)**
Soil class (USDA)	Silty Clay Loam	Silty Clay Loam	Silty Clay Loam	Silty Clay	Silty Clay Loam	Silty Clay Loam	Silty Clay Loam	Clay Loam
sand [%]	15	9	9	11	15	7	13	23
silt [%]	52	52	56	46	48	54	52	46
clay [%]	33	39	35	43	37	39	35	31
Organic matter [%]	4.3	3.3	1.8	0.82	0.56	0.56	0.47	0.34
Total organic C [%]	2.5	1.9	1.0	0.47	0.32	0.32	0.27	0.20
pH [a]	6.0	6.0	6.3	6.6	6.8	7.0	7.2	7.4
CEC [meq $100g^{-1}$]	18.9	21.0	23.0	23.8	22.6	21.6	19.0	16.6
Moisture (gravimetric) at 1/3 bar [%]	33.9	34.9	36.8	39.8	37.9	35.9	33.5	29.6

CEC = Cation exchange capacity
[a] Measured in saturated paste

B. STUDY DESIGN

1. Experimental conditions

At each test site there were two test plots; a control bare soil plot (Plot 1) and a treated bare soil plot (Plot 2). The treated plot was divided into three replicate areas to provide three replicate samples (Subplot 1, 2, and 3). The control plot was not subdivided, but was separated from the treated plot by a buffer zone of at least 15 m width.

The formulated product was broadcast applied to bare soil in two (IL test site) or three applications (NY, ND, OK, WA and CA test sites) at target application rates of 150 g a.s. ha^{-1} (NY, IL, ND, WA, and CA test sites) or 200 g a.s. ha^{-1} (OK test site), which resulted in the maximum proposed label use rate for the crop represented at each test site. Actual application rates of BAS 750 F at each trial site were determined from the sprayer calibration and pass times. Details of the application are presented in Table 7.1.2.2.1-15.

Table 7.1.2.2.1-15: Application parameters of field trial sites treated with BAS 750 F

Trial, location	Formulated product	No. of applications (interval)	Application rate per treatment		Application date
			Nominal [g a.s. ha^{-1}]	Actual [g a.s. ha^{-1}]	
R140591, New York	BAS 750 UA F (SC)	3 (7 days)	150	150 152 147	17-Jun-14 24-Jun-14 01-Jul-14
R140592, North Dakota	BAS 750 01 F (EC)	3 (7 days)	150	141 150 148	19-Jul-14 26-Jul-14 02-Aug-14
R140593, Washington	BAS 750 UA F (SC)	3 (7 days)	150	149 149 148	17-Jun-14 24-Jun-14 01-Jul-14
R140594, California	BAS 750 UA F (SC)	3 (7 days)	150	148 149 148	15-Aug-14 22-Aug-14 29-Aug-14
R140595, Oklahoma	BAS 750 01 F (EC)	3 (14 days)	200	196 197 199	12-Aug-14 26-Aug-14 09-Sep-14
R140596, Illinois	BAS 750 01 F (EC)	2 (7 days)	150	157 152	13-Aug-14 20-Aug-14

Treated application verification (AV) samples were generated in the field to confirm the amount of test compound applied to a given area. The methodology used Speedisks® as the spray capture system. Three treated/spray samples (AV-Sprays for each plot) were generated at the application event. The results of the application verification experiment will be reported in the final report and are not presented at this time in the interim report.

After application of the test substance, the test plots were maintained in ways that minimized the disturbance of the plots. The bare soil plots, treated and control, were kept weed free so that the maximum amount of test substance could reach the soil surface.

Seasonal weather data were collected at each test site for the duration of the study period, beginning just prior to application. In addition, historical weather data (average monthly minimum, maximum, and average air temperatures and monthly precipitation totals) were submitted for at least a thirty-year period from a reliable source located no more than approximately 20 miles (32 km) from the test site.

Sprinkler irrigation was provided to ensure that the entire area could be irrigated with even water distribution. Irrigation was applied to supplement normal precipitation so that the plots would receive 110% of historical average rainfall for the study period.

A summary of already available monthly weather data (temperature and precipitation, as well as volumes of the supplementary irrigation) is presented in Table 7.1.2.2.1-16.

Table 7.1.2.2.1-16: Summary of monthly air temperature, precipitation, and irrigation at each field trial site – interim data

Trial	R140591			R140592		
Location	New York			North Dakota		
Month/ Year	T_{mean} Air [°C]	Precipitation [mm]	Irrigation [mm]	T_{mean} Air [°C]	Precipitation [mm]	Irrigation [mm]
Jun-14	20.0	37.3	38.4	n.d.	n.d.	n.d.
Jul-14	19.8	181.6	0.0	21.4	19.6	9.9
Aug-14	19.5	100.3	19.1	20.9	114.0	0.0
Sep-14	16.3	26.9	94.5	15.9	36.8	0.0
Oct-14	11.9	55.6	76.2	8.8	13.7	0.0
Nov-14	3.5	49.5	0.0	-5.4	43.4	0.0
Dec-14	1.0	7.9	0.0	-6.4	6.4	0.0
Jan-15	-6.6	43.7	0.0	-9.1	1.0	0.0
Feb-15	-10.1	8.6	0.0	-13.9	0.5	0.0
Mar-15	-1.6	35.6	0.0	0.8	7.6	0.0
Apr-15	7.6	98.6	0.0	8.2	26.9	0.0
May-15	17.1	95.3	0.0	12.7	187.5	0.0
Jun-15	18.2	137.9	0.0	19.6	76.7	0.0
Jul-15	21.1	62.7	38.1	22.3	73.2	0.0
Aug-15	n.d.	n.d.	n.d.	20.3	37.1	0.0
Trial	**R140593**			**R140594**		
Location	**Washington**			**California**		
Month/ Year	**T_{mean} Air [°C]**	**Precipitation [mm]**	**Irrigation [mm]**	**T_{mean} Air [°C]**	**Precipitation [mm]**	**Irrigation [mm]**
Jun-14	18.4	1.3	59.4	n.d.	n.d.	n.d.
Jul-14	24.5	0.5	297.2	n.d.	n.d.	n.d.
Aug-14	23.1	9.7	219.5	25.6	0.0	50.8
Sep-14	17.8	9.4	114.3	24.2	0.0	184.2
Oct-14	12.4	19.3	11.4	19.2	0.0	88.9
Nov-14	2.1	23.1	0.0	11.6	29.7	12.7
Dec-14	1.0	25.4	0.0	8.0	74.4	0.0
Jan-15	0.0	21.6	0.0	5.0	4.8	76.2
Feb-15	5.3	15.0	0.0	9.6	26.2	38.1
Mar-15	9.0	26.9	0.0	14.8	1.5	69.9
Apr-15	10.6	9.7	0.0	16.4	13.2	101.6
May-15	17.7	17.0	106.4	19.4	9.4	88.9
Jun-15	23.3	0.3	182.9	25.9	0.3	152.4
Jul-15	25.8	0.0	197.9	27.0	3.3	241.3
Aug-15	n.d.	n.d.	n.d.	26.3	0.0	171.5
Trial	**R140595**			**R140596**		
Location	**Oklahoma**			**Illinois**		
Month/ Year	**T_{mean} Air [°C]**	**Precipitation [mm]**	**Irrigation [mm]**	**T_{mean} Air [°C]**	**Precipitation [mm]**	**Irrigation [mm]**
Aug-14	28.5	0.3	89.4	23.6	49.3	0.0
Sep-14	23.1	1.5	53.3	18.4	93.0	0.0
Oct-14	18.3	13.2	17.8	12.3	91.9	0.0
Nov-14	6.7	6.6	0.0	1.8	47.5	0.0
Dec-14	4.7	6.6	0.0	0.9	44.2	0.0
Jan-15	3.1	48.3	0.0	-3.4	8.4	0.0
Feb-15	2.5	17.8	19.8	-6.7	9.1	0.0
Mar-15	10.7	76.2	25.4	3.2	42.4	0.0
Apr-15	15.8	127.0	0.0	12.8	67.8	0.0
May-15	18.1	439.4	0.0	19.4	124.2	0.0
Jun-15	26.1	53.3	22.1	22.7	178.8	0.0
Jul-15	27.6	129.5	0.0	23.4	82.8	16.3
Aug-15	26.4	55.9	0.0	22.3	58.4	17.3

Weather data refer to time period from start of trial (day of application) until end of trial (day of last sampling)
n.d. = Not determined

2. Sampling

There were 16 (IL test site) or 18 (NY, ND, OK, WA and CA test sites) scheduled sampling intervals in the treated plots: Prior to and immediately after each test substance application and then 3, 7, 15, 30, 60, 90, 180, 270, 390, 510, 630, and 750 days after last application (DALA). The sampling events available for the interim report are summarized in Table 7.1.2.2.1-17. In the control plots, six sampling intervals were scheduled: Prior to the first test substance application (-T1) and then 3, 15, 60, 90, and 390 days after last application.

Table 7.1.2.2.1-17: Sampling intervals of the treated plots following first application at each field trial site – interim data

R140591, New York

Event	Date	DA1A	DALA
T1	17-Jun-14	0	--
-T2	23-Jun-14	6	--
T2	24-Jun-14	7	--
-T3	30-Jun-14	13	--
T3	01-Jul-14	14	0
3	04-Jul-14	17	3
7	08-Jul-14	21	7
15	16-Jul-14	29	15
30	31-Jul-14	44	30
60	30-Aug-14	74	60
90	29-Sep-14	104	90
180	29-Dec-14	195	181
270	24-Mar-15	280	266
390	27-Jul-15	405	391

R140592, North Dakota

Event	Date	DA1A	DALA
T1	19-Jul-14	0	--
-T2	25-Jul-14	6	--
T2	26-Jul-14	7	--
-T3	01-Aug-14	13	--
T3	02-Aug-14	14	0
3	05-Aug-14	17	3
7	09-Aug-14	21	7
15	20-Aug-14	32	18
30	03-Sep-14	46	32
60	02-Oct-14	75	61
90	31-Oct-14	104	90
270	26-May-15	311	297
390	26-Aug-15	403	389

R140593, Washington

Event	Date	DA1A	DALA
T1	17-Jun-14	0	--
-T2	23-Jun-14	6	--
T2	24-Jun-14	7	--
-T3	30-Jun-14	13	--
T3	01-Jul-14	14	0
3	04-Jul-14	17	3
7	08-Jul-14	21	7
15	16-Jul-14	29	15
30	31-Jul-14	44	30
60	29-Aug-14	73	59
90	29-Sep-14	104	90
180	10-Dec-14	176	162
270	27-Mar-15	283	269
390	21-Jul-15	399	385

R140594, California

Event	Date	DA1A	DALA
T1	15-Aug-14	0	--
-T2	22-Aug-14	7	--
T2	22-Aug-14	7	--
-T3	28-Aug-14	13	--
T3	29-Aug-14	14	0
3	01-Sep-14	17	3
7	05-Sep-14	21	7
15	13-Sep-14	29	15
30	28-Sep-14	44	30
60	28-Oct-14	74	60
90	19-Nov-14	96	82
180	25-Feb-15	194	180
270	26-May-15	284	270
390	24-Aug-15	374	360

R140595, Oklahoma

Event	Date	DA1A	DALA
T1	12-Aug-14	0	--
-T2	25-Aug-14	13	--
T2	26-Aug-14	14	--
-T3	08-Sep-14	27	--
T3	09-Sep-14	28	0
3	11-Sep-14	30	2
7	16-Sep-14	35	7
15	24-Sep-14	43	15
30	09-Oct-14	58	30
60	06-Nov-14	86	58
90	08-Dec-14	118	90
180	11-Mar-15	211	183
270	10-Jun-15	302	274
390	21-Aug-15	374	346

R140596, Illinois

Event	Date	DA1A	DALA
T1	13-Aug-14	0	--
-T2	19-Aug-14	6	--
T2	20-Aug-14	7	0
3	23-Aug-14	10	3
7	27-Aug-14	14	7
15	04-Sep-14	22	15
30	19-Sep-14	37	30
60	20-Oct-14	68	61
90	14-Nov-14	93	86
180	17-Mar-15	216	209
270	18-May-15	278	271
390	25-Aug-15	377	370

T = Day of application (immediately after treatment)
-T = Day before the first application
DA1A = days after first application
DALA = days after last application

At each sampling event in the treated plots, five cores were taken to a depth of 48 inches (~122 cm) in each subplot (replicate sampling). This resulted in 15 cores being collected in the treated plot during a sampling event (except on days of application T1, T2, and T3 events when a duplicate set of 0-3 inch cores were taken).

Immediately after sampling and before freezing, all soil cores were sectioned into segments of three inches (7.6 cm) (0-3 inches and 3-6 inches) or six inches (15.2 cm) (6-12 inches to 42-48 inches and pooled by depth.

All soil specimens stored under freezer conditions at about -18°C and remained frozen until processing and/or analysis of the samples.

3. Description of analytical procedure

Analysis of soil core samples was conducted using BASF Analytical Method L0214/01 (Version October 29, 2013). Details on the method are given in the full interim study report. Soil samples were extracted twice with acetonitrile:water 70:30 and the extracts were combined. The extracts were analyzed for parent BAS 750 F and the metabolites M750F001 (1,2,4-triazole) and M750F003. Analysis was performed using LC-MS/MS. The limit of quantitation (LOQ) and limit of detection (LOD) for residues of BAS 750 F and its metabolites were 0.002 mg/kg (ppm) and 0.0004 mg/kg (ppm), respectively. Results of soil analysis were reported on a “dry weight” basis for residue determination.

4. Kinetic evaluation

A preliminary kinetic evaluation of the dissipation of BAS 750 F from the time of the last application through 346 - 391 days after the last application was conducted.

The goodness-of-fit of the kinetic models was assessed by visual inspection and statistical measures, as recommended by the FOCUS Kinetics guidance. A kinetic model was considered appropriate if the residuals were randomly distributed around zero, the χ^2 error value was ideally <15% (though often higher for field studies) and the estimated degradation parameters differed significantly from zero.

The software package KinGUI version 2.2012.202.925 was used for parameter fitting.

The areic mass (g ha^{-1}) of analyte in each soil core segment was calculated from the dry weight analytical concentration values considering the length of each segment and the undisturbed soil bulk density. The total areic mass was calculated as the sum over all soil core segments. Analytical values <LOD were treated as zero. Analytical values between the LOD and LOQ were used as reported. Values for all the three replicate plots were used in the kinetic analysis for BAS 750 F.

Assessment of dissipation of the metabolites M750F003 and 1,2,4-triazole was not attempted due to the low and/or variable levels of these products observed during the analyzed sampling intervals. Further, 1,2,4-triazole levels were similar in pre application and post application samples at some sites, indicating pre-existing levels of 1,2,4 triazole due to historic use of other triazole containing pesticides or agricultural products or natural occurrence.

5. Ecoregion Crosswalk

An ecoregion comparison for six test sites was conducted to determine which locations in Europe, if any, have similar environmental conditions. All work was conducted using the Organisation for Economic Co-operation and Development (OECD) Europe – North America Soil Geographic Information for Pesticide Studies (ENASGIPS) v2.3.2 application.

The underlying premise of ENASGIPS is that the field dissipation behavior of a pesticide in a region depends primarily on environmental factors, such as soils and climate. If these environmental factors are similar between regions, then field dissipation of a pesticide is also expected to be similar in those regions.

ENASGIPS compares ecoregions based on five parameters: mean annual temperature, mean annual precipitation, mean soil pH, mean soil organic carbon, and soil texture. Both holistic (including all parameters) and weights of evidence (including a subset of parameters) ecoregion comparisons were made for the six test sites. As dissipation or degradation of BAS 750 F is known not be influenced by soil pH this parameter was not included in the ecoregion comparison for the weights of evidence assessment.

II. RESULTS AND DISCUSSION

1. Residues in field soil samples

Mean procedural recoveries from control soil samples fortified with BAS 750 F and its metabolites at LOQ and 100 × LOQ were generally found to be within an acceptable range, which is mean values of recoveries between 70-120%. A summary of the individual procedural recovery results is provided in the study report.

Field soil samples taken from different depths were analyzed to a maximum of about 390 days after the last application. The available analytical results for the interim report are summarized in Table 7.1.2.2.1-18 to Table 7.1.2.2.1-23. All residue values presented in these tables are related to the dry weight of the soil and were not corrected for procedural recoveries.

Table 7.1.2.2.1-18: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – New York site (R140591) – interim results

Compound	Soil depth [inch]	Targeted days after last application														
		-T1	T1	-T2	T2	-T3	T3	3	7	15	30	60	90	180	270	390
BAS 750 F	0-3	<LOD	0.16	0.063	0.16	0.064	0.18	0.077	0.14	0.12	0.087	0.12	0.11	0.14	0.459	0.064
	3-6	<LOD	0.00067	<LOD	0.0036	0.0065	0.0014	0.11	0.0016	0.0018	0.00088	0.0034	0.003	0.016	0.067	0.0039
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.012	0.0021
M750F001 (1,2,4-triazole)	0-3	0.0024	0.0015	0.0022	0.0023	0.0021	0.0022	0.0018	0.0023	0.0022	0.002	0.0021	0.0022	0.0023	0.0023	0.0024
	3-6	0.0024	0.0014	0.0017	0.0015	0.0016	0.0017	0.0019	0.0016	0.0014	0.0018	0.0018	0.0023	0.0021	0.0031	0.0015
	6-12	0.00094		0.001	0.001	0.0012	0.0016	0.00068	0.0007	0.0011	0.001	0.00071	0.0013	0.001	0.0013	0.0008
M750F003	0-3	<LOD	<LOD	0.0021	0.0023	0.0013	0.0019	0.0012	0.0023	0.0025	0.0018	0.0022	0.0024	0.0047	0.0076	0.0022
	3-6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0011	<LOD	<LOD	<LOD	<LOD	<LOD	0.00069	0.00178	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Table 7.1.2.2.1-19: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – North Dakota site (R140592) – interim results

Compound	Soil depth [inch]	Targeted days after last application													
		-T1	T1	-T2	T2	-T3	T3	3	7	15	30	60	90	270	390
BAS 750 F	0-3	<LOD	0.08	0.1	0.16	0.14	0.3	0.19	0.33	0.24	0.22	0.19	0.15	0.17	0.09
	3-6	<LOD	<LOD	<LOD	0.00062	0.000849	0.0068	0.00083	0.0023	0.0011	<LOD	0.00081	0.00085	0.004	0.011
	6-12	<LOD		<LOD	<LOD	<LOD	0.0017	0.0033	0.0013	0.0015	0.0082	0.0014	0.0027	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	0.0018	<LOD	0.005	0.001	<LOD	<LOD	<LOD	<LOD	
M750F001 (1,2,4-triazole)	0-3	0.0033	0.0041	0.0049	0.0046	0.0047	0.0051	0.0056	0.0051	0.0048	0.0056	0.0053	0.0046	0.0054	0.0054
	3-6	0.0028	0.003	0.0036	0.0032	0.0036	0.0041	0.0041	0.004	0.004	0.0033	0.004	0.0027	0.0035	0.0022
	6-12	0.00174		0.0021	0.00197	0.00146	0.0022	0.002	0.00234	0.0022	0.0027	0.0032	0.00287	0.0019	0.0009
	12-18	0.0013		0.0013	0.00089	0.00093	0.0012	0.00079	0.0015	0.00085	0.0014	0.0013	0.0013	0.00076	
M750F003	0-3	<LOD	<LOD	0.0021	0.0017	0.0035	0.0045	0.0038	0.0064	0.0054	0.0045	0.0038	0.0035	0.0048	0.0015
	3-6	<LOD	<LOD	<LOD	<LOD	0.00021	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Table 7.1.2.2.1-20: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – Washington site (R140593) – interim results

Compound	Soil depth [inch]	Targeted days after last application														
		-T1	T1	-T2	T2	-T3	T3	3	7	15	30	60	90	180	270	390
BAS 750 F	0-3	<LOD	0.11	0.065	0.15	0.13	0.24	0.19	0.2	0.2	0.21	0.17	0.15	0.14	0.15	0.052
	3-6	<LOD	0.00025	0.00031	0.00061	0.00031	0.00076	0.0016	0.0016	0.00079	0.0011	0.0016	0.0035	0.00043	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0016	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.00052	<LOD	0.002	<LOD	
M750F001 (1,2,4-triazole)	0-3	<LOD	<LOD	0.00042	<LOD	0.00069	0.00049	0.00088	0.00075	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	3-6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
M750F003	0-3	<LOD	<LOD	0.0005	0.00047	<LOD	0.00052	0.001	0.0013	<LOD	<LOD	<LOD	<LOD	0.00065	0.00068	<LOD
	3-6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.00036	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Table 7.1.2.2.1-21: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – California site (R140594) – interim results

Compound	Soil depth [inch]	Targeted days after last application														
		-T1	T1	-T2	T2	-T3	T3	3	7	15	30	60	90	180	270	390
BAS 750 F	0-3	<LOD	0.17	0.033	0.25	0.095	0.17	0.12	0.12	0.1	0.14	0.089	0.12	0.13	0.07	0.04
	3-6	<LOD	0.0052	0.0038	0.0053	0.0038	0.0024	0.0047	0.0059	0.00046	0.0027	0.0028	0.0017	0.0023	0.001	0.0003
	6-12	<LOD		0.014	0.0042	0.016	0.044	0.01	0.011	0.0092	0.015	0.0091	0.0021	0.011	0.007	0.0027
	12-18	<LOD		0.001	<LOD	<LOD	0.00055	<LOD	0.00089	<LOD	0.00058	<LOD	<LOD	<LOD	<LOD	
M750F001 (1,2,4-triazole)	0-3	<LOD	0.0007	0.00083	0.0018	0.00086	0.00093	0.0018	0.0017	0.0012	0.0014	<LOD	<LOD	0.00067	0.0064	0.0021
	3-6	<LOD	<LOD	<LOD	<LOD	0.00066	0.00095	<LOD	0.00058	0.00041	0.00046	<LOD	<LOD	<LOD	0.000442	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0012	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
M750F003	0-3	<LOD	<LOD	0.0013	0.0023	0.0016	0.0015	0.0022	0.0014	0.0011	0.00068	0.00034	0.00074	0.0015	0.0006	0.00048
	3-6	<LOD	<LOD	<LOD	<LOD	0.00053	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Table 7.1.2.2.1-22: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – Oklahoma site (R140595) – interim results

Compound	Soil depth [inch]	Targeted days after last application														
		-T1	T1	-T2	T2	-T3	T3	3	7	15	30	60	90	180	270	390
BAS 750 F	0-3	<LOD	0.11	0.093	0.25	0.17	0.25	0.28	0.28	0.3	0.24	0.27	0.31	0.2	0.15	0.08
	3-6	<LOD	0.00084	<LOD	0.0037	0.0039	<LOD	<LOD	0.00054	0.006	0.0024	<LOD	0.0045	0.0068	<LOD	<LOD
	6-12	<LOD		<LOD	0.00063	0.00074	0.0028	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	0.00042	0.0012	0.0025	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
M750F001 (1,2,4-triazole)	0-3	<LOD	<LOD	<LOD	<LOD	0.00041	<LOD	0.0014	0.001	0.0014	0.0016	0.001	0.0012	0.0014	0.0102	0.0011
	3-6	<LOD	<LOD	<LOD	<LOD	0.00056	0.00041	<LOD	<LOD	0.00074	0.00089	0.0012	0.00075	0.001	0.0133	<LOD
	6-12	0.001		0.00092	0.0013	0.0014	0.0013	0.0017	0.00096	0.00099	0.00097	0.0012	0.00084	0.0011	0.0262	0.0011
	12-18	0.00031		<LOD	0.00067	0.0007	<LOD	0.00053	<LOD	<LOD	0.0006	0.00069	<LOD	0.00088	0.0107	
M750F003	0-3	<LOD	<LOD	0.00053	0.00085	0.00078	0.0008	0.0008	0.0017	0.0016	0.0014	0.001	0.0022	0.0018	0.0008	<LOD
	3-6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.00042	<LOD	<LOD
	6-12	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Table 7.1.2.2.1-23: Residues of BAS 750 F and metabolites [mg kg^{-1} dry weight] in treated soil samples (mean of three replicates) – Illinois site (R140596) – interim results

Compound	Soil depth [inch]	Targeted days after last application												
		-T1	T1	-T2	T2	3	7	15	30	60	90	180	270	390
BAS 750 F	0-3	<LOD	0.13	0.16	0.24	0.25	0.24	0.20	0.19	0.20	0.055	0.055	0.06	0.03
	3-6	<LOD		0.028	0.036	0.017	0.025	0.015	0.015	0.087	0.022	0.0017	0.0012	0.0023
	6-12	<LOD		0.0035	0.04	0.0033	0.0024	0.016	0.00077	0.005	0.0022	0.0005	<LOD	0.00075
	12-18	<LOD		<LOD	<LOD	0.0006	0.00062	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
M750F001 (1,2,4-triazole)	0-3	0.00076	0.00087	0.0012	0.0012	0.0015	0.0016	0.0019	0.0015	0.0014	0.0014	0.00047	0.00093	0.00184
	3-6	0.00083		0.00086	0.00074	0.00079	0.00092	0.0010	0.0010	0.0013	0.0013	0.00088	0.00070	0.0015
	6-12	0.00085		0.00053	0.00073	0.00072	0.0010	0.00095	0.00061	0.0012	0.0014	0.00082	0.00072	0.0008
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.00043	<LOD	0.0006	0.00058
M750F003	0-3	<LOD	<LOD	0.0026	0.0024	0.0034	0.0050	0.0058	0.0047	0.0055	0.0038	0.0026	0.0019	0.0010
	3-6	<LOD		0.00044	<LOD	<LOD	<LOD	0.00054	<LOD	0.0025	0.002	<LOD	<LOD	<LOD
	6-12	<LOD		<LOD	0.00062	0.00022	<LOD	0.00045	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	12-18	<LOD		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	

T = Application dates
LOQ = 0.002 mg kg^{-1}
LOD = 0.0004 mg kg^{-1}

Prior to the first application (-T1), residue concentrations of BAS 750 F and its metabolite M750F003 were below LOD in all samples taken at all sites. Residue concentrations of metabolite M750F001 were below LOD at the sites WA (R140593) and the CA (R140594) in samples at all depths. In the sites NY (R140591), ND (R140592), OK (R140595) and IL (R140596), M750F001 was detected before the first application (-T1).

BAS 750 F dissipated slowly in all trials. The interim analytical data showed that about 10 to 40% of the applied amount remained as parent after 390 days after the last application.

The metabolites M750F001 and M750F003 were detected at all trial sites. Quantifiable amounts (>0.002 mg kg^{-1}) of M750F001 were found at the trial sites NY (R140591), ND (R140592), CA (R140594) and OK (R140595). Quantifiable amounts of M750F003 were found at the trial sites NY (R140591), ND (R140592), CA (R140594) and IL (R140596).

3. Kinetic evaluation

The SFO kinetic model was selected as the most appropriate to describe the dissipation of BAS 750 F at all six sites. The preliminary kinetic endpoints ($DisT_{50}$ and $DisT_{90}$) are summarized in Table 7.1.2.2.1-24. These should be viewed with caution as the results in the final report may differ.

Table 7.1.2.2.1-24: Summary of preliminary kinetic endpoints for BAS 750 F

Trial, location	Soil type (USDA)	pH (H_2O)	Kinetic model	χ^2 [%]	$DisT_{50}$ [d]	$DisT_{90}$ [d]
R140591, New York	silt loam	5.0	SFO	15.1	281	933
R140592, North Dakota	clay	7.5	SFO	16.9	286	951
R140593, Washington	loamy sand	8.3	SFO	10.1	286	951
R140594, California	loamy sand	7.6	SFO	23.7	266	884
R140595, Oklahoma	sandy loam	7.1	SFO	11.6	292	969
R140596, Illinois	silty clay loam	6.0	SFO	20.4	101	335

4. Ecoregion Crosswalk

Results from the holistic similarity assessment showed that matching ecoregions exceeding the 80% similarity level exist in Europe for the California, Illinois, New York, Oklahoma and Washington study sites. Four to 16 matching ecoregions were found for each site. No matching ecoregions were found for the North Dakota study site using the 80% similarity criteria in the holistic assessment.

Results from the weights of evidence similarity assessment showed that five (California, Illinois, New York, Oklahoma and Washington) out of the six US TFD study sites, 3 to 16 similar ecoregions exceeding the 80% similarity level exist in Europe.

III. CONCLUSION

The dissipation of BAS 750 F in soil under field conditions was investigated at six sites in the U.S. As sampling and analysis are still ongoing, interim results were reported.

BAS 750 F dissipated slowly in all trials with preliminary $DisT_{50}$ values from 101 to 292 days.

The metabolites M750F001 and M750F003 were detected at all trial sites. Quantifiable amounts (>0.002 mg kg^{-1}) were found at four of the six trial sites. M750F001 was observed in some sites prior to or at application.

The ecoregion crosswalk for the six trial sites showed that five (California, Illinois, New York, Oklahoma and Washington) out of the six terrestrial field dissipation trial sites have similar ecoregions in Europe. Therefore, results for those five trial sites of the US terrestrial field dissipation study of BAS 750 F are applicable to Europe.

Report:	CA 7.1.2.2.1/4 Studenroth S.,Pape L., 2015 a Kinetic evaluation of a field dissipation study with BAS 750 F conducted in 2013 to 2015: Determination of best-fit and modeling endpoints according to FOCUS 2015/1249176
Guidelines:	FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014, EFSA Guidance to obtain DegT50 values in soil (2014)
GLP:	no

EXECUTIVE SUMMARY

The degradation behavior of the fungicide BAS 750 F in soil has been investigated in a field dissipation study including six field trials located in Denmark, Germany (two trials, East and West), France, Italy and Spain. The purpose of this evaluation was to analyze the degradation kinetics of BAS 750 F observed in the six soils according to the current guidance of the FOCUS workgroup on degradation kinetics, under consideration of the recommendations provided in the EFSA guidance to obtain $DegT_{50}$ values in soil for modeling purposes.

In a first step, a kinetic evaluation was performed in order to derive best-fit field degradation parameters for BAS 750 F. The best-fit kinetic model was selected based on a visual and statistical assessment under consideration of the FOCUS guidance for deriving trigger endpoints.

In a second step, a kinetic evaluation was performed to derive degradation parameters that can be used as modeling endpoints. Prior to kinetic analysis, the sampling intervals of the field studies were normalized to reference conditions (20°C, pF2) regarding soil moisture and temperature according to the time-step normalization technique. Kinetic evaluation was performed on the time-step normalized data set, and degradation parameters that can be used as modeling endpoints were derived. The respective degradation parameters were derived based on a visual and statistical assessment under the consideration of the EFSA Guidance as well as the recommendations of the FOCUS kinetics working group.

The kinetic evaluation for derivation of best-fit endpoints showed that the degradation behavior of BAS 750 F was best described in four field trials using the SFO kinetic model (trial Denmark, Germany-East/West, and Italy) and in two field trials using DFOP model (trial France and Spain). The best-fit endpoints ranged from 145.4 to 846.6 days for the $DegT_{50}$ and from 616.1 to >1000 days for the $DegT_{90}$.

The SFO model was most appropriate to derive modeling endpoints for BAS 750 F. Resulting normalized $DegT_{50}$ ranged from 96.5 to 610.8 days.

The study design was compliant with EFSA's recommendations for obtaining $DegT_{50}$ values in soil from field studies for modeling purposes, as dissipation caused by surface processes like photolysis or volatilization was excluded by covering all plots with a sand layer. Hence, the reported best-fit endpoints represent a conservative estimate of the dissipation behavior of BAS 750 F in soil while the reported modeling endpoints are suitable for use in environmental fate models.

I. MATERIAL AND METHODS

The kinetic evaluation was conducted for six field trials with BAS 750 F from the data of one field dissipation study, which can be found in CA 7.1.2.2.1/1 and CA 7.1.2.2.1/2. The trials were situated in different regions of Europe (Denmark, Germany (two trials), France, Italy and Spain) considering a range of different soils and climatic conditions. Detailed soil characteristics in each trial are reported in the cited study. Applications were made to bare soil using a calibrated boom sprayer. Immediately after application and before subsequent soil sampling all plots were covered with a thin layer of sand to protect the applied product from surface processes like photolysis or volatilization, and to exclude any potential impact on the degradation of the test item caused by any of these processes. Soil samples were taken at day 0, immediately after application down to 10 cm soil depth and at 12 consecutive sampling dates after application down to a maximum soil depth of 50 cm from three individual subplots.

Prior to analysis by LC-MS/MS, the samples were extracted with acetonitrile/water (70/30, v/v). The limit of quantification was 0.002 mg kg^{-1} for BAS 750 F. The limit of detection (LOD) was set at 0.0004 mg kg^{-1} (20% of LOQ).

BAS 750 F residues were almost exclusively found in the top 0-20 cm soil layer in all six trials. No residues above the limit of quantification (LOQ) were detected below 20 cm in any sample at any site. Therefore, it can be concluded that BAS 750 F does not show any significant tendency to move into deeper soil layers.

Kinetic modeling strategy

The appropriate kinetic model was identified considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. In a first step, a kinetic evaluation was performed on the original data set in order to derive best-fit field degradation parameters for BAS 750 F (trigger endpoints). In a second step, a kinetic evaluation was performed on a time-step normalized data set to derive degradation parameters that can be used as modeling endpoints.

Normalization procedure

The suitability of field dissipation data for normalization was proven following the evaluation criteria for normalization as compiled by the Dutch regulatory authority (CTGB criteria).

The normalization procedure was carried out based on the recommendations of FOCUS [*FOCUS (2006)*] for all field trials by reducing or increasing day lengths depending on soil temperature and moisture by means of correction factors (f_{temp} and f_{moist}). For DAT 0, no normalization was considered and application was assumed to occur at the time point zero.

Temperature correction factors (f_{temp}) were determined to account for differences between actual daily soil temperatures as calculated by FOCUS-PEARL 4.4.4 and a reference temperature of 20°C using a Q_{10} value of 2.58.

Moisture correction factors (f_{moist}) were determined to account for differences between actual daily soil moisture as calculated by FOCUS-PEARL 4.4.4 and the reference soil moisture at field capacity (pF 2).

Normalized sampling days after application (DAT_{norm}) were calculated by cumulatively summing up normalized day lengths.

Table 7.1.2.2.1-25 shows the field sampling dates for the trial locations and the normalized (20°C, pF2) day lengths based on soil moisture and soil temperature data as simulated by FOCUS-PEARL 4.4.4.

Table 7.1.2.2.1-25: Time-step normalized (temperature and moisture) sampling days

Bogense, Denmark (L130556)		Lentzke, Germany (L130557)	
DAT	**D_{norm}**	**DAT**	**D_{norm}**
0	0	0	0
6	3.3	6	3.8
13	7.3	14	10.4
29	17.0	33	26.0
61	45.7	56	50.0
92	68.3	85	78.1
124	85.2	118	97.1
174	103.2	176	117.8
245	118.0	272	137.2
363	159.5	355	171.5
487	250.6	476	272.3
615	286.9	590	308.9
713	315.0	715	342.0
Goch-Nierswalde, Germany (L130558)		**Stotzheim, France North (L130559)**	
DAT	**D_{norm}**	**DAT**	**D_{norm}**
0	0	0	0
7	5.9	7	6.9
13	9.6	14	14.7
27	19.9	30	28.7
59	51.6	62	69.1
95	78.5	91	94.0
125	92.8	120	111.4
185	113.0	175	131.5
248	129.6	238	145.5
361	180.7	366	208.4
474	267.3	471	301.6
613	310.7	591	344.6
710	343.7	720	393.1
Poggio Renatico, Italy (L130560)		**Utrera, Spain (L130561)**	
DAT	**D_{norm}**	**DAT**	**D_{norm}**
0	0	0	0
7	7.0	6	8.9
13	14.9	13	15.5
29	34.8	29	32.8
56	77.2	58	83.3
90	126.8	92	163.3
120	156.4	127	246.3
183	188.2	183	328.8
285	217.7	230	357.3
351	257.0	353	441.7
475	405.1	478	661.1
600	463.4	591	807.6
714	515.9	713	880.7

DAT = Days after treatment

Kinetic models included in the evaluations

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance [*FOCUS (2006)*] were tested, i.e. single-first order (SFO), double first-order in parallel (DFOP) and first-order multi-compartment (FOMC).

The goodness-of-fit of the kinetic models was assessed by visual inspection and statistical measures, as recommended by the FOCUS Kinetics guidance. A kinetic model was considered appropriate if the residuals were randomly distributed around zero, the χ^2 error value was ideally <15% and the estimated degradation parameters differed significantly from zero.

Data handling and software for kinetic evaluation

As surface processes had been excluded by covering the soil with sand in the field study, all data points were considered in this evaluation regardless of the 10 mm rain criterion described in the EFSA guidance document [*EFSA (2014): Guidance Document for evaluating laboratory and field dissipation studies to obtain $DegT_{50}$ values of active substances of plant protection products and transformation products of these active substances in soil. EFSA Journal 2014;12(5):3662, 38 pp.*].

For the evaluation, the residue data in g ha^{-1} cumulated over the whole sampling depth were taken from the study report. Values below LOQ (0.002 mg kg^{-1}) were set to $0.5 \times$ LOQ according to FOCUS [*FOCUS (2006)*].

The software package KinGUI version 2.2012.224.1704 was used for parameter fitting. The error tolerance and the number of iterations of the optimization tool (IRLS) were set to 10^{-6} and 100, respectively.

The data sets submitted to kinetic analysis are provided in the original evaluation report.

II. RESULTS AND DISCUSSION

The kinetic evaluation for derivation of best-fit endpoints showed that the degradation behavior of BAS 750 F was best described in four field trials using SFO kinetic model (trial Denmark, Germany-East/West, and Italy) and in two field trials using DFOP kinetic model (trial France and Spain). For all trials, the SFO kinetic model was appropriate to derive modeling endpoints.

Best-fit models and corresponding endpoints ($DegT_{50}$ and $DegT_{90}$) and the appropriate modeling endpoints of BAS 750 F are summarized in Table 7.1.2.2.1-26.

Table 7.1.2.2.1-26: Summary of best-fit and modeling field degradation endpoints for BAS 750 F

Field trial	Soil type (USDA)	Best-fit endpoints				Modeling endpoints		
		Kinetic model	χ^2 [%]	$DegT_{50}$ [d]	$DegT_{90}$ [d]	Kinetic model	χ^2 [%]	Normalized $DegT_{50}$ [d]
L130556 (Denmark)	Sandy loam	SFO	9.2	185.5	616.1	SFO	9.4	96.5
L130557 (Germany-East)	Loamy sand	SFO	8.9	350.6	>1000	SFO	9.0	184.0
L130558 (Germany-West)	Silt loam	SFO	16.2	267.6	889.1	SFO	17.5	146.7
L130559 (France)	Silty clay loam	DFOP	8.4	145.4	870.2	SFO	6.2	128.6
L130560 (Italy)	Silty clay loam	SFO	9.4	846.6	>1000	SFO	8.5	610.8
L130561 (Spain)	Loamy sand	DFOP	6.3	200.5	971.6	SFO [a]	14.2	313.0

[a] Endpoint was derived with the initial concentration fixed to the mean of the measured values.

III. CONCLUSION

Kinetic evaluation of six field trials with BAS 750 F, originating from one field dissipation study, was conducted in order to derive reliable best-fit and normalized modeling endpoints according to the current guidance of the FOCUS workgroup on degradation kinetics.

The non-normalized best-fit field half-lives ($DegT_{50}$) for BAS 750 F ranged from 145.4 to 846.6 days. The corresponding $DegT_{90}$ values ranged from 616.1 to >1000 days.

Modeling endpoints for BAS 750 F could be derived from SFO kinetics for all field trials. Kinetic evaluation of the time-step normalized data set (20°C, pF2) resulted in normalized field half-lives ($DegT_{50}$) between 96.5 and 610.8 days.

Following are found studies in support of the field data. Storage stability as well as extractability studies are found below.

Report:	CA 7.1.2.2.1/5 Staudenmaier H.,Dalkmann P., 2015 b Investigation of the extractability of BAS 750 F in samples from 14C soil degradation studies 2015/1182724
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In this study, the extractability from soil of BAS 750 F and its soil metabolites was investigated. Two different extraction procedures were applied: the extraction procedure according to the residue analytical method L0214/01 and the extraction procedure that was used in soil metabolism / degradation studies.

Samples containing residues of BAS 750 F were taken from three previously conducted soil metabolism / degradation studies, where BAS 750 F had been applied in three radiolabeled forms (triazole-3(5)-^{14}C-, chlorophenyl-U-^{14}C-, and trifluoromethylphenylring-U-^{14}C-label). The selection criterion for the soil samples was the occurrence of the metabolites M750F001 (Reg. No. 87084) and M750F003 (Reg. No. 5924326) besides the active substance BAS 750 F.

Aliquots of the selected soil samples were extracted applying both extraction procedures. Using the residue analytical method, soil samples were extracted two times with acetonitrile / water (70/30, v/v). The extraction method used in the metabolism / degradation studies comprised extractions of soil samples two times with acetonitrile, two times with acetonitrile / water (80/20, v/v), and two times with acetonitrile / water (50/50, v/v).

The individual extracts were analyzed by liquid scintillation counting (LSC). For each extraction procedure, sample extracts were pooled according to the solvent used (acetonitrile and acetonitrile / water). The combined acetonitrile, as well as the combined acetonitrile / water extracts were concentrated and analyzed by radio-HPLC.

The total extractability of radioactive residues was in the range of 66.3 to 87.7% TAR for the different samples and extractions.

The extractability of radioactive residues was very similar for both the extraction scheme used for residue analysis and the extraction scheme used in the soil metabolism / degradation studies, irrespective of the extracted soil, the incubation time, and the position of the radiolabel. Differences between the two different extraction schemes were generally lower than 2% TAR.

HPLC analyses of the soil extracts revealed very similar results for the two different extraction schemes. For all three analytes, BAS 750 F and its soil metabolites M750F001 and M750F003, differences comprised no more than 2.9% TAR.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: ^{14}C-BAS 750 F

Description: triazole label: carbon-14 labeled at the triazole-3(5) position
chlorophenyl label: carbon-14 uniformly labeled in the chlorophenyl ring
trifluoromethylphenyl label: carbon-14 uniformly labeled in the trifluoromethylphenyl ring
specific activities:
triazole label: 5.46 MBq/mg
chlorophenyl label: 7.878 MBq/mg
trifluoromethylphenyl label: 8.288 MBq/mg

Lot/Batch #: triazole label: 1062-2001
chlorophenyl label: CFQ41561
trifluoromethylphenyl label: CFQ42039

Purity: triazole label: 98.8% (radiochemical), 98.9% (chemical)
chlorophenyl label: 98.9% (radiochemical), 99.1% (chemical)
trifluoromethylphenyl label: 98.3% (radiochemical), 96.3% (chemical)

CAS#: 1417782-03-6

Reg. No.: 5834378

Development code: BAS 750 F

2. Soil

The soil samples were obtained from two aerobic soil metabolism studies (DocID 2014/1275177 and 2015/1003306) and from one soil degradation study (DocID 2014/1275178). The soil samples were derived from the soils New Jersey (Batch Nos. 13/1720/01 and 14/1720/01) and Li10 (Batch No. 13/1680/04). Details on the soil parameters are given in the respective sections.

B. STUDY DESIGN

1. Test procedure

During this study, selected soil samples from three previously conducted soil metabolism / degradation studies, where BAS 750 F had been applied in three radiolabeled forms (triazole-3(5)-14C-, chlorophenyl-U-14C-, and trifluoromethylphenylring-U-14C-label), were extracted. Two different extraction procedures were applied: the extraction procedure according to the residue analytical method L0214/01 [*see KCA 4.1.2/1 2015/1039006*] and the extraction procedure that had been used in the soil metabolism / degradation studies.
The objective of the present study was to determine the efficiency of the analytical method to extract BAS 750 F and its potential soil metabolites from soils by using radiolabeled samples from recently performed crop metabolism studies.
The present study provides information on (1) the overall extractability of the radiolabeled material and (2) the quantification of the parent compound BAS 750 F and its potential metabolites in the extracts.

2. Description of analytical procedures

Extraction method applied for soil residue analysis (BASF Method No. L0214/01)

For workup, the soil sample was removed from the freezer and defrosted at room temperature. A 5 g aliquot of soil (dry weight) was weighed into a centrifuge tube. The soil was consecutively extracted with 2 x 40 mL acetonitrile / water (70/30, v/v) on a laboratory shaker at 225 rpm for 30 min. After each extraction step, the sample was centrifuged at 4000 rpm for 10 min.

Deviating from BASF Method No. L0214/01, supernatants were decanted separately into 50 mL volumetric flasks that were subsequently filled to the calibration mark with acetonitrile / water (70/30, v/v). Aliquots were measured by LSC. To obtain sufficient amounts of radioactivity for HPLC analyses, the whole volume of soil extracts was processed. Method deviations were considered as reasonable adaptions for the measurement and balancing by radio-analytical methods.

Extraction method applied in the soil metabolism / degradation studies

The extraction methods applied in the three soil metabolism / degradation studies are identical. However, in the present study only 5 g of soil (dry weight equivalent) were extracted. The soil to extraction solution ratio of 1/1 (v/v) remained unchanged.

For workup, the soil sample was removed from the freezer and defrosted at room temperature. A 5 g aliquot of soil (dry weight) was weighed into a centrifuge tube. The soil was consecutively extracted with 2 x 5 mL acetonitrile, 2 x 5 mL acetonitrile / water (80/20, v/v), and 2 x 5 mL acetonitrile / water (50/50, v/v) on a laboratory shaker at 150 rpm for 30 min. After each extraction step, the sample was centrifuged at 4000 rpm for 10 min. The supernatants were decanted into 5 mL volumetric flasks and the flasks were filled to the calibration mark with the according solvent. Three aliquots of each extract were analyzed by LSC.

General workup for HPLC analysis

Prior to HPLC analysis, the obtained acetonitrile and acetonitrile / water extracts obtained by applying both extraction methods were combined according to the used solvent. The combined extracts were concentrated to dryness by means of a rotary evaporator at 40°C). The remainder was dissolved in small volumes of acetonitrile / water (70/30, v/v; soil residue method) or acetonitrile or acetonitrile / water (60/40, v/v; extraction method from the soil metabolism studies) and transferred into a volumetric flask that was filled to the calibration mark with the respective solvent. Aliquots were analyzed by LSC.

Aliquots of the concentrated extracts were centrifuged and measured by LSC to determine the recovery and analyzed by radio-HPLC.

LSC and HPLC measurement

LSC was performed on a Tri-Carb 2910 TR (Perkin Elmer Life Sciences, Germany). A scintillator, IRGA-Safe PLUS (Perkin Elmer Life Sciences, Germany) was used. The external standardization method with a built-in β-radiation source was used for the determination of counting efficiencies.

II. RESULTS AND DISCUSSION

The extractability of radioactive residues from soil using two different extraction protocols and HPLC results for extracts are summarized in Table 7.1.2.2.1-27.

Table 7.1.2.2.1-27: Extractability and HPLC results for extracts of soil

Original study (DocID) / label / soil (Batch No.)	Days after treatment [1]	Extraction method	Extract [% TAR]	$t_R \approx 4.4$ min M750F001 [% TAR]	$t_R \approx 26.5$ min M750F003 [% TAR]	$t_R \approx 38.2$ min BAS 750 F [% TAR]
2014/1275177 / triazole / New Jersey (13/1720/01)	14	Metabolism method	87.2	1.4	1.1	84.4
		Residue analytical method	87.7	2.1	1.2	83.6
	90	Metabolism method	71.7	4.0	0.6	66.8
		Residue analytical method	70.9	6.1	0.7	63.9
2014/1275177 / chlorophenyl / New Jersey (13/1720/01)	90	Metabolism method	66.5	- [2]	- [2]	64.4
		Residue analytical method	66.3	- [2]	- [2]	65.6
2015/1003306 / trifluoromethylphenyl / New Jersey (14/1720/01)	30	Metabolism method	84.2	- [2]	1.5	79.3
		Residue analytical method	82.6	- [2]	2.1	79.0
2014/1275178 / triazole / Li10 (13/1680/04)	91	Metabolism method	85.6	1.5	0.7	82.4
		Residue analytical method	84.0	1.3	1.4	80.1

TAR = Total applied radioactivity
t_R = Retention time
[1] Referring to the original study
[2] Not detected since the metabolite contains no radiolabel

The total extractability of radioactive residues was in the range of 66.3 to 87.7% TAR for the different samples and extractions.

The extractability of radioactive residues was very similar for both the extraction scheme for residue analysis and the extraction scheme used in the soil metabolism / degradation studies, irrespective of the extracted soil, the incubation time, and the position of the radiolabel. Differences between the two different extraction schemes were generally lower than 2% TAR.

The parent compound BAS 750 F was detected in amounts between 63.9% and 84.4% TAR in the different samples and extractions. The two metabolites M750F001 and M750F003 were detected only in low amounts ranging from 1.3% to 6.1% TAR for the metabolite M750F001 and from 0.7% to 2.1% TAR for the metabolite M750F003. The occurrence of the soil metabolites is in accordance with the respective soil metabolism / degradation studies, where maximum amounts around 5% TAR (M750F001) or even below 5% TAR (M750F003) were observed.

HPLC analyses of the soil extracts revealed very similar results for the two different extraction schemes. For all three analytes, differences comprised no more than 2.9% TAR.

III. CONCLUSION

Both, the extraction scheme used in the soil metabolism / degradation studies and the extraction applied in the soil residue analytical method showed very similar results with regard to the extractability of BAS 750 F and its soil metabolites M750F001 and M750F003.

Report:	CA 7.1.2.2.1/6 Brewin S., 2015 a Storage stability of residues of BAS 750 F- Reg.No. 5834378 and its metabolite Reg.No. 5924326 in soil when stored at approximately -20°C for 540 days - Interim Report 2015/1050221
Guidelines:	2004/10/EC of 11 February 2004, OECD 506 (Oct. 2007), EPA 860.1380, SANCO/3029/99 rev. 4, OECD-ENV/JM/MONO/(2007)17
GLP:	yes (certified by Department of Health of the Government of the United Kingdom, United Kingdom)

This amendment was necessary for correcting typographical errors. Update study title presented on pages 1, 2, 3, and to correct a typographical error on page 11. It does not have any adverse impact on the data of the final report.

Report:	CA 7.1.2.2.1/7 Brewin S., 2015 b Interim report Amendment No. 1: Storage stability of residues of BAS 750 F- Reg.No. 5834378 and its metabolite Reg.No. 5924326 in soil when stored at approximately -20°C for 540 days - Interim Report 2015/1249072
Guidelines:	2004/10/EC of 11 February 2004, OECD 506 (Oct. 2007), EPA 860.1380, SANCO/3029/99 rev. 4, OECD-ENV/JM/MONO/(2007)17
GLP:	yes (certified by Department of Health of the Government of the United Kingdom, United Kingdom)

In the present study, the storage stability of BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326 (M750F003) in soil was investigated in six soils under deep frozen conditions (≤ -18°C). The soils used for set up the stability samples were derived from six field dissipation trials of a parallel field study [*CA 7.1.2.2.1/1, BASF DocID 2015/1046920*]. Stored samples have been tested for stability up to 240 days as the study is still ongoing and further analysis will be performed after 650 days of storage.

The stability samples were fortified at a concentration level of 0.02 mg kg^{-1} (10fold LOQ). Each soil aliquot was spiked individually with a single test item, two replicates per field soil type and analyzed time point were prepared. At different intervals (0, 27/28, 56/57, 90/91, 118/119 and 241/242 days) the stability samples were analyzed using BASF method L0214/01. The limit of quantification of the method is 0.002 mg kg^{-1}. The analytical method was validated in a previous study.

The storage stability of BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326 (M750F003) was proven in all six tested soils over the tested period of 240 days at ≤ -18°C, with the exception of Reg.No. 5924326 in soil from trial L130559, which showed some slight apparent degradation during this period. Further analysis will be performed after 360, 480 and 650 days of storage.

I. MATERIAL AND METHODS

A. MATERIALS

Test materials:	BAS 750 F	M750F003
Reg. No.	5834378	5924326
Batch No.:	L84-238, L85-12	L84-250
Purity:	99.7%, 99.4%	99.6%
CAS #:	-	-

B. STUDY DESIGN

1. Experimental Conditions

Six different soil types (sandy loam, loamy sand, silt loam, silty clay loam, silty clay loam and loamy sand), originating from the terrestrial dissipation study [*CA 7.1.2.2.1/1, BASF DocID 2015/1046920*] were used in the storage stability study. The soil samples were untreated and stored refrigerated at approximately 4°C until the day of fortification.

5 g of soil were weighed in 50 mL polypropylene-tubes and fortified individually at 0.02 mg/kg (10fold LOQ) with fortification solutions of Reg.No. 5834378 and Reg.No. 5924326 (separate systems for each analyte). For each storage interval and each soil type a set of at least eight samples (two samples for analysis of Reg.No. 5834378, two samples for analysis of Reg.No. 5924326, two control matrix samples and 2 procedural recovery samples, fortified with both analytes on the day of analysis) were prepared.

The soil samples were stored at approximately -20°C and analyzed after different intervals (0, 30, 60, 90, 120, and 240 days). Sampling interlay 360, 480 and 650 days are not included in this report.

2. Description of analytical procedures

The soil samples were analysed for residues of BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326 (M750F003) using method L0214/01.
The soil samples (5 g) are extracted twice with 40 mL of an acetonitrile/water solution (70/30, v/v) by mechanical shaking (30 minutes at 200 rpm). The samples are centrifuged (10 minutes at 3500 rpm), and combined aliquots of the supernatant are analysed for BAS 750 F (Reg.No. 5834378) and Reg. No. 5924326 (M750F003) using LC-MS/MS.

The method has a limit of quantitation of 2 µg/kg in soil, for each analyte.

The performance of the analytical method was confirmed by simultaneous analysis of two freshly prepared fortified specimens from each trial on each date of analysis.

II. RESULTS AND DISCUSSION

The residues in the soil samples were determined immediately after fortification and after storage for up to 240 days. At each time point two control samples, two stability samples fortified with BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326 (M750F003), respectively. No significant interferences (> 30% of LOQ) were observed in the samples at the retention time and mass transitions of both analytes.

From the results, recovery rates were calculated which were corrected for the mean recovery rates obtained from the respective freshly fortified specimens (same sampling date and soil). The recovery values in percent at the various dates of analysis are shown in Table 7.1.2.2.1-28 to Table 7.1.2.2.1-31.

Table 7.1.2.2.1-28: Results of storage stability samples of BAS 750 F (Reg.No. 5834378) in soil samples

Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery uncorrected [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L130556	0	0.0183	92	**85**	94	98	100
		0.0157	79			84	
	30	0.0172	86	**92**	101	85	108
		0.0193	97			96	
	60	0.0189	95	**99**	115	82	116
		0.0207	104			90	
	90	0.0186	93	**92**	113	83	108
		0.0182	91			81	
	120	0.0172	86	**89**	102	85	105
		0.0181	91			89	
	240	0.0146	73	**77**	89	82	91
		0.0159	80			90	
L130557	0	0.0190	95	**96**	98	97	100
		0.0191	96			98	
	30	0.0197	99	**99**	103	96	103
		0.0198	99			96	
	60	0.0198	99	**99**	107	93	103
		0.0198	99			93	
	90	0.0163	82	**88**	106	77	92
		0.0188	94			89	
	120	0.0194	97	**93**	103	94	97
		0.0175	88			85	
	240	0.0162	81	**81**	85	96	84
		0.0159	80			94	
L130558	0	0.0178	89	**91**	95	94	100
		0.0185	93			98	
	30	0.0186	93	**93**	100	93	102
		0.0186	93			93	
	60	0.0201	101	**102**	110	92	112
		0.0204	102			93	
	90	0.0178	89	**90**	101	88	99
		0.0179	90			89	
	120	0.0164	82	**84**	92	89	92
		0.0171	86			93	
	240	0.0153	77	**80**	88	87	88
		0.0165	83			94	
L130559	0	0.0219	110	**108**	107	103	100
		0.0213	107			100	
	30	0.0190	95	**98**	98	97	91
		0.0199	100			102	
	60	0.0209	105	**108**	109	96	100
		0.0222	111			102	
	90	0.0175	88	**94**	103	85	87
		0.0198	99			96	
	120	0.0174	87	**88**	99	88	81
		0.0177	89			90	
	240	0.0156	78	**81**	87	90	75
		0.0166	83			96	

* Recovery of storage stability sample corrected by procedural recovery sample at same time point

** Percent of initial was calculated from **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

Table 7.1.2.2.1-29: Results of storage stability samples of BAS 750 F (Reg.No. 5834378) in soil samples

Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery uncorrected [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L130560	0	0.0194	97	**100**	110	88	100
		0.0203	102			93	
	30	0.0189	95	**96**	104	91	96
		0.0192	96			93	
	60	0.0201	101	**100**	111	91	100
		0.0197	99			89	
	90	0.0202	101	**97**	113	90	97
		0.0184	92			82	
	120	0.0171	86	**90**	104	82	90
		0.0186	93			90	
	240	0.0158	79	**80**	85	93	80
		0.0161	81			95	
L130561	0	0.0197	99	**102**	105	94	100
		0.0208	104			99	
	30	0.0193	97	**99**	99	98	97
		0.0203	102			103	
	60	0.0208	104	**106**	114	91	104
		0.0215	108			95	
	90	0.0183	92	**92**	110	83	90
		0.0183	92			83	
	120	0.0189	95	**93**	107	89	91
		0.0181	91			85	
	240	0.0164	82	**84**	93	88	100
		0.0170	85			92	

* Recovery of storage stability sample corrected by procedural recovery sample at same time point
** Percent of initial was calculated from **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

Table 7.1.2.2.1-30: Results of storage stability samples of Reg.No. 5924326 in soil samples

Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery uncorrected [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L130556	0	0.0172	86	**94**	96	90	100
		0.0204	102			107	
	30	0.0187	94	**100**	104	90	106
		0.0210	105			101	
	60	0.0194	97	**103**	116	84	110
		0.0215	108			93	
	90	0.0174	87	**96**	110	79	102
		0.0210	105			96	
	120	0.0179	90	**93**	104	86	99
		0.0192	96			93	
	240	0.0144	72	**79**	89	81	84
		0.0172	86			97	
L130557	0	0.0191	96	**95**	99	108	100
		0.0187	94			105	
	30	0.0202	101	**99**	97	104	104
		0.0193	97			100	
	60	0.0200	100	**102**	116	86	107
		0.0206	103			89	
	90	0.0192	96	**99**	105	92	104
		0.0204	102			97	
	120	0.0173	87	**90**	100	87	95
		0.0186	93			93	
	240	0.0159	80	**81**	88	91	85
		0.0162	81			92	
L130558	0	0.0191	96	**99**	99	97	100
		0.0203	102			103	
	30	0.0196	98	**100**	105	94	101
		0.0201	101			96	
	60	0.0209	105	**111**	115	91	112
		0.0233	117			102	
	90	0.0179	90	**87**	109	82	88
		0.0166	83			76	
	120	0.0176	88	**91**	101	87	92
		0.0186	93			92	
	240	0.0147	74	**76**	90	82	77
		0.0157	79			87	
L130559	0	0.0199	100	**97**	99	101	100
		0.0189	95			96	
	30	0.0141	71	**69**	104	68	71
		0.0133	67			64	
	60	0.0125	63	**63**	110	57	65
		0.0126	63			58	
	90	0.0106	53	**56**	109	49	58
		0.0118	59			54	
	120	0.0108	54	**56**	99	55	58
		0.0114	57			58	
	240	0.0074	37	**40**	88	42	41
		0.0083	42			47	

* Recovery of storage stability sample corrected by procedural recovery sample at same time point

** Percent of initial was calculated from **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

Table 7.1.2.2.1-31: Results of storage stability samples of Reg.No. 5924326 in soil samples

Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery uncorrected [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L130560	0	0.0189	95	**103**	104	91	100
		0.0220	110			106	
	30	0.0187	94	**94**	102	92	91
		0.0187	94			92	
	60	0.0202	101	**104**	109	93	101
		0.0212	106			97	
	90	0.0183	92	**96**	115	80	93
		0.0201	101			88	
	120	0.0189	95	**96**	101	94	93
		0.0192	96			95	
	240	0.0148	74	**77**	87	85	75
		0.0158	79			91	
L130561	0	0.0187	94	**95**	96	98	100
		0.0191	96			100	
	30	0.0192	96	**97**	107	90	102
		0.0195	98			91	
	60	0.0211	106	**105**	111	95	111
		0.0209	105			94	
	90	0.0202	101	**99**	112	90	104
		0.0192	96			86	
	120	0.0185	93	**95**	108	86	100
		0.0193	97			90	
	240	0.0155	78	**78**	96	81	82
		0.0154	77			80	

* Recovery of storage stability sample corrected by procedural recovery sample at same time point
** Percent of initial was calculated from **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

The storage stability of BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326 was proven in all six tested soils over the tested period of 240 days at ≤ -18°C, with the exception of Reg.No. 5924326 in soil from trial L130559, which showed some slight apparent degradation during this period. Further analysis will be performed after 360, 480 and 650 days of storage.

For BAS 750 F (Reg.No. 5834378) and Reg.No. 5924326, the uncorrected recoveries in the six different soil samples after 240 days of storage ranged from 77% to 84% and from 76% to 81% of the fortified concentration, respectively, only in the soil from trial L130559, the recovery of Reg.No. 5924326 accounted only for 40% after 240 days of storage.

III. CONCLUSION

Residues can be regarded as stable if the mean recovery at a given storage period is in the range of 70 % up to 120 % of the fortified value. It was demonstrated that both analytes are stable in the 6 soil types when stored frozen at approximately -20ºC for a period of up to 240 days, with the exception of Reg. No. 5924326 in soil from trial L130559, which showed some slight apparent degradation during this period. The mean concentration declined from 0.0194 mg/kg on day 0 to 0.0079 mg/kg on day 240.

Report:	CA 7.1.2.2.1/8 Geschke S., 2015 a Determination of storage stability of BAS 555 F (Metconazole) and its metabolite 1,2,4-Triazole in soil 2015/1204922
Guidelines:	EEC 7032/VI/95 rev. 5, OECD 506 (Oct. 2007), EPA 860.1380
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

In the following study, the storage stability of metconazole (BAS 555 F) cis-isomer (Reg. No. 4079468) and trans-isomer (Reg. No. 4079654), as well as the metabolite 1,2,4-(1H)-triazole (Reg. No. 87084) in soil samples was investigated. Since in the context here, only the storage stability of 1,2,4 (1H) triazole is of interest, the executive summary of the study is confined only to its results.

EXECUTIVE SUMMARY

In the present study, the storage stability of the metabolite 1,2,4-(1H)-triazole (Reg. No. 87084) in soil was investigated in six soils under deep frozen conditions (≤ -18°C). The soils used for set up the stability samples were derived from six field dissipation trials of a parallel field study [*field study in support of Metconazole, not found in this dossier, BASF DocID 2015/1000221*]. Stored samples have been tested for stability up to 720 days.

The stability samples were fortified at a concentration level of 0.02 mg kg^{-1} (10fold LOQ). Each soil aliquot was spiked individually with a single test item, two replicates per field soil type and analysed time point were prepared. At different intervals (0, 32, 60, 120, 242, 361, 540 and 720 days) the stability samples were analyzed using BASF method L0203/01. The limit of quantification of the method is 0.002 mg kg^{-1}. The validity of the analytical method was confirmed within the storage stability study.

The storage stability of 1,2,4-(1H)-triazole was proven in all six tested soils over a period of at least 720 days at ≤ -18°C. The recoveries for 1,2,4-(1H)-triazole in the soil samples after 720 days of storage under frozen conditions ranged from 72% to 92% of the initial concentration., after 720 days of storage.

I. MATERIAL AND METHODS

A. MATERIALS

Test materials: 1,2,4-(1H)-triazole
Reg. No. 87084

Batch No.: AC10194-134
Purity: 99.0%
CAS #: 288-88-0

B. STUDY DESIGN

3. Experimental Conditions

Six different soil types (sandy loam, loamy sand, silt loam, silt, silt loam and sand), originating from the terrestrial dissipation study [*field study for Metconazole not found in this dossier, BASF DocID 2015/1000221*] were used in the storage stability study. The soil samples were untreated and stored refrigerated in the dark (1 – 10°C) until the day of fortification.

5 g of soil were weighed in 50 mL polypropylene-tubes and fortified individually at 0.02 mg kg^{-1} (10fold LOQ) with fortification solutions of cis-metconazole, trans-metconazole and 1,2,4-(1H)-triazole (separate systems for each analyte). The test items in acetonitrile/water (70/30, v/v) respectively demineralized water were distributed drop wise onto the soil, avoiding the formation of concentration “hot-spots”. The solvent was allowed to evaporate for approximately 5 minutes and then the tube was closed and placed into the deep freezer. For each storage interval, soil type and analyte a set of at least four samples (two samples for analysis plus two backup samples) were prepared. Additionally, soil samples without treatment were stored deep-frozen to serve as control samples and for method validation purposes (freshly fortified recovery samples).

The soil samples were stored at approximately -18°C and analyzed after different intervals (0, 32, 60, 120, 242, 361, 540 and 720 days). Minimum and maximum temperature in the freezer over the entire storage period was continuously recorded and were in the range of -25.3°C to -16.0°C.

4. Description of analytical procedures

For the determination of the metabolite 1,2,4-(1H)-triazole, BASF Method L0203/01 was used. Therefore, the soil samples were extracted twice with 40 mL acetonitrile/water (70/30, v/v) by mechanical shaking for 30 minutes at 225 rpm. After centrifugation the extract solution was decanted. Both extracts were combined and analysed by HPLC-MS/MS. Two characteristic mass transitions per compound (one for quantification, one for confirmation) were monitored. All results from the quantifier mass transition were reported. The limit of quantification (LOQ) of the method was 0.002 mg kg^{-1}.

The validity of the analytical method was analysed within the storage stability study. The accuracy of the method was proven by simultaneous analysis of two freshly prepared fortified specimens from each trial on each date of analysis. The fortification level was the same as for the storage stability specimens. Overall mean recoveries ranged from 84 % to 97 % for 1,2,4-(1H)-triazole with relative standard deviation ranging from 5.4% to 10%.

In addition, untreated control samples were analysed at the date of analysis in duplicate. Residues of 1,2,4-(1H)-triazole were detectable in the range of below the limit of detection (< 30 % of LOQ) to below the LOQ (< 0.002 mg/kg). Therefore, interferences in some control samples were determined and blank correction in the recovery data was needed.

Significant matrix effects (> 20%) were not found in HPLC-MS/MS analysis for all six soil types, hence analysis was accomplished using calibration standards prepared in acetonitrile/water (70/30, v/v).

II. RESULTS AND DISCUSSION

The residues in the soil samples were determined immediately after fortification and after storage for up to 720 days. Two samples were analysed at each defined time point. Significant interferences (> 30% of LOQ) were observed in some samples at the retention time and mass transitions considered for 1,2,4-(1H)-triazole. Therefore, interferences in some control samples were determined and blank correction in the stored sample data was needed.

From the results, recovery rates were calculated which were corrected for the mean recovery rates obtained from the respective freshly fortified specimens (same sampling date and soil). The recovery values in percent of 1,2,4-(1H)-triazole at the various dates of analysis are shown in Table 7.1.2.2.1-32

Table 7.1.2.2.1-32: Results of storage stability samples of 1,2,4 (1H)-triazole in soil samples

Field Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery uncorrected [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L120312	0	0.0194	97	**97**	94	103	100
		0.0193	97			103	
	32	0.0160	80	**80**	82	98	82
		0.0159	80			98	
	60	0.0161	81	**80**	85	95	82
		0.0156	78			92	
	120	0.0154	77	**76**	74	104	78
		0.0150	75			101	
	242	0.0133	67	**72**	98	68	74
		0.0151	76			78	
	361	0.0140	70	**71**	75	93	73
		0.0143	72			96	
	540	0.0142	71	**73**	94	76	75
		0.0148	74			79	
	720	0.0142	71	**72**	72	99	74
		0.0146	73			101	
L120313	0	0.0181	91	**99**	96	95	100
		0.0214	107			111	
	32	0.0197	99	**93**	95	104	93
		0.0172	86			91	
	60	0.0159	80	**78**	90	89	78
		0.0150	75			83	
	120	0.0154	77	**77**	92	84	77
		0.0151	76			83	
	242	0.0157	79	**79**	90	88	79
		0.0156	78			87	
	361	0.0155	78	**75**	86	91	76
		0.0143	72			84	
	540	0.0153	77	**75**	82	94	75
		0.0143	72			88	
	720	0.0163	82	**76**	86	95	77
		0.0139	70			81	
L120314	0	0.0175	88	**88**	98	90	100
		0.0176	88			90	
	32	0.0188	94	**94**	100	94	107
		0.0187	94			94	
	60	0.0169	85	**83**	90	94	94
		0.0162	81			90	
	120	0.0159	80	**84**	85	94	95
		0.0176	88			104	
	242	0.0174	87	**80**	102	85	90
		0.0143	72			71	
	361	0.0140	70	**71**	80	88	80
		0.0142	71			89	
	540	0.0236	118	**94**	86	137	106
		0.0137	69			80	
	720	0.0165	83	**77**	88	94	88
		0.0141	71			81	

* Mean recovery of storage stability sample corrected by procedural recovery sample at same time point
** Percent of initial was calculated from two **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

Table 7.1.2.2.1-33: Results of storage stability samples of 1,2,4 (1H)-triazole in soil samples

Field Trial	Storage Time [d]	Residue [mg kg^{-1}]	Recovery [%]	Mean Recovery [%]	Mean Procedural Recovery [%]	Corrected Recovery * [%]	Recovery [% of initial] **
L120315	0	0.0198	99	**99**	101	98	100
		0.0196	98			97	
	32	0.0161	81	**82**	86	94	83
		0.0163	82			95	
	60	0.0156	78	**81**	83	94	82
		0.0166	83			100	
	120	0.0146	73	**75**	80	91	76
		0.0151	76			95	
	242	0.0145	73	**76**	89	82	77
		0.0156	78			88	
	361	0.0153	77	**75**	79	97	76
		0.0144	72			91	
	540	0.0164	82	**84**	91	90	85
		0.0170	85			93	
	720	0.0160	80	**81**	82	98	82
		0.0162	81			99	
L120316	0	0.0179	90	**92**	100	90	100
		0.0185	93			93	
	32	0.0192	96	**95**	100	96	104
		0.0188	94			94	
	60	0.0176	88	**88**	86	102	96
		0.0176	88			102	
	120	0.0157	79	**79**	83	95	86
		0.0156	78			94	
	242	0.0148	74	**76**	86	86	83
		0.0155	78			91	
	361	0.0140	70	**71**	72	97	77
		0.0142	71			99	
	540	0.0162	81	**83**	91	89	90
		0.0168	84			92	
	720	0.0180	90	**90**	97	93	98
		0.0177	89			92	
L120317	0	0.0190	95	**94**	97	98	100
		0.0183	92			95	
	32	0.0210	105	**105**	108	97	112
		0.0209	105			97	
	60	0.0198	99	**95**	102	97	102
		0.0182	91			89	
	120	0.0181	91	**87**	94	97	93
		0.0166	83			88	
	242	0.0151	76	**78**	97	78	83
		0.0157	79			81	
	361	0.0156	78	**77**	79	99	82
		0.0151	76			96	
	540	0.0179	90	**89**	103	87	95
		0.0175	88			85	
	720	0.0182	91	**92**	94	97	98
		0.0186	93			99	

* Mean recovery of storage stability sample corrected by procedural recovery sample at same time point
** Percent of initial was calculated from two **uncorrected** samples. The mean recovery at 0 days was set to 100 % of initial.

The storage stability of 1,2,4-(1H)-triazole was proven in all six tested soils over the tested period of 720 days at ≤ -18°C.
The uncorrected recovery values for 1,2,4-(1H)-triazole, after 540 days of storage, ranged from 72% to 92% of the initial concentration.

III. CONCLUSION

Residues can be regarded as stable if the mean recovery at a given storage period is in the range of 70 % up to 120 % of the fortified value. The results of the storage stability study show that 1,2,4-(1H)-triazole (Reg. No. 87084) is stable under deep-frozen conditions (≤ -18°C) in soil for at least 720 days of storage.

CA 7.1.2.2.2 Soil accumulation studies

Report:	CA 7.1.2.2.2/1 Schäufele M., 2015b Accumulation behavior of BAS 750 F in soil under field conditions in the United Kingdom following repeated application onto winter wheat over several years 2015/1076325
Guidelines:	NAFTA Guidance Document for Conducting Terrestrial Field Dissipation Studies, Regulatory Directive DIR2006-01, March 2006 EPA (Environmental Protection Agency) US: Fate, Transport and Transformation Test Guidelines, OPPTS 835.6100, Terrestrial Field Dissipation, October 2008 SETAC – Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides, March 1995 SANCO/3029/99 rev. 4 (11/07/00): Guidance document for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, Official Journal of the European Union L 93, Volume 56, 03 April 2013
GLP:	yes (certified by The Department of Health of the Government of the United Kingdom)

EXECUTIVE SUMMARY

The accumulation behavior of the fungicide BAS 750 F and its metabolite 1,2,4-triazole (M750F001) in soil after several years of use under field conditions is under investigation at one site in the United Kingdom representative of Northern EU conditions. The objective of the study is to determine the residue concentrations and the determination of the plateau concentration of BAS 750 F in soil after repeated application to the same area cropped with wheat in several successive years. The site represents a typical region of agricultural practice representative for growing wheat, which is among the most important crops for the use of BAS 750 F. The trial site consists of an untreated and a treated plot, the latter being subdivided into 3 subplots that are assigned for replicates. This is an on-going study, which was initiated in April 2014 and is scheduled to run for seven years. The data reported in the summarized interim report cover the fieldwork and weather data until the end of December 2014 and are intended to provide a general overview of the study design and set up.

The product BAS 750 01 F, formulated as an emulsifiable concentrate (EC), was broadcast sprayed to spring wheat twice a year at BBCH 33 (first application) and BBCH 39-51 (second application) at a nominal rate of 150 g a.s. ha^{-1} per application using a target water volume of 241 L ha^{-1} (first application) and 211 L ha^{-1} (second application). The applications were conducted using a calibrated tractor mounted boom sprayer. The actual application rates for each application determined by the actual time sprayed was 249 L ha^{-1} (first application) and 215 L ha^{-1} (second application) with an average of 232 L ha^{-1}. This resulted in an application rate of the active substance of 153 and 152 g a.s. ha^{-1}, with an average of 152 g a.s. ha^{-1}.

Trial maintenance, such as tillage, fertilization, sowing, harvest and additional crop protection measures were conducted in line with local agricultural practice. No additional products containing the active substance BAS 750 F or azole fungicides were used on the trial area. Moreover, no soil disinfectants or ammonium fertilizer containing 1,2,4-triazole (nitrification inhibitor; M750F001) were used. No irrigation was performed.

At three times in 2014, soil specimens were sampled down to a maximum soil depth of 60 cm as follows: One day before the first application from designated subsubplots (control and treated), three days after the second application from designated subsubplots (control and treated), and on 02 Oct 2014 after harvest, but before ploughing the trial area. No cultivation was performed between harvest and sampling on 02 Oct 2014.

Soil cores were cut into 10 cm sections. Soil segments of the same depth and subplot from a defined sampling event were pooled and homogenized and a representative sub-sample of each depth was taken for residue analysis. All soil specimens were stored at about -18°C within a maximum of 5 hours and 15 minutes after start of sampling and remained frozen until analysis.

In order to demonstrate stability of the residues in soil during storage and shipment, shipment verification specimens were prepared at the second sampling (after second application) by fortifying untreated soil from the field sites with known amounts of BAS 750 F. These specimens were stored and shipped under the same conditions as the actual residue specimens.

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. Therefore, no analytical data are reported within the summarized interim study report to date. An assessment about the risk of accumulation of BAS 750 F in soil will be made once enough data are available. The study will be continued until an assessment can be made.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material

Test item (formulation):	BAS 750 01 F
Active substance (a.s.):	BAS 750 F (Reg. No. 5834378)
Type of formulation:	EC
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Batch No.:	FD-140113-0006 (certificate of analysis: 703791_1)
Content of a.s.:	98.9 g L^{-1} (nominal 100 g L^{-1})
Expiration date:	February 29, 2016

2. Test site

The accumulation of BAS 750 F under field conditions was investigated at one site in the United Kingdom, representative of Northern EU conditions. The site represents a typical region of agricultural practice for growing cereals, which are among the most important crops for the use of BAS 750 F. The trial area had been under cultivation for many years. Before commencement of the first sampling, the trial site was cropped with spring wheat. The trial is located in the United Kingdom, near Stratton Audley on a clay soil (according to USDA classification) with an organic carbon content of 2.4%, a pH value ($CaCl_2$) of 7.0, cation exchange capacity of 32.8 meq 100 g^{-1} dry soil and a maximum water holding capacity of 65.4 g water 100 g^{-1} dry soil (top soil).

B. STUDY DESIGN

1. Experimental treatments

The product, formulated as an emulsifiable concentrate (EC), was broadcast sprayed to wheat using a calibrated tractor mounted boom sprayer. The test item was applied at a nominal rate of 150 g a.s. ha^{-1} per application using a target water volume of 241 L ha^{-1} (first application) and 211 L ha^{-1} (second application). The target application timings were BBCH 25-29 (first application) and BBCH 37-39 (second application). The actual application timings in the following year (2014) were at BBCH 33 (first application) and at BBCH 39-51 (second application) with average BBCH 45. Both applications were delayed due to adverse weather conditions. The actual application rates for each application determined by the actual time sprayed were 153 g a.s. ha^{-1} and 152 g a.s. ha^{-1}.

Application verification

The application dose was verified by means of sampling Petri dishes filled with untreated soil from the field. The Petri dishes were placed on the treated plots (5 in each subplot) before each application. On completion of the application, the Petri dishes were stored in a freezer at ≤ -18°C.

Agronomic measures
Details to previous crops, fertilization and crop protection measures can be found in the interim study report. No products containing the test item active substance BAS 750 F, any azole fungicides or soil disinfectants had been used on the test plots in the last three years. Moreover, no ammonium fertilizer containing 1,2,4-triazole (nitrification inhibitor) was used. The grubbing of the soil was not deeper than 20 cm. The crops were harvested according to good agricultural practice.
Air temperature, soil temperature, air humidity, precipitation, wind velocity, and global radiation were recorded daily at an on-site weather station. Air temperatures were measured at 2 m height, soil temperatures at 10 cm depth. Details can be found in the interim study report or the interim field phase report.

2. Sampling and storage

Replicate soil specimens (10 per treated subplot and control plot) were taken before the first application (-1 days after first treatment; DAFT), after the second application (20 DAFT) and after crop harvest, but before ploughing (121 DAFT). Soil cores were collected to a depth of 60 cm at all sampling events. The specimens were taken randomly at each sampling occasion from the control and from each of the three treated subplots A, B and C. Soil cores were taken in one run (0-60 cm) using a tractor mounted hydraulic soil corer, fitted with plastic tubes of 125 cm length and an inner diameter of 4.5 cm. Details are summarized in the interim study report.

All main soil cores collected were cut into 10 cm segments and pooled by depth at the test site in frozen stage. Soil cores did not defrost during the segmentation process. Segments of the same sampling event, subplot and soil depth were combined.

All soil specimens were deep-frozen at a temperature around or below -18°C within less than 5 hours 15 minutes time. In addition to the main sampling described above, a second complete sampling (double sampling) was carried out. The reserve samples were not sectioned into 10 cm segments but directly placed and kept into the freezers at the field test site.

3. Analytical procedure

Residue analysis of BAS 750 F and 1,2,4-triazole (M750F001) in field specimens is performed at Envigo CRS Limited, Eye, Suffolk, IP23 7PX, United Kingdom.

II. RESULTS AND DISCUSSION

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. Therefore, no analytical data are reported within this interim study report or within any analytical phase report to date.

III. CONCLUSION

In April 2014, a soil accumulation study was started at a site in The United Kingdom. The trial site area was cropped with spring wheat. The data presented in this first interim report cover the field part of the study until end of 2014 and are intended to provide a general overview on the study design.

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. An assessment about the risk of accumulation of BAS 750 F in soil will be made once enough data are available. The study will be continued until an assessment can be made.

Report:	CA 7.1.2.2.2/2 Schäufele M., 2015c Accumulation behavior of BAS 750 F in soil under field conditions in Germany following repeated application onto winter barley over several years 2015/1076326
Guidelines:	NAFTA Guidance Document for Conducting Terrestrial Field Dissipation Studies, Regulatory Directive DIR2006-01, March 2006 EPA (Environmental Protection Agency) US: Fate, Transport and Transformation Test Guidelines, OPPTS 835.6100, Terrestrial Field Dissipation, October 2008 SETAC – Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides, March 1995 SANCO/3029/99 rev. 4 (11/07/00): Guidance document for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, Official Journal of the European Union L 93, Volume 56, 03 April 2013
GLP:	yes (certified by The Department of Health of the Government of the United Kingdom)

EXECUTIVE SUMMARY

The accumulation behavior of the fungicide BAS 750 F and its metabolite 1,2,4-triazole (M750F001) in soil after several years of use under field conditions is under investigation at one site in Germany representative of Northern EU conditions. The objective of the study is the determination of the residue concentrations and the determination of the plateau concentration in soil after repeated applications of BAS 750 F to the same area cropped with barley in several successive years. The site represents a typical region of agricultural practice representative for growing barley, which is among the most important crops for the use of BAS 750 F. The trial site consists of an untreated and a treated plot, the latter being subdivided into 3 subplots that are assigned for replicates. This is an on-going study, which was initiated in April 2014 and is scheduled to run for seven years. The data reported in the summarized interim report cover the fieldwork and weather data until the end of December 2014 and are intended to provide a general overview on the study design.

The product BAS 750 01 F, formulated as an emulsifiable concentrate (EC), was broadcast sprayed onto spring barley twice a year at BBCH 23-29 (first application) and BBCH 45-49 (second application) at a nominal rate of 150 g a.s. ha^{-1} per application using a target water volume of 200 L ha^{-1}. The applications were conducted using a calibrated research boom sprayer. The actual application rates for each application were determined by quantifying the amount of spray solution discharged and ranged from 190 to 203 L ha^{-1} (first application) and from 188 to 196 L ha^{-1} (second application) with an average of 193 L ha^{-1}.

This resulted in an application rate of the active substance from 141 to 147 g a.s. ha^{-1} with average of 144 g a.s. ha^{-1} at the first application and from 139 to 146 g a.s. ha^{-1}, with an average of 143 g a.s. ha^{-1} at the second application.

Trial maintenance, such as tillage, fertilization, sowing, harvest and additional crop protection measures were conducted in line with local agricultural practice. No additional products containing the active substance BAS 750 F or any azole containing fungicides were used on the trial area. Moreover, no soil disinfectants or ammonium fertilizer containing 1,2,4-triazole (nitrification inhibitor; M750F001) were used. No irrigation was performed.

At three times in 2014, soil specimens intended for residue analysis were sampled down to a maximum soil depth of 60 cm. Soil cores were taken one day before the first application, one day after the second application, and on 21 Aug 2014 - after harvest and light cultivation, but before ploughing the trial area. Soil cores were collected from designated subsubplots (control and treated).

Soil cores were cut into 10 cm sections. Soil segments of the same depth and subplot from a defined sampling event were pooled and homogenized and a representative sub-sample of each depth was taken for residue analysis. All soil specimens were stored at about -18°C within a maximum of 4 hours and 40 minutes after sampling and remained frozen during the storage and shipping period.

In order to demonstrate stability of the residues in soil during storage and shipment, shipment verification specimens were prepared at the second sampling (after second application) by fortifying untreated soil from the field sites with known amounts of BAS 750 F. These specimens were stored and shipped under the same conditions as the actual residue specimens.

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. Therefore, no analytical data are reported within the first interim study report. An assessment about the risk of accumulation of BAS 750 F in soil will be made once enough data are available. The study will be continued until an assessment can be made.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material

Test item (formulation):	BAS 750 01 F
Active substance (a.s.):	BAS 750 F (Reg. No. 5834378)
Type of formulation:	EC
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Batch No.:	FD-140113-0006 (certificate of analysis: 703791_1)
Content of a.s.:	98.9 g L^{-1} (nominal 100 g L^{-1})
Expiration date:	February 29, 2016

2. Test site

The accumulation of BAS 750 F under field conditions was investigated at one site in Germany, representative of Northern EU conditions. The site represents a typical region of agricultural practice representative for growing cereals, which are among the most important crops for the use of BAS 750 F. The trial area had been under cultivation for many years. Before commencement of the first sampling, the trial site was cropped with spring barley. The trial is located in Germany, near Lentzke on a loamy sand soil (according to USDA classification) with an organic carbon content of 0.8%, a pH value ($CaCl_2$) of 6.2, cation exchange capacity of 4.6 meq 100 g^{-1} dry soil and a maximum water holding capacity of 29.9 g water 100 g^{-1} dry soil (top soil).

B. STUDY DESIGN

1. Experimental treatments

The product, formulated as an emulsifiable concentrate (EC), was broadcast sprayed to barley using a calibrated boom sprayer. The test item was applied at a nominal rate of 150 g a.s. ha^{-1} per application using a target water volume of 200 L ha^{-1}. The target application timings were BBCH 25-29 (first application) and BBCH 37-39 (second application). The actual application timing of the first application in 2014 was at BBCH 23-29, slightly before the target crop growth stage. It was decided to conduct this application at this stage, because of perfect weather conditions at that day. The second application was conducted at BBCH 45-49, after the target crop growth stage. Unstable weather conditions did not allow conducting the application at the target crop growth stage. The actual application rates for each application were determined by quantifying the amount of spray discharged and ranged from 141 to 147 g a.s. ha^{-1} with an average of 144 g a.s. ha^{-1} (first application) and from 139 to 146 g a.s. ha^{-1} with an average of 143 g a.s. ha^{-1} (second application).

Application verification
The application dose was verified by means of sampling Petri dishes filled with untreated soil from the field. The Petri dishes were placed on the treated plots (5 in each subplot) before each application. On completion of the application, the Petri dishes were stored in a freezer.

Agronomic measures
Details to previous crops, fertilization and crop protection measures can be found in the interim study report. No products containing the test item active substance BAS 750 F, any azole fungicides or soil disinfectants had been used on the test plots in the last three years. Moreover, no ammonium fertilizer containing 1,2,4-triazole (nitrification inhibitor) was used. The grubbing of the soil was not deeper than 20 cm. No catch crops were planted between the time of harvest and replanting of winter barley in autumn 2014. The trial site remained fallow between harvest in summer and sowing of new crop in autumn 2014.
Air temperature, soil temperature, air humidity, precipitation, wind velocity, and global radiation were recorded daily at an on-site weather station. Air temperatures were measured at 2 m height, soil temperatures at 10 cm depth. Details can be found in the interim study report or the interim field phase report.

2. Sampling and storage

Replicate soil specimens intended for residue analysis (10 per treated subplot and control plot) were taken before the first application (-1 DAFT), after the second application (30 DAFT) and after crop harvest, but before ploughing (113 DAFT). Soil cores were collected to a depth of 60 cm at all sampling events.

The specimens were taken randomly at each sampling occasion. Further specimens were collected from the control and from each of the three treated subplots A, B and C. Soil cores were taken using a tractor mounted hydraulic soil corer (Humax), fitted with plastic tubes of 30 cm length and an inner diameter of 5.0 cm. Sampling of these cores were conducted in two runs. Details are summarized in the interim study report.

All main soil cores collected were cut into 10 cm segments on the field right after sampling and before freezing. Segmentation was conducted with a knife. Segments of the same sampling event, subplot and soil depth were combined. All soil specimens were deep-frozen at a temperature around or below -18°C within less than 4 hours 40 minutes time. In addition to the main sampling described above, a second complete sampling (double sampling) was carried out. The reserve samples were not sectioned into 10 cm segments but directly placed and kept into the freezers at the field test site.

3. Analytical procedure

Residue analysis of BAS 750 F and 1,2,4-triazole (M750F001) in field specimens is performed at Envigo CRS Limited, Eye, Suffolk, IP23 7PX, United Kingdom.

II. RESULTS AND DISCUSSION

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. Therefore, no analytical data are reported within this interim study report or within any analytical phase report to date.

III. CONCLUSION

In April 2014, a soil accumulation study was started at a site in Germany. The trial site area is cropped with spring barley. The data presented in this first interim report cover the field part of the study until end of 2014 and are intended to provide a general overview on the study design.

Analysis is ongoing. Due to the limited amount of analytical data, it is not possible to make any scientific statements or conclusions. An assessment about the risk of accumulation of BAS 750 F in soil will be made once enough data are available. The study will be continued until an assessment can be made.

Assessment of persistence of BAS 750 F in soil

In the following it will be discussed if BAS 750 F fulfils the P in soil criterion within the PBT (persistence, bioaccumulation and toxicity) and the vP criterion in the vPvB (very persistent very bioaccumulative) assessment, which are defined according to Section 3.7.2.1. and 3.7.3.1, respectively, of Annex II of EC Regulation 1107/2009 as follows:

An active substance, safener or synergist fulfils the persistence criterion where:
- *The half-life in soil is higher than 120 days.*

An active substance, safener or synergist fulfils the 'very persistent' criterion where:
- *the half-life in soil is higher than 180 days.*

The relevant endpoints for the persistence assessment were identified based on the DG SANCO working document on "Evidence Needed to Identify POP, PBT and vPvB Properties for Pesticides" [*SANCO 2012. DG SANCO Working Document on "Evidence Needed to Identify POP, PBT and vPvB Properties for Pesticides". Brussels: European Commission Health and Consumers Directorate-General. Report 25.09.2012 - rev. 3.*]. According to this document, when available, field degradation half-lives are relevant for the P and vP assessment.

The degradation of BAS 750 F was investigated in a laboratory soil degradation study in four aerobic soils (see chapter 7.1.1). Additionally, the degradation of BAS 750 F was investigated under field conditions. Field plots were set up in representative growing regions of Europe. Plots were covered by a layer of sand to exclude surface processes and to enable a straightforward generation of modeling $DegT_{50}$ as input for calculation of predicted environmental concentrations as recommended by EFSA (2014) [*EFSA Guidance Document for evaluating laboratory and field dissipation studies to obtain DegT50 values of active substances of plant protection products and transformation products of these active substances in soil. EFSA Journal 2014:12(5):3662*]. A kinetic evaluation was performed in order to derive best-fit field degradation parameters for BAS 750 F according to the FOCUS kinetics guidance (2006, 2014). An additional kinetic evaluation was performed to derive degradation parameters that can be used as input for modelling according to the EFSA (2014) guidance. The geometric mean normalized $DegT_{50}$ of BAS 750 F from field studies is 200.0 days. This half-life describes the degradation rate in bulk soil: degradation due to surface processes is not included. Details are presented in chapter 7.1.2.

As the laboratory $DegT_{50}$ of BAS 750 F exceeds 60 days, an additional terrestrial field dissipation study was run at six different sites (in the US) without covering the plots (surface processes included) according to NAFTA guidelines. Plot locations corresponded closely to the growing regions for the intended GAP though the test sites were not cropped. At the time of the writing of this dossier the study was incomplete. A comprehensive interim reporting of the data at hand has been presented in this dossier. A kinetic evaluation of the data was not performed. Details are presented in chapter 7.1.2.

Due to the exclusion of surface processes $DegT_{50}$ derived from data collected in European field studies are appropriate for an initial conservative assessment of persistence of BAS 750 F in soil. Considering the geomean $DegT_{50}$ of 200 days derived from these studies, BAS 750 F fulfils the criterion for both P and vP. However, the final evaluation of the data collected in the non-covered US studies can provide more realistic DT_{50} values, which may be used for the refinement of the persistence assessment of this compound.

Thus, based on available data presented in this dossier BAS 750 F meets the requirements set forth for classification as P and vP in soil.

CA 7.1.3 Adsorption and desorption in soil

CA 7.1.3.1 Adsorption and desorption

CA 7.1.3.1.1 Adsorption and desorption of the active substance

Report:	CA 7.1.3.1.1/1 Vasques A.C., 2015 a Adsorption / desorption behavior of 14C-BAS 750 F on different US, Japanese and European soils 2014/3017870
Guidelines:	OECD 106 (2000), EPA 835.1230, POP-PA.1005, SOP-PA.1005, POP-SG. 023 Manual de Radioprotecao da Unidade de Estudos Ambientais
GLP:	yes (certified by Instituto Nacional de Metrologia, Normalizacao e Qualidade Industrial - INMETRO, Rio de Janeiro, Brazil)

EXECUTIVE SUMMARY

The adsorption/desorption characteristics of triazole-3(5)-^{14}C-labeled BAS 750 F were studied in eight different US, Japanese and European soils, representing a range of soil types, to provide data for the evaluation of the environmental fate, including the potential for leaching, in the environment.

During the preliminary study, an adsorption test of the BAS 750 F on the surface of the experimental vessels, the soil/solution ratio determination and the equilibrium test were conducted by the indirect method. However, samples from the equilibrium test were analyzed directly, additionally, to demonstrate stability. Therefore, recoveries of ^{14}C-BAS 750 F for all test soils were investigated after 48 h shaking applying the direct method. Recoveries (mean of two replicates) ranged from 90.0% (FPR soil) to 96.5 % (GI soil) of the total of test item applied. Since at least >90% recoveries were obtained, the compound can be considered to be stable under the applied test conditions. As a result, the indirect method could be applied.

For the determination of the adsorption isotherm, the soils were treated at five target concentrations; 1.0, 0.5, 0.1, 0.05 and 0.01 µg mL^{-1} in the adsorption solution. The desorption phase was carried out in two steps by incubating the soil residue from the adsorption phase with fresh 0.01 M $CaCl_2$ solution containing no test substance. The ratio of soil mass and test solution volume was 1/10. The tests period used was 24 hours for all soils except La Gironda soil for which it was 48 hours. Concentrations of test substance were determined in the supernatant solutions and calculated in the remaining soil residue at each stage.

From the measured isotherms the Freundlich adsorption and desorption constants (K_F) were determined, as well as the values corrected for the organic carbon content (K_{FOC}).

Table 7.1.3.1.1-1: Summary of adsorption and desorption isotherms tests of BAS 750 F on eight soils

Soil Name	Soil Type (USDA)	pH (H_2O)	Org. C [%]	Adsorption			Desorption 1			Desorption 2		
				K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]	K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]	K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]
Indiana	Loam	5.7	1.22	48.46	0.95	3972.29	60.57	0.87	4964.39	82.77	0.85	6784.21
New Jersey	Loam	6.8	1.00	35.61	0.96	3560.75	55.12	0.92	5512.06	71.09	0.86	7109.39
Obhiro	Loam	6.9	3.40	126.14	1.01	3709.90	183.03	0.94	5383.27	300.82	0.89	8847.69
Fiorentino Poggio Renatico 1	Loam	8.2	1.00	31.43	0.92	3143.03	46.47	0.88	4647.21	72.15	0.83	7214.67
La Gironda	Sandy clay loam	8.3	1.22	24.53	0.94	2010.28	39.10	0.93	3205.28	60.02	1.07	4919.91
Li10	Loamy sand	6.9	0.95	36.34	1.02	3824.78	44.63	0.90	4697.83	60.96	0.87	6417.22
LUFA 5M	Sandy loam	7.4	1.10	35.83	1.00	3251.56	46.59	0.89	4228.21	60.17	0.82	5460.28
LUFA 2.1	Sand	6.5	0.60	29.59	1.00	4930.94	45.85	0.92	7640.87	66.11	0.90	11019.15

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in one ^{14}C-labeled form.

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Label position:	triazole-3(5)-^{14}C
Molecular mass:	397.78 g mol^{-1} (unlabeled)
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$
Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%, see certificate of analysis attached to the final report
Purity:	98.9%

2. Soils

The study was conducted with eight different soils originating from the US (two soils), Japan (one soil), Italy (one soil), Spain (one soil), and Germany (three soils). The physico-chemical properties of the soils are provided in Table 7.1.3.1.1-2.

Table 7.1.3.1.1-2: Soil characteristics

Soil designation	Indiana	New Jersey	Obhiro	Fiorentino Pog. Ren. 1	La Gironda	Li10	LUFA 5M	LUFA 2.1
	USA		Japan	Italy	Spain	Germany		
DIN 4220 Particle size distribution *[%]* sand 0.063 – 2 mm silt 0.002 – 0.063 mm clay < 0.002 mm textural class	 n.d. n.d. n.d. n.d.	 n.d. n.d. n.d. n.d.	 n.d. n.d. n.d. n.d.	 41.7 41.6 16.7 loamy sand	 48.0 24.3 27.7 sandy clay loam	 81.7 13.9 4.3 silty sand	 n.d. n.d. n.d. n.d.	 89.5 8.2 2.3 sand
USDA Particle size distribution *[%]* sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 35 44 21 loam	 30 44 26 loam	 37.5 37.9 24.6 loam	 49.4 33.9 16.7 loam	 49.2 23.0 27.7 sandy clay loam	 83.5 12.2 4.3 loamy sand	 58 28 14 sandy loam	 90.8 6.9 2.3 sand
Organic C *[%]*	1.22	1.0	3.4	1.00	1.22	0.95	1.10	0.60
pH [H_2O]	5.7	6.8	6.9	8.2	8.3	6.9	7.4	6.5
pH [$CaCl_2$]	n.d.	n.d.	6.1	7.4	7.4	6.2	7.3	5.6
Cation exchange capacity *[$cmol^+ kg^{-1}$]*	11.6	8.3	17.2	11.8	26.3	5.5	10.6	-0.7
Max. water holding capacity *[g per 100 g dry weight]*	41.8	42.6	77.8	29.7	39.2	23.2	35.3	23.1
Microbial biomass (from certificate) *[mg C per 100 g dry soil]*	7.22	5.86	n.d.	n.d.	n.d.	23.6	11.69	n.d.

n.d. = not determined

B. STUDY DESIGN

1. Experimental conditions

Tier 1: Adsorption Preliminary Tests

The adsorption of the test substance to the vessel surface was investigated at the highest concentration (1.0 µg mL^{-1}) in duplicate vessels with no soil present (blank). Samples were analyzed after 24 and 48 hours shaking.

The solution to soil ratio to be used for the main experiment was investigated using the soils Obhiro (JP; OC content 1.00%) and Fiorentino Poggio Renatico 1 (FPR; OC content 3.4%), at the highest test substance solution concentration (1.0 µg mL^{-1}). Soils with organic carbon content out of this range – Li10 (0.95%) and LUFA 2.1 (0.60%) – were made available later. There was considered no need for conducting additional soil/solution ratio tests with the soil Li10 or LUFA 2.1. Adsorption experiments were conducted with duplicate vessels prepared at solution/soil ratios of 1:1, 5:1, and 10:1. The vessels were mixed at approximately 20°C for approximately 24 hours in the dark. The solutions were then separated by centrifugation and analyzed by liquid scintillation counting (LSC) and radio-HPLC.

Equilibration time experiments were performed with each soil at a solution to soil ratio of 10/1 at the highest proposed test substance concentration (1.0 µg mL^{-1}). Adsorption experiments were set up using the solution/soil ratio determined above using JP and FPR soils. After intervals of 4, 8, 24, 32 and 48 hours, single glass centrifuge tubes, containing soil portions of each of the eight soils, were removed and centrifuged. Supernatants were measured by LSC. Soils incubated for 48 hours were extracted; extracts and supernatants were analyzed by LSC and (for 48 h samplings) radio-HPLC.

Tier 2 was not required as soils were pre-selected and a range of concentrations were investigated under Tier 3.

Tier 3: Adsorption and Desorption Isotherms

A solution to soil ratio of 10/1 and equilibration time of 24 hours was chosen for the main experiment for all soils, except for La Gironda soil, which was equilibrated for 48 hours.

Portions of the eight soils were weighed into centrifuge tubes. The soils were treated with the test item dissolved in 0.01 M $CaCl_2$ solution to achieve nominal concentrations of 1.0, 0.5, 0.1, 0.05 and 0.01 µg mL^{-1} in the test vessels. The test was conducted in duplicate at each test substance concentration. Controls were prepared with only the treatment solution (no soil) in the tube and soil blanks were prepared by weighing soil in the tube and applying 0.01 M $CaCl_2$ solution (no test item). Following application the vessels were mixed and stored at 20 ± 2°C and in the dark for the equilibration time of 24 hours (La Gironda 48 hours). At the end of the equilibration period, each sample tube was centrifuged to separate the phases. For selected samples, the soil was additionally extracted (see section 2, Description of Analytical Procedures). Afterwards, the supernatants and extracts were analyzed by LSC and radio-HPLC.

Following the adsorption procedure, fresh 0.01 M $CaCl_2$ solution was added to each vessel to replace the solution removed, including the sample blanks. The soil/solution mixtures were then mixed again for 24 hours (La Gironda 48 hours) equilibration time under the same conditions as described for adsorption. Solution and soil were separated by centrifugation and the desorption process repeated once more. After each centrifugation, the supernatant solution was analyzed by LSC and radio-HPLC.

2. Description of analytical procedures

As mentioned above, supernatants derived from the adsorption as well as from desorption phases were analyzed by LSC and radio-HPLC.

The extraction of the soil samples enabled collection of data on extraction efficiency, to evaluate the nature of items adsorbed to soil and to determine stability of test item on soil. For the first batch of equilibrium tests conducted with the soils Indiana, New Jersey, JP and FPR, both samplings 32 and 48 hours were extracted, for comparison and determination of extraction method. For the remaining equilibrium tests, only 48-hour sampling was extracted. For the isotherms determination test, only samples originally treated with the highest concentration solution (1.0 µg mL^{-1}) were extracted. Following the adsorption phase, the soil residues were extracted once with methanol and twice with methanol/water (1/1, v/v) by shaking for 30 minutes, followed by centrifugation at least by 2,500 rpm for 10 minutes. The extracts were analyzed by LSC and radio-HPLC.

If the recovery was below 90% of the total applied radioactivity (TAR), the extracted soil was combusted. To that purpose, the extracted soil was air dried and homogenized before combustion. Aliquots were combusted in a sample oxidizer. The trapped $^{14}CO_2$ was analyzed by LSC.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Recovery rate determinations were carried out for sampling 48 hours from equilibrium test considering all soils. Mean recovery rates from 90.0% (FPR soil) to 96.5% TAR (La Gironda soil) were obtained considering both, the aqueous and the soil phases.

B. FINDINGS

Tier 1: Adsorption Preliminary Tests

From the control run in parallel to tests, it was demonstrated that the test item in 0.01 M $CaCl_2$ solution in absence of soil shaking at 150 rpm during 48 h was stable.

After the preliminary test, a solution to soil ratio 10/1 was chosen, because it provided adsorption higher than 50%, but still enabled enough radioactivity in both phases (soil and solution) for accurate measurements (the mean % adsorption at this solution to soil ratio was 83.0% and 70.5% for soil JP and soil FPR, respectively).

Treated blank samples, comprising test vessels and treated solutions without soil, incubated alongside with the test vessels demonstrated that there was no significant adsorption of the test substance to the test vessels.

In the equilibration time experiments, for all soils, except soil La Gironda, equilibrium was achieved within 24 hours. For soil La Gironda, adsorption equilibrium was not observed before 48 hours shaking. Therefore, for soil La Gironda, the test period used for conduction of isotherm test was 48 hours.

Tier 3: Adsorption Isotherms

Adsorption isotherm testing for BAS 750 F resulted in Freundlich adsorption coefficients (K_F^{ads}) for the eight soils in the range of 24.53 to 126.14 mL g^{-1}, resulting in organic carbon normalized values (K_{FOC}^{ads}) from 2010 to 4931 mL g^{-1}.

Desorption isotherm testing for BAS 750 F resulted in Freundlich desorption coefficients for the eight soils in the range from 39.10 to 183.03 mL g^{-1} (K_F^{des1}) for the first desorption step and in the range from 60.02 to 300.82 mL g^{-1} (K_F^{des2}) for the second desorption step. The organic carbon normalized values ranged of 3205 to 7641 mL g^{-1} (K_{FOC}^{des1}) and from 4920 to 11019 mL g^{-1} (K_{FOC}^{des2}).

A summary of the experimental results is provided in Table 7.1.3.1.1-3.

Table 7.1.3.1.1-3: Summary of adsorption and desorption isotherms tests of BAS 750 F on eight soils

Soil Name	Soil Type (USDA)	pH (H_2O)	Org. C [%]	Adsorption			Desorption 1			Desorption 2		
				K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]	K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]	K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]
Indiana	Loam	5.7	1.22	48.46	0.95	3972.29	60.57	0.87	4964.39	82.77	0.85	6784.21
New Jersey	Loam	6.8	1.00	35.61	0.96	3560.75	55.12	0.92	5512.06	71.09	0.86	7109.39
Obhiro	Loam	6.9	3.40	126.14	1.01	3709.90	183.03	0.94	5383.27	300.82	0.89	8847.69
Fiorentino Poggio Renatico 1	Loam	8.2	1.00	31.43	0.92	3143.03	46.47	0.88	4647.21	72.15	0.83	7214.67
La Gironda	Sandy clay loam	8.3	1.22	24.53	0.94	2010.28	39.10	0.93	3205.28	60.02	1.07	4919.91
Li10	Loamy sand	6.9	0.95	36.34	1.02	3824.78	44.63	0.90	4697.83	60.96	0.87	6417.22
LUFA 5M	Sandy loam	7.4	1.10	35.83	1.00	3251.56	46.59	0.89	4228.21	60.17	0.82	5460.28
LUFA 2.1	Sand	6.5	0.60	29.59	1.00	4930.94	45.85	0.92	7640.87	66.11	0.90	11019.15

III. CONCLUSION

The adsorption behavior of BAS 750 F was determined on eight different soils originating from the US (two soils), Japan (one soil), Italy (one soil), Spain (one soil), and Germany (three soils). The soils covered a range of pH from 5.7 to 8.3 (measured in H_2O) and a range of organic carbon content from 0.60 to 3.4%. The soils were classified by USDA scheme into five different textural classes: three loams, one sandy clay loam, one sandy loam, one loamy sand, and one sand.

The Freundlich adsorption coefficient K_F covered a range from 24.53 mL g^{-1} to 126.14 mL g^{-1} for the eight soils. The K_{FOC} values ranged from 2010 mL g^{-1} to 4931 mL g^{-1}.

The Freundlich desorption coefficients covered a range from 39.10 to 183.03 mL g^{-1} (K_F^{des1}) for the first desorption step and from 60.02 to 300.82 mL g^{-1} (K_F^{des2}) for the second desorption step. The organic carbon normalized values ranged from 3205 to 7641 mL g^{-1} (K_{FOC}^{des1}) and from 4920 to 11019 mL g^{-1} (K_{FOC}^{des2}).

CA 7.1.3.1.2 Adsorption and desorption of metabolites, breakdown and reaction products

Report: CA 7.1.3.1.2/1
Szegedi, K., 2015 b
Estimation of adsorption coefficients of metabolites of BAS 750F with QSAR
2015/1260816
Guidelines: <none>
GLP: no

EXECUTIVE SUMMARY

Adsorption coefficients (K_{oc}) were estimated for metabolites of BAS 750 F that occurred in studies with BAS 750 F in aqueous systems. The QSAR methods implemented in the KocWIN (EPISuite) tool were used.

Predicted values were found to be in a reasonable range and thus appropriate for further use in the exposure assessments.

I. MATERIAL AND METHODS

Adsorption coefficients (K_{oc}) were estimated for the metabolites M750F003, M750F005, M750F006, M750F007 and M750F008 of BAS 750 F that occurred in the study on aqueous photolysis and in the water/sediment study with BAS 750 F [*CA 7.2.1.2/1, DocID 2015/7000233; CA 7.2.2.3/1, DocID 2015/1000941*]. The QSAR methods implemented in the KocWIN 2.00 (EPISuite) tool were used [*US EPA (2000-2012) EPI Suite*]. Values obtained with the molecular connectivity index (MCI) were reported.

II. RESULTS AND DISCUSSION

The resulting K_{oc} values are given in the table below.

Table 7.1.3.1.2-1: Estimated K_{oc} values for metabolites of BAS 750 F

Metabolite code	K_{oc} [mL g^{-1}]
M750F003	597.6
M750F005	7863
M750F006	4919
M750F007	3938
M750F008	17240

III. CONCLUSION

Predicted values are in the range of K_{oc} values observed for several similar structures, and thus appropriate for further use in the exposure assessments.

CA 7.1.3.2 Aged sorption

No studies were conducted for aged sorption.

CA 7.1.4 Mobility in soil

Report: CA 7.1.4/1
Sandt H.J. van de, 2015 a
Determination of foliar DT50 of Triazole (BAS 750 F) after application of BAS 750 01 F to wheat surfaces
2015/1130156
Guidelines: EPA 875.2100
GLP: yes
(certified by Ministry of Health, Welfare and Sport, Utrecht, The Netherlands)

EXECUTIVE SUMMARY

A foliar DT_{50} study was conducted with the fungicide BAS 750 01 F in a raw agricultural commodity of wheat to determine the decline of residues on plant leaves after application that are available for dislodging by a rainfall event. Default modelling parameter values may potentially be superseded by such experimentally derived, measured values. While currently no guideline exists for such an experimental study to supplant the conservative default, a similar study type in which a guideline exists is the Dislodgeable Foliar Residue for worker exposure from the EPA. This guideline was used as a template for this study with slight modifications to make the data more appropriate for environmental exposure assessments.

The test system was cultivated indoors and grown to the desired BBCH growth stage to give sufficient leaves to collect as well as mimic the desired growth stage set in the GAP (BBCH 30-39). The subsequent dislodging procedure is independent from the crop growth stage and therefore the Foliar DT_{50} values derived are considered valid for use in environmental data modeling.

The test item is applied using equipment that simulates commercial application to wheat. Whole leaf samples were taken at the given intervals and treated with the dislodging procedure.

The amount of residue on the leaf surfaces was determined with a dislodging procedure utilizing Aerosol OT-B (0.01%) as the dislodging solution. Subsequent analysis of the dislodging solution was performed via LC-MS/MS to indirectly determine the amount of BAS 750 F residues on the leaf surface.

Kinetic evaluation showed that the SFO model gave the best fit of the experimental data. The suitable DT_{50} value for modeling purposes is 2.1 days.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test item (formulation):	BAS 750 01 F
Active substance (a.s.):	BAS 750 F (Reg. No. 5834378)
Type of formulation:	EC
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
CAS Number:	1417782-03-6
Batch No.:	FD-140113-0006
Content of a.s.:	100 g L^{-1} (nominal 100 g L^{-1}), actual 98.9 g L^{-1}
Expiration date:	February 29, 2016

Internal code:	BAS 750 F
Chemical Formula:	$C_{18}H_{15}ClF_3N_3O_2$
Molecular wt.:	397.8
Reg. No.:	5834378
Batch No.:	L85-12
Purity:	99.4%

2. Test system

The trial was designed with one treated plot and one non-treated (used for procedural recoveries) of wheat plants (variety, Trappe). The treated plot consisted of 102 pots with ± 35 plants each. From these 102 pots, leaves were selected for samples at random points throughout the plot. The plants were grown to growth stage BBCH 30 before application.

Crop Details

Test Crop and Variety/Cultivar:	Wheat – Trappe
Sowing Date(s):	22/09/2014
Plant density:	± 35 plants/pot (ø24 cm)
Crop height:	± 30 cm
Pot distance in the row:	0.325 m
Pot distance between de row:	0.50 m

Plot Details

General Plot Description:	Greenhouse
Outdoor/ Indoor Situation:	Indoor test conditions
Plot Dimensions (treated plot):	3.0 m (width) x = 12,0 m (length) = 36 m² 102 pots with ± 35 plants/pot (ø24 cm) were treated
Plot Dimensions (untreated plot):	12 pots, ± 35 plants/pot (ø24 cm)
Number of Control Plots:	1
Number of Treated Plots:	1

Application Details

Table 7.1.4-1: Application details for BAS 750 01 F applied to wheat

Plot No.	Application No.	Application date	Spray volume (L ha^{-1})	Actual product rate (L ha^{-1})	Nominal rate of active ingredient (g a.s. ha^{-1})	Actual rate of active ingredient (g a.s. ha^{-1})	Growth stage (BBCH)
2	1	10/11/2014	199	0.993	99.34	98.25	30

Application Equipment:	Compressed air with spray boom
Boom Width (m):	3.0
Nozzle Type:	Teejet flat fan XR 11003 VS
Number of Nozzles:	6
Pressure (bar):	3.0

B. STUDY DESIGN

1. Experimental conditions

The application was carried out in the greenhouse at growth stage BBCH 30 with a hand carried compressed air sprayer with 3.0 m spraying boom. The spray boom was fitted with 6 flat fan nozzles XR 11003 VS at 3 bar. The intended spray volume was 200 L ha^{-1}.

The application method was foliar application, chosen to simulate good agricultural practice (GAP). The spray solution was prepared by diluting the required quantity of the test item with tap water. The application rate was verified by sprayer calibration and confirmed by measuring the remaining volume. The rate achieved was calculated to be within ± 10% of that specified in the study plan.

The relevant test conditions such as relative air humidity, air temperature were all monitored during the study. Detailed information may be found in the original study report and/or the raw data.

2. Sampling

Whole leaf samples were taken after drying of the spray solution at 2 HAA (Hours After Application), 6 HAA, 24 HAA, 48 HAA, 4 DAA (Days After Application) and 7 DAA. Each sampling point consisted of 5 replicates. Each replicate consisted of leaves (BBCH 30) selected randomly from the plots to give ~20 g (80 leaves) of fresh weight leaf material. The leaves were cut from the stems at their base by the use of scissors. The leaves were then placed into pre-labelled containers before extraction.

Samples (extract solutions) were stored at -18 °C and shipped via overnight courier packed with dry ice. Complete details and records on storage and shipping can be found in the raw data associated with the study report.

3. Description of analytical procedures

Extraction of the cut leaf samples was performed immediately after collection. The residues of BAS 750 F were dislodged from the surface with the following procedure:

The dislodging solution was prepared by diluting 0.1 g of a Aerosol OT-B powder to 1000 mL using distilled deionized water. The dislodging solution was used within 6 hours.

200 mL of the dislodging solution (Water / Aerosol OT-B (0.01%)) was added to the bottle containing approximately 20 g of leaf material and the bottle was subsequently transferred to a reciprocating table shaker. The bottles were shaken at 250 rpm for a period of 10 minutes. The dislodging solution was decanted into a beaker and the bottle with the remaining leaves was subjected to a second dislodging process with another 200 mL of fresh dislodging solution (250 rpm, 10 minutes). The dislodging solution was also decanted from the bottle and the two dislodging solutions were combined in the beaker and thoroughly mixed. The extraction procedure employed is a relatively mild extraction technique but is thought to reflect accurately residues in which would be readily removed from the surface of the leaf due to environmental effects (i.e. rainfall event). While an overall mild extraction technique, the surfactant based dislodging solution is thought to represent a worst case extraction medium when compared with actual rainfall.

The residues that could be “extracted” by the dislodging procedure were considered to be available for wash off, bearing in mind that degradation as well as strong adsorption to plant surfaces as uptake into plant leaves could contribute to the reduction of the “available” residue fraction.

Two aliquots of 20 mL were obtained from the thoroughly mixed solutions and labelled as ship and retain specimens. These specimens were transferred to freezer storage before shipment to the analytical facility.

Fortification samples were generated to confirm the stability of BAS 750 F under the storage and shipping conditions. Two fortification solutions, one higher (1000x LOQ) and one lower (10x LOQ) concentration, were generated at the analytical facility and sent to the field test site (De Bredelaar). The solutions were then used to spike blank dislodging solutions at the desired concentrations to create the field fortifications.

BAS 750 F residues were determined by means of LC-MS/MS. For the analysis of the dislodging solutions the samples were only diluted with methanol. The limit of quantitation was set for dislodging solution at 5 µg L^{-1}.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as modelling endpoints. Kinetic analysis and calculations of DT_{50} values for BAS 750 F was performed using data (residues dislodgeable from plant leaf surfaces) obtained from the in-life phase and following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*] The software package KinGUI (version 2.2014.224.1704) was used for parameter fitting. The error tolerance and the number of iterations of the optimization tool were set to 10^{-6} and 100, respectively.

II. RESULTS AND DISCUSSION

For each sampling time point 5 replicates were analyzed. The results presented in Table 7.1.4-2 are mean values (*n*=5) of actual measured amounts from the dislodging solutions and were not corrected for the results obtained from the field fortifications. Residues of BAS 750 F were < LOQ immediately prior to the application (-0). Two hours after the application, 0.457 mg L^{-1} were observed and the residues of BAS 750 F decreased continuously to 0.037 mg L^{-1} at 168 hours (=7 days) after application.

Table 7.1.4-2: BAS 750 F in Dislodging solutions

	Sampling time (hours after application), (*n*=5)						
	- 0	**2 HAA**	**6 HAA**	**24 HAA**	**48 HAA**	**95 HAA**	**168 HAA**
Dislodging solution [mg L^{-1}]							
Rep 1	<LOQ	0.428	0.404	0.325	0.278	0.130	0.036
Rep 2	<LOQ	0.472	0.408	0.327	0.285	0.091	0.046
Rep 3	<LOQ	0.483	0.444	0.338	0.307	0.078	0.034
Rep 4	<LOQ	0.411	0.440	0.350	0.261	0.102	0.033
Rep 5		0.489	0.437	0.386	0.244	0.102	0.035
Average	<LOQ	0.457	0.427	0.345	0.275	0.101	0.037
Coefficient of variation		7.6 %	4.5 %	7.2 %	8.7%	19.0 %	14.2 %

HAA = Hours after application; LOQ = Limit of quantification

During the analysis of the study specimens, procedural recoveries were determined at two spiking levels. The mean recovery was 81.4% (n=2).

Field fortification experiments were conducted on the day of the crop treatment to investigate the stability of BAS 750 F under field specimen transport and storage conditions. The field fortification recoveries ranged from 84.6 % to 113.4 % (n=6). Non treated leaves were treated with extract solution and subsequently spiked with a known amount of BAS 750 F.

The kinetic evaluation (Table 7.1.4-3) showed that the SFO model fits the experimental data of the greenhouse trial (visually and statistically).

Table 7.1.4-3 Statistical and visual assessment of different kinetic models for BAS 750 F in the greenhouse trial R14-275-01

Step in FOCUS flowchart	Kinetic model	χ^2 error [%]	p (t-test)	Visual assessment	DT_{50} [d]	DT_{90} [d]
SFO	SFO	5.3	k: <0.01	Good	2.1	7.1
⇨ The SFO visual fit is good; the residuals are randomly scattered around zero. The χ^2 error is low; the parameter k is significantly different from zero. ⇨ **Conclusion:** Use **SFO** to derive **modeling endpoints**.						

The visual assessments of the residual plots show that SFO kinetics describes the best fit. The estimated dissipation rate constant is significantly different from zero as indicated by low p-values (t-test). The estimated DT_{50} value of 2.1 days is suitable for modeling.

III. CONCLUSION

The results obtained in the study demonstrate that the foliar DT_{50} of BAS 750 F was reliably determined for wheat surfaces. After analyzing the data under the guidance from the FOCUS kinetics working group the best fit was found using SFO kinetics. A foliar DT_{50} of 2.1 days was derived.

CA 7.1.4.1 Column leaching studies

CA 7.1.4.1.1 Column leaching of the active substance

Column leaching studies on the mobility of BAS 750 F in soil are not required since reliable K_{FOC} values for BAS 750 F were determined within the batch equilibrium experiments presented in the above chapters.

CA 7.1.4.1.2 Column leaching of metabolites, breakdown and reaction products

Column leaching studies on the mobility of M750F001 in soil are not required since reliable K_{FOC} values are available for this compound.

CA 7.1.4.2 Lysimeter studies

Mobility of BAS 750 F can be evaluated based on the information presented in the current section. Risk of leaching of BAS 750 F and its metabolites can be assessed based on the information provided in chapter CP 9.1.4. Accordingly, the applicant considers these studies as not necessary for the current evaluation.

CA 7.1.4.3 Field leaching studies

A field leaching study for the active substance BAS 750 F was not performed since it is not required under Commission Regulation (EU) No 283/2013 in accordance with Regulation (EC) No 1107/2009.

CA 7.2 Fate and behaviour in water and sediment

The route and rate of degradation of BAS 750 F was investigated relative to the aquatic environment.

CA 7.2.1 Route and rate of degradation in aquatic systems (chemical and photochemical degradation)

CA 7.2.1.1 Hydrolytic degradation

Report:	CA 7.2.1.1/1 Hassink J., 2015 b BAS 750 F: Aqueous hydrolysis at four different pH values 2015/1046919
Guidelines:	OECD 111, EPA 835.2120, JMAFF No 12 Nosan No 8147
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive summary

The hydrolytical stability of BAS 750 F at pH 4, 5, 7 and 9 over a study period of 30 days at 25°C was investigated. No hydrolysis of BAS 750 F was observed, 98-101% TAR were still present in the test systems after 30 days incubation at 25°C. There was no change in the isomer ratio of the test item. No estimation of half-life was attempted as BAS 750 F was found to be stable under the test conditions.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in one ^{14}C-labeled form.

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

Triazole-3(5)-^{14}C-label

Batch No.:	1062-2101
Specific radioactivity of a.s.:	5.57 MBq mg^{-1}
Radiochemical purity:	99.2% (95.3%, determined within the study, Reg. No. 5863469 was identified as impurity)

Unlabeled

Internal code:	BAS 750 F
Reg. No.:	5834378
Common name:	-
Batch No.:	L85-12
Purity:	99.4%

2. Test system

All buffer solutions were prepared from commercially available buffer concentrates (Titrisol, Merck, Darmstadt, Germany) by 10-fold dilution and sterile filtration. The following buffer concentrates were used:

pH 4: Titrisol 1.09884 (citrate – HCl)
pH 5: Titrisol 1.09885 (citrate – NaOH)
pH 7: Titrisol 1.09887 (phosphate)
pH 9: Titrisol 1.09889 (boric acid/KCl – NaOH)

B. STUDY DESIGN

1. Experimental conditions

3.33 mL application solution (containing about 150 µg ^{14}C-BAS 750 F) were given into 500 mL of the diluted buffer, corresponding to a final concentration of about 0.3 mg L^{-1}. Subsets of 50 mL were used for hydrolysis.

The sterile samples were stored in a climatic chamber at the required temperature of 25°C for 30 days. Sterility of the solutions was checked at each sampling time for the respective sample prior to analysis. The pH was checked for each sample after analysis.

2. Sampling

The sampling was performed 0, 3, 10, 17, 21, 25 and 30 days after treatment.

3. Description of analytical procedures

All samples of the test solutions were analysed without a work-up. All samples were measured for radioactivity by liquid scintillation counting (LSC) and were analysed by radio-HPLC to determine the amount of test item and potential metabolites. Furthermore, the isomers of BAS 750 F were separated by a chiral HPLC method.

4. Calculation of the degradation rate

No estimation of half-life was attempted as BAS 750 F was found to be stable under the test conditions.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Total recoveries of radioactivity are summarized in Table 7.2.1.1-1. During the testing period of 30 days the material balance was in the range of 96.3% to 102.1% of the total applied radioactivity (TAR). No loss of radioactivity occurred.

Table 7.2.1.1-1: Recovery of radioactivity after treatment with ^{14}C-labeled BAS 750 F and incubation at pH 4-9 and 25°C

Days after treatment	pH 4 [% TAR] [a]	pH 5 [% TAR] [a]	pH 7 [% TAR] [a]	pH 9 [% TAR] [a]
0	100.0	100.0	100.0	100.0
3	99.1	97.1	101.0	98.6
10	100.2	100.4	102.1	101.7
17	98.7	97.4	97.7	98.3
21	96.3	101.4	99.8	99.9
25	99.7	99.8	101.2	100.6
30	100.9	98.3	98.9	99.6

TAR = total applied radioactivity
[a] Concentration of day 0 was set to 100% TAR

B. TRANSFORMATION OF PARENT COMPOUND

Results of radio-HPLC analyses are presented in Table 7.2.1.1-2 and Table 7.2.1.1-3.

No significant degradation products above 5% TAR occurred beside an impurity (identified as Reg.No. 5863469 - M750F006) that was already present in the test item at the beginning of the study, no significant increase of its concentration occurred.

Furthermore, no significant change in the isomer ratio of BAS 750 F was observed for each test (see Table 7.2.1.1-4).

Table 7.2.1.1-2: Radio-HPLC analysis after treatment with ^{14}C-labeled BAS 750 F and incubation at pH 4 and pH 5 and 25°C

Days after treatment	pH 4				pH 5			
	Reg.No 5863469 [% TAR	BAS 750 F [% TAR]	Others [a] [% TAR	Sum [% TAR]	Reg.No 5863469 [% TAR]	BAS 750 F [% TAR]	Others [b] [% TAR]	Sum [% TAR
0	4.7	95.3	-	100.0	5.3	94.7	-	100.0
3	5.5	92.7	0.8	99.0	5.3	91.1	0.7	97.1
10	4.8	95.4	-	100.2	5.5	94.3	0.7	100.5
17	5.8	92.3	0.7	98.8	5.7	90.4	1.3	97.4
21	4.4	91.3	0.7	96.4	4.9	96.0	0.6	101.5
25	6.1	93.6	-	99.7	5.0	94.8	-	99.8
30	6.2	93.8	0.9	100.9	5.2	92.1	1.0	98.3

TAR = total applied radioactivity
[a] Sum of other peaks, each peak less than 1% TAR
[b] Sum of other peaks, each peak less than 2% TAR

Table 7.2.1.1-3: Radio-HPLC analysis after treatment with ^{14}C-labeled BAS 750 F and incubation at pH 7 and pH 9 and 25°C

Days after treatment	pH 7				pH 9			
	Reg.No 5863469 [% TAR	BAS 750 F [% TAR]	Others [a] [% TAR	Sum [% TAR]	Reg.No 5863469 [% TAR]	BAS 750 F [% TAR]	Others [a] [% TAR]	Sum [% TAR
0	3.7	96.3	-	100.0	5.0	95.0	-	100.0
3	5.4	94.8	0.8	101.0	5.4	93.2	-	98.6
10	4.7	96.4	1.0	102.1	4.6	97.1	-	101.7
17	5.3	91.4	1.1	97.8	4.9	92.0	1.5	98.4
21	4.5	94.5	0.8	99.8	5.2	93.7	1.0	99.9
25	4.9	96.4	-	101.3	4.9	95.7	-	100.6
30	5.6	93.3	-	98.9	5.0	93.1	1.4	99.5

TAR = total applied radioactivity
[a] Sum of other peaks, each peak less than 2% TAR

Table 7.2.1.1-4: Chiral radio-HPLC analysis after treatment with ^{14}C-labeled BAS 750 F and incubation at pH 7-9 and 25°C

Days after treatment	pH	BAS 750 F		
		Isomer I [% TAR]	Isomer II [% TAR]	Sum [% TAR]
0	4	49.4	45.6	95.0
	5	47.8	48.4	96.2
	7	48.5	48.0	96.5
	9	50.2	46.3	96.5
30	4	50.1	47.3	97.4
	5	47.2	46.7	93.9
	7	46.4	48.7	95.1
	9	49.1	46.6	95.7

TAR = total applied radioactivity

III. CONCLUSION

BAS 750 F was stable in aqueous solution at pH 4, 5, 7 and 9 (25°C). No degradation products with equal or more than 2% TAR were detected, no change in the isomer ratio of the test item was observed.

CA 7.2.1.2 Direct photochemical degradation

The direct photolytic degradation of BAS 750 F was investigated in a buffered system (pH 7) under continuous irradiation for 15 days. Fast degradation of BAS 750 F (DT_{50} = 2.3 days) was observed and multiple metabolites were detected. Four of these metabolites were observed in amounts above 5% TAR and the structures were elucidated. Four other minor photolysis products were observed in amounts below < 3% TAR and structure elucidation was not further pursued. From these data an aquatic photolysis degradation scheme was proposed and kinetic analyses were performed on BAS 750 F. In addition, it was possible to propose dissipation half-lives for two of the metabolites, M750F005 and M750F006, which were 34.8 and 12.4 days, respectively. Chiral analysis of the parent was performed in the study and no chiral inversion or selectivity was observed.

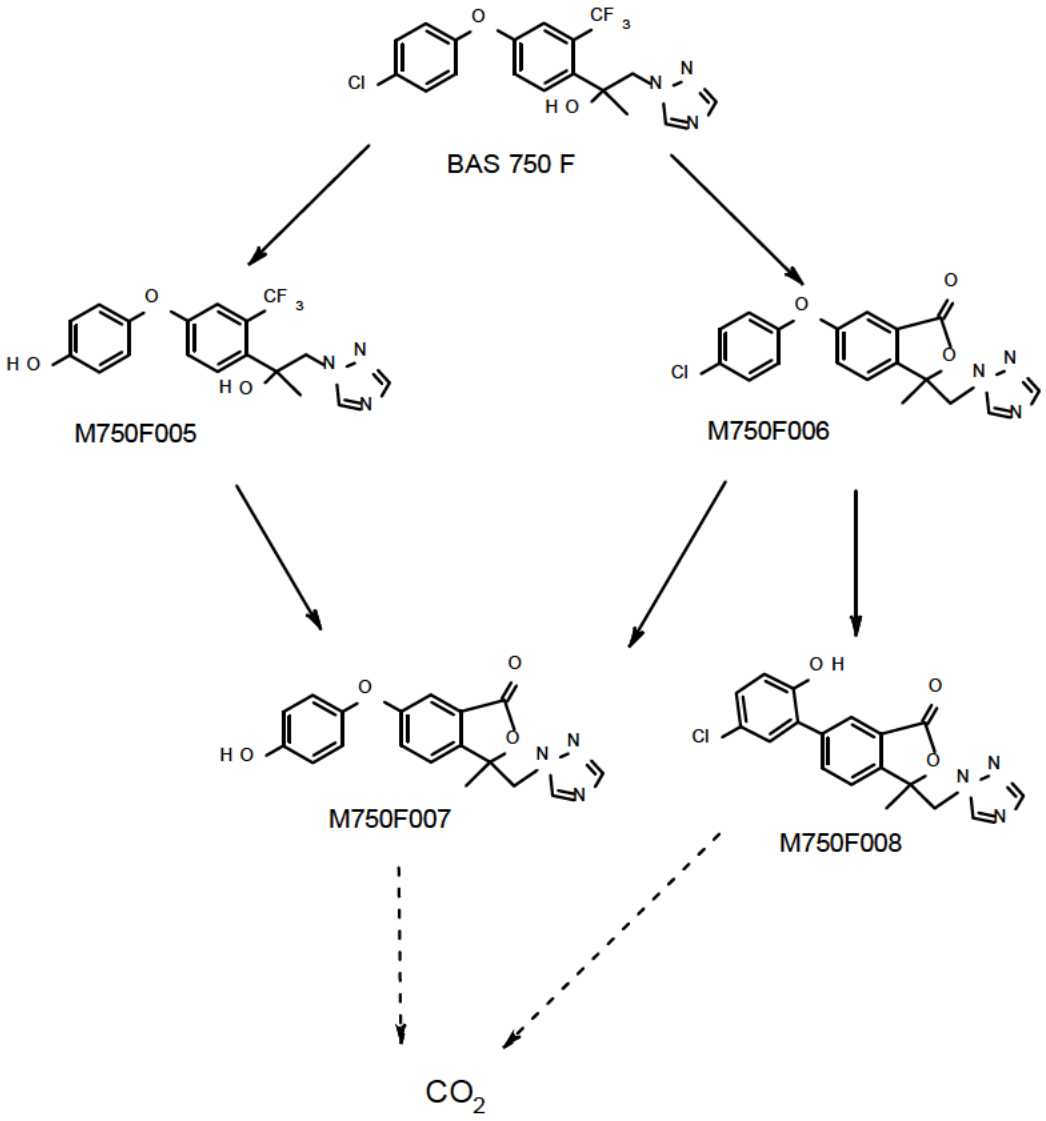


Figure 7.2.1.2-1: Proposed Aqueous Photolysis Scheme

Report:	CA 7.2.1.2/1 Zhixing Y., 2015 a Aqueous Photolysis of 14C-BAS 750 F 2015/7000233
Guidelines:	EPA 835.2240, OECD 316 (Photodegradation in Water), FIFRA 40 CFR 160
GLP:	yes (certified by United States Environmental Protection Agency)

Executive summary

The aqueous photolysis of ^{14}C-BAS 750 F was conducted under sterile conditions with [chlorophenyl-U-^{14}C] and [triazole-3(5)-^{14}C] labelled test item in pH 7 buffer solution at 25 ± 1°C. The concentration of ^{14}C-BAS 750 F in the buffer solution was approximately 0.7 mg L^{-1}. This test concentration is higher than the guideline recommended "half the water solubility", but because of the low water solubility of BAS 750 F (0.81 mg L^{-1}) this was deemed necessary to achieve a concentration suitable enough for identification of the degradation products. This change is not believed to adversely affect the study outcome. The treated buffer solutions were continuously exposed to artificial sunlight in a SUNTEST® apparatus for 15 days. The light spectrum and intensity of the Xenon arc lamp was measured at the beginning and end of the irradiation period and the measured intensity and spectrum was comparable to natural sunlight at 40 N latitude. An actinometer solution [4-nitroacetophenone (PNAP)] was irradiated concurrently with the buffer solutions of test substances. During the irradiation, sterile air was purged over the samples and through traps for the collection of volatiles. Dark control samples consisted of sterile pH 7 buffer solutions treated with similar amounts of chlorophenyl and triazole-radiolabeled BAS 750 F were stored in a dark incubator maintained at 25 ± 1°C.

After 0, 0.25, 1, 2, 3, 6, 9, 13 and 15 days of irradiation, samples of the chlorophenyl and triazole-radiolabeled BAS 750 F treated buffer solutions were analyzed by liquid scintillation counting (LSC) and high performance liquid chromatography (HPLC). The dark control samples were analyzed at the same sampling times by LSC and HPLC as the irradiated samples.

The material balance for the irradiated samples ranged between 92.6-100.0% total applied radioactivity (TAR) (chlorophenyl label) and 95.6-104.8% TAR (triazole label). The volatiles produced were negligible. The material balance for the dark control samples ranged between 90.1-100.0% TAR (chlorophenyl label) and 90.0-100.0% TAR (triazole label).

Rapid degradation of ^{14}C-BAS 750 F was observed in the irradiated samples with a half-life of 2.3 days. No degradation of ^{14}C-BAS 750 F was observed in the dark control samples. The quantum yield of BAS 750 F was determined to be 3.5×10^{-1}.

^{14}C-BAS 750 F degraded to several products. Four of them, observed with both radiolabels, reached amounts above 5% TAR and were identified by LC-MS/MS (M750F007, M750F005, M750F006, and M750F008). The maximum levels observed for M750F007, M750F005, M750F006, and M750F008 were 43.9%, 32.2%, 30.7%, and 7.3% TAR, respectively. All other degradation products formed were less than 5% TAR.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

The test item BAS 750 F was used in two ^{14}C-labeled forms.

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

1. [chlorophenyl-U-^{14}C] label

Batch No.:	CFQ41561
Specific radioactivity of a.s.:	7.878 MBq mg^{-1}
Radiochemical purity:	98.9%, see certificate of analysis
Purity:	99.1%

2. [triazole-3(5)-^{14}C] label

Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%, see certificate of analysis
Purity:	98.9%

Unlabeled

Internal code:	BAS 750 F
Reg. No.:	5834378
Common name:	NA
Batch No.:	L84-238
Purity:	99.7%

2. Buffer solution

A pH 7 Buffer solution was used and prepared as follows: Boric acid (0.621 g) was transferred to a bottle and mixed with water (1 L) until all of the boric acid was in solution. After mixing, the pH of the solution was measured (pH 6) and adjusted to a pH of 7.08 by dropwise addition of NaOH (1 N). After preparation, the buffer was autoclaved.

B. STUDY DESIGN

1. Experimental conditions

Photolysis was carried out in an Atlas SUNTEST® CPS+ unit with a xenon lamp equipped with filters to mimic sunlight (wavelengths < 290 nm were filtered out). Continuous illumination was employed throughout the study.

The stock solutions were prepared by transferring a small amount of chlorophenyl or triazole labeled BAS 750 F to a 25 mL glass volumetric flask and diluting to volume with ACN. The flasks were vortexed to ensure a homogenous solution. The concentrations were checked by LSC and found to be approximately 0.13 mg mL^{-1} and 0.18 mg mL^{-1} for the Stk0001 (chlorophenyl label) and the Stk0002 (triazole label) solutions respectively.

Two 500 mL aliquots of pH 7 buffer solution were dosed using an auto pipette with 2.628 mL and 1.999 mL of Stk0001 and Stk0002, respectively. These were thoroughly mixed using manual shaking and sonication to ensure homogenous distribution and then divided into two photolysis vessels containing 200 mL of buffered, dosed solution. These were then transferred to the photolysis apparatus.

The photolysis setup consisted of a rectangular hollow box made of Plexiglas® equipped with a coolant inlet and outlet. The box consisted of 6 wells to house 6 photolysis glass vessels. Each glass vessel had an air inlet, air outlet and a quartz glass disc at the top. Two photolysis glass vessels were filled with 200 mL triazole-labelled BAS 750 F treated sterile pH 7 buffer solution, two photolysis glass vessels were filled with 200 mL chlorophenyl-labelled BAS 750 F treated sterile pH 7 buffer solution, and two photolysis glass vessels were filled with 200 mL of the actinometer solution (mixture of 4-nitroacetophenone and pyridine used for quantum yield determination). Each duplicate set of vessels was connected to trapping solutions for the collection of volatile radioactivity (1 N NaOH for CO_2). The concentration of the test substance in the buffer solutions was approximately 0.7 mg L^{-1}. The temperature of the photolysis solution was maintained at approximately 25 ± 1°C during irradiation by circulating cold water.

Control solutions of the test substance (^{14}C-BAS 750 F) in sterile test buffer (pH 7) were placed in sterilized containers, sealed and maintained in the dark at 25 ± 1°C. Aliquots from these dark control solutions were taken concurrently with the irradiated samples for analysis by LSC and HPLC.

Sterility was checked on the day of dosing and at the completion of the experiment period. No microbial growth was detected.

2. Sampling

Samples were collected at 0, 0.25, 1, 2, 3, 6, 9, 13 and 15 days after treatment (DAT) during the irradiation period and analyzed by LSC and HPLC to establish the rate of photolytic degradation of the parent and the formation and decline of the photolysis products. Selected samples were also analyzed by LC-MS in order to facilitate the identification of the degradation products. The trapping solutions were analyzed by LSC at each sampling interval with the exception of 0 DAT.

3. Description of analytical procedures

Each time-course sample (including volatile traps) were analyzed by LSC (in triplicate) to determine the amount of radioactivity present in the solutions. Aliquots from each time-point were also analyzed by HPLC. Aliquots of the dark control samples were analyzed concurrently with the irradiated samples. Actinometer solutions used for quantum yield determination were also taken at each sampling time and analyzed by HPLC. LOQ of the LSC method was 0.459% TAR (3.173µg L^{-1}) and LOD was defined as 2/3 of the LOQ or 0.306% TAR (2.116 µg L^{-1}). LOQ for the HPLC method was 0.312% TAR (2.158 µg L^{-1}) and LOD was defined as 2/3 of the LOQ or 0.208% TAR (1.439 µg L^{-1}).

4. Kinetic evaluation

A kinetic evaluation was performed in accordance with FOCUS kinetic guidance based on the results of the irradiated test systems. For this purpose, the results from the two radiolabels were combined and evaluated as one data set. Besides the parent compound BAS 750 F, the metabolites M750F005 and M750F006, which showed a decline in concentration were included in the evaluation. The metabolites M750F007 and M750F008 were not included, as they were either increasing at the end of the study (M750F007), or there were too few sampling points beyond the maximum reported value (M750F008).

II. RESULTS AND DISCUSSION

A. MATERIAL BALANCE

Chlorophenyl-label:
The material balance results for the irradiated samples of the chlorophenyl-labelled BAS 750 F treated buffers are given in Table 7.2.1.2-1. It ranged from 92.6-100.0% TAR. The amount of radioactivity collected in the volatile traps remained negligible throughout the study.

The material balance results for the dark control samples of the chlorophenyl-labelled BAS 750 F treated buffers are given in Table 7.2.1.2-2. The mean total radioactivity (% TAR) ranged from 90.1-100.0% TAR.

Triazole- label:
The material balance results for the irradiated samples of the triazole-labelled BAS 750 F treated buffers are given in Table 7.2.1.2-3. The mean total radioactivity (% TAR) ranged from 95.6-104.8% TAR. The % TAR collected in the volatile traps remained negligible throughout the study.

The material balance results for the dark control samples of the triazole- labelled BAS 750 F treated buffers are given in Table 7.2.1.2-4. The mean total radioactivity (% TAR) ranged from 90.0-100.0% TAR.

Table 7.2.1.2-1: Material balance of irradiated test systems for chlorophenyl-labelled BAS 750 F

DAT	Buffer [%TAR]	Volatiles traps [%TAR]	Material balance %TAR
0 rep 1	98.8	< LOQ	98.8
0 rep 2	101.2	< LOQ	101.2
0 mean	100.0	< LOQ	100.0
0.25 rep 1	91.7	< LOQ	91.7
0.25rep 2	95.1	< LOQ	95.1
0.25 mean	93.4	< LOQ	93.4
1 rep 1	94.2	< LOQ	94.2
1 rep 2	91.0	< LOQ	91.0
1 mean	92.6	< LOQ	92.6
2 rep 1	94.4	< LOQ	94.4
2 rep 2	97.0	< LOQ	97.0
2 mean	95.7	< LOQ	95.7
3 rep 1	95.4	< LOQ	95.4
3 rep 2	94.6	< LOQ	94.6
3 mean	95.0	< LOQ	95.0
6 rep 1	98.3	< LOQ	98.3
6 rep 2	98.7	< LOQ	98.7
6 mean	98.5	< LOQ	98.5
9 rep 1	97.9	< LOQ	97.9
9 rep 2	98.4	< LOQ	98.4
9 mean	98.1	< LOQ	98.1
13 rep 1	97.8	< LOQ	97.8
13 rep 2	97.3	< LOQ	97.3
13 mean	97.6	< LOQ	97.6
15 rep 1	98.0	< LOQ	98.0
15 rep 2	97.0	< LOQ	97.0
15 mean	97.5	< LOQ	97.5

TAR = Total applied radioactivity
DAT = Days after treatment
LOQ = Limit of quantification (0.459% TAR)

Table 7.2.1.2-2: Material balance of non-irradiated test systems for chlorophenyl-labelled BAS 750 F

DAT	Buffer [%TAR]	Volatiles traps [%TAR]	Material balance %TAR
0 rep 1	98.8	NA	98.8
0 rep 2	101.2	NA	101.2
0 mean	100.0	NA	100.0
0.25 rep 1	94.7	NA	94.7
0.25 rep 2	94.8	NA	94.8
0.25 mean	94.8	NA	94.8
1 rep 1	92.1	NA	92.1
1 rep 2	93.1	NA	93.1
1 mean	92.6	NA	92.6
2 rep 1	91.0	NA	91.0
2 rep 2	89.1	NA	89.1
2 mean	90.1	NA	90.1
3 rep 1	92.9	NA	92.9
3 rep 2	90.8	NA	90.8
3 mean	91.9	NA	91.9
6 rep 1	94.3	NA	94.3
6 rep 2	89.8	NA	89.8
6 mean	92.1	NA	92.1
9 rep 1	91.7	NA	91.7
9 rep 2	92.6	NA	92.6
9 mean	92.1	NA	92.1
13 rep 1	92.9	NA	92.9
13 rep 2	93.2	NA	93.2
13 mean	93.0	NA	93.0
15 rep 1	95.0	NA	95.0
15 rep 2	96.3	NA	96.3
15 mean	95.6	NA	95.6

TAR = Total applied radioactivity
DAT = Days after treatment
NA = Not applicable

Table 7.2.1.2-3: Material balance of irradiated test systems for triazole-labelled BAS 750 F

DAT	Buffer [%TAR]	Volatiles traps [%TAR]	Material balance %TAR
0 rep 1	99.8	< LOQ	99.8
0 rep 2	100.2	< LOQ	100.2
0 mean	100.0	< LOQ	100.0
0.25 rep 1	99.2	< LOQ	99.2
0.25 rep 2	96.6	< LOQ	96.6
0.25 mean	97.9	< LOQ	97.9
1 rep 1	95.9	< LOQ	95.9
1 rep 2	96.3	< LOQ	96.3
1 mean	96.1	< LOQ	96.1
2 rep 1	96.1	< LOQ	96.1
2 rep 2	95.1	< LOQ	95.1
2 mean	95.6	< LOQ	95.6
3 rep 1	97.3	< LOQ	97.3
3 rep 2	97.3	< LOQ	97.3
3 mean	97.3	< LOQ	97.3
6 rep 1	100.2	< LOQ	100.2
6 rep 2	101.7	< LOQ	101.7
6 mean	100.9	< LOQ	100.9
9 rep 1	101.6	< LOQ	101.6
9 rep 2	102.5	< LOQ	102.5
9 mean	102.1	< LOQ	102.1
13 rep 1	103.2	< LOQ	103.2
13 rep 2	102.1	< LOQ	102.1
13 mean	102.7	< LOQ	102.7
15 rep 1	104.6	< LOQ	104.6
15 rep 2	105.1	< LOQ	105.1
15 mean	104.8	< LOQ	104.8

TAR = Total applied radioactivity
DAT = Days after treatment
LOQ = Limit of quantification (0.459% TAR)

Table 7.2.1.2-4: Material balance of non-irradiated test systems for triazole-labelled BAS 750 F

DAT	Buffer [%TAR]	Volatiles traps [%TAR]	Material balance %TAR
0 rep 1	99.8	NA	99.8
0 rep 2	100.2	NA	100.2
0 mean	100.0	NA	100.0
0.25 rep 1	93.1	NA	93.1
0.25 rep 2	94.1	NA	94.1
0.25 mean	93.6	NA	93.6
1 rep 1	93.4	NA	93.4
1 rep 2	90.2	NA	90.2
1 mean	91.8	NA	91.8
2 rep 1	92.4	NA	92.4
2 rep 2	94.7	NA	94.7
2 mean	93.5	NA	93.5
3 rep 1	90.6	NA	90.6
3 rep 2	89.4	NA	89.4
3 mean	90.0	NA	90.0
6 rep 1	90.0	NA	90.0
6 rep 2	91.4	NA	91.4
6 mean	90.7	NA	90.7
9 rep 1	91.2	NA	91.2
9 rep 2	92.6	NA	92.6
9 mean	91.9	NA	91.9
13 rep 1	92.3	NA	92.3
13 rep 2	93.9	NA	93.9
13 mean	93.1	NA	93.1
15 rep 1	99.4	NA	99.4
15 rep 2	95.6	NA	95.6
15 mean	97.5	NA	97.5

TAR = Total applied radioactivity
DAT = Days after treatment
NA = Not applicable

B. TRANSFORMATION OF PARENT COMPOUND

Quantitative distribution of [^{14}C]-BAS 750 F and photodegradation products in irradiated test systems

Chlorophenyl- label:
The results of the HPLC analysis for the irradiated chlorophenyl-labelled BAS 750 F treated buffer solutions are shown in Table 7.2.1.2-5. The HPLC analysis results showed that BAS 750 F was degraded very rapidly to a large number of products. BAS 750 F accounted for ~1.8% TAR at the end of study (15 DAT). The number of the degradation products increased with time and the degradation products M750F007, M750F005, M750F006, and M750F008 were observed in levels >5% TAR. The maximum levels observed for M750F007, M750F005, M750F006, and M750F008 were 36.1% TAR (15 DAT), 30.1% TAR (9 DAT), 30.7% TAR (9 DAT), and 7.0% TAR (13 DAT), respectively. Four other degradation products were observed at levels of < 2% TAR and one of them (t_R of ~34.2 min) was an impurity from the chlorophenyl-labelled BAS 750 F test substance detected at every time point throughout the study (including 0 DAT).

Triazole-label:
The results of the HPLC analysis for the irradiated triazole- labelled BAS 750 F treated buffer solutions are shown in Table 7.2.1.2-6. The HPLC analysis results also demonstrated that BAS 750 F was degraded very rapidly to a large number of products. BAS 750 F accounted for ~0.9% TAR at the end of study (15 DAT). The number of the degradation products increased with time and the degradation products M750F007, M750F005, M750F006, and M750F008 were observed at levels >5% TAR. The maximum levels observed for M750F007, M750F005, M750F006, and M750F008 were 43.9% TAR (15 DAT), 32.2% TAR (6 DAT), 30.2% TAR (6 DAT), and 7.3% TAR (13 DAT), respectively. A few other degradation products, including M750F002 and M750F003, were observed at levels of ≤3% TAR (identified by HPLC and retention time matching to known standards). The product with t_R of ~4.9 min (maximum level of ~3% TAR) was very likely M750F001 (1,2,4-triazole) based on its retention time as compared to the reference standard.

Quantitative distribution of [^{14}C]-BAS 750 F and photodegradation products in the non-irradiated (dark control) test systems

Chlorophenyl- label:
The results of the HPLC analysis for the non-irradiated chlorophenyl-labelled BAS 750 F treated buffer solutions are shown in Table 7.2.1.2-7. BAS 750 F is stable in the dark control samples. Only the parent compound was observed in the HPLC chromatograms with the exception of a few minor degradates or a slight impurity (degradates < 1% TAR and the impurity with t_R of ~34.2 min <2% TAR).

Triazole- label:
The results of the HPLC analysis for the non-irradiated triazole-labelled BAS 750 F treated buffer solutions are shown in Table 7.2.1.2-8. HPLC analysis demonstrated that BAS 750 F was stable in the dark control samples.

Table 7.2.1.2-5: HPLC quantitation of ^{14}C-residues in the irradiated test system for chlorophenyl-labelled BAS 750 F

	%TAR								
Compound t_R [min]	**~16.0**	**M750F007 ~24.2**	**M750F005 ~26.3**	**M750F008 ~26.7**	**~27.4**	**M750F006 ~28.9**	**~29.7**	**BAS 750 F ~31.2**	**~34.7**
DAT									
0 rep1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	96.9	1.9
0 rep2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	99.5	1.7
0 mean	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	98.2	1.8
0.25 rep1	< LOQ	< LOQ	4.6	< LOQ	< LOQ	3.5	< LOQ	81.6	2.0
0.25 rep2	< LOQ	< LOQ	4.5	< LOQ	< LOQ	4.2	< LOQ	84.9	1.5
0.25 mean	< LOQ	< LOQ	4.6	< LOQ	< LOQ	3.8	< LOQ	83.3	1.8
1 rep1	< LOQ	< LOQ	12.3	1.9	< LOQ	11.8	< LOQ	66.8	1.4
1 rep2	< LOQ	< LOQ	11.9	1.6	< LOQ	10.7	< LOQ	65.3	1.5
1 mean	< LOQ	< LOQ	12.1	1.7	< LOQ	11.2	< LOQ	66.1	1.4
2 rep1	< LOQ	1.8	17.7	3.6	< LOQ	17.1	< LOQ	52.8	1.4
2 rep2	< LOQ	2.4	19.7	2.7	< LOQ	17.8	< LOQ	53.2	1.3
2 mean	< LOQ	2.1	18.7	3.1	< LOQ	17.4	< LOQ	53.0	1.3
3 rep1	< LOQ	3.1	22.5	2.6	0.9	22.8	1.0	41.2	1.3
3 rep2	< LOQ	3.8	24.1	3.3	< LOQ	20.7	< LOQ	41.1	1.6
3 mean	< LOQ	3.5	23.3	2.9	0.4	21.8	0.5	41.1	1.4
6 rep1	< LOQ	10.1	29.2	5.3	< LOQ	28.4	1.1	23.0	1.3
6 rep2	< LOQ	13.1	30.2	5.6	< LOQ	29.6	1.4	18.0	0.8
6 mean	< LOQ	11.6	29.7	5.4	< LOQ	29.0	1.3	20.5	1.0
9 rep1	1.5	16.2	30.2	5.3	< LOQ	30.9	1.9	11.2	0.7
9 rep2	1.1	20.8	29.9	5.6	< LOQ	30.5	1.5	8.2	0.8
9 mean	1.3	18.5	30.1	5.4	< LOQ	30.7	1.7	9.7	0.8
13 rep1	1.0	31.3	27.9	7.0	1.0	24.3	1.3	3.5	0.6
13 rep2	1.4	33.2	26.0	7.1	< LOQ	25.6	1.5	2.6	< LOQ
13 mean	1.2	32.2	27.0	7.0	0.5	24.9	1.4	3.0	0.3
15 rep1	1.3	35.2	26.9	7.0	< LOQ	22.8	2.0	2.2	0.5
15 rep2	1.7	37.0	30.2	4.4	< LOQ	20.5	1.7	1.4	< LOQ
15 mean	1.5	36.1	28.6	5.7	< LOQ	21.7	1.9	1.8	0.3

TAR = Total applied radioactivity
t_R = HPLC retention time
DAT = Days after treatment
LOQ = Limit of quantification (0.312% TAR)

Table 7.2.1.2-6: HPLC quantitation of ^{14}C-residues in the irradiated test system for triazole-labelled BAS 750 F

	% TAR								
Compound t_R [min]	**~4.9**	**M750F002 ~20.1**	**M750F003 ~22.4**	**M750F007 ~24.2**	**M750F005 ~26.3**	**M750F008 ~26.7**	**M750F006 ~28.9**	**BAS 750 F ~31.2**	**All Other Peaks***
DAT									
0 rep1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	99.8	< LOQ
0 rep2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	100.2	< LOQ
0 mean	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	100.0	< LOQ
0.25 rep1	< LOQ	< LOQ	< LOQ	< LOQ	3.8	< LOQ	3.5	91.8	< LOQ
0.25 rep2	< LOQ	< LOQ	< LOQ	< LOQ	3.5	< LOQ	3.1	90.0	< LOQ
0.25 mean	< LOQ	< LOQ	< LOQ	< LOQ	3.6	< LOQ	3.3	90.9	< LOQ
1 rep1	1.3	< LOQ	< LOQ	1.1	12.4	1.5	12.3	67.2	< LOQ
1 rep2	1.1	< LOQ	< LOQ	1.0	12.2	1.0	12.7	68.2	< LOQ
1 mean	1.2	< LOQ	< LOQ	1.1	12.3	1.3	12.5	67.7	< LOQ
2 rep1	< LOQ	< LOQ	0.7	2.1	20.7	2.8	19.2	50.5	< LOQ
2 rep2	< LOQ	< LOQ	0.8	2.5	20.3	3.2	19.4	48.9	< LOQ
2 mean	< LOQ	< LOQ	0.7	2.3	20.5	3.0	19.3	49.7	< LOQ
3 rep1	1.3	0.5	1.1	4.4	24.8	4.0	24.6	36.5	< LOQ
3 rep2	1.5	0.4	1.4	5.6	25.9	4.1	23.6	34.8	< LOQ
3 mean	1.4	0.5	1.2	5.0	25.4	4.1	24.1	35.7	< LOQ
6 rep1	< LOQ	2.1	1.1	14.4	32.3	4.5	29.7	16.1	< LOQ
6 rep2	1.1	1.0	1.1	15.6	32.1	5.9	30.6	14.4	< LOQ
6 mean	0.6	1.5	1.1	15.0	32.2	5.2	30.2	15.2	< LOQ
9 rep1	1.7	1.6	1.3	24.1	31.5	6.7	29.0	5.8	< LOQ
9 rep2	1.9	1.9	1.2	26.2	30.1	5.7	28.7	4.8	2.0
9 mean	1.8	1.8	1.3	25.1	30.8	6.2	28.9	5.3	1.0
13 rep1	3.6	2.4	1.2	37.0	27.4	6.7	23.4	1.6	< LOQ
13 rep2	2.3	3.9	< LOQ	40.0	24.4	7.9	22.1	1.6	< LOQ
13 mean	2.9	3.2	0.6	38.5	25.9	7.3	22.7	1.6	< LOQ
15 rep1	3.0	3.4	1.3	41.8	24.9	6.8	21.0	0.9	1.4
15 rep2	2.7	3.2	1.8	46.1	24.2	5.4	20.9	0.9	< LOQ
15 mean	2.8	3.3	1.5	43.9	24.6	6.1	21.0	0.9	0.7

TAR = Total applied radioactivity
t_R = HPLC retention time
DAT = Days after treatment
LOQ = Limit of quantification (0.312% TAR)

Table 7.2.1.2-7: HPLC quantitation of ^{14}C-residues in the dark control test system for chlorophenyl-labelled BAS 750 F

	%TAR			
Compound t_R [min]	**M750F005 ~26.3**	**M750F006 ~28.9**	**BAS 750 F ~31.2**	**~34.7**
DAT				
0 rep1	< LOQ	< LOQ	96.9	1.9
0 rep2	< LOQ	< LOQ	99.5	1.7
0 mean	< LOQ	< LOQ	98.2	1.8
0.25 rep1	< LOQ	< LOQ	93.0	1.7
0.25 rep2	< LOQ	< LOQ	93.4	1.4
0.25 mean	< LOQ	< LOQ	93.2	1.6
1 rep1	< LOQ	< LOQ	90.8	1.4
1 rep2	0.9	< LOQ	90.8	1.4
1 mean	0.4	< LOQ	90.8	1.4
2 rep1	< LOQ	< LOQ	89.7	1.3
2 rep2	0.7	0.9	86.3	1.2
2 mean	0.3	0.4	88.0	1.3
3 rep1	1.1	< LOQ	90.0	1.8
3 rep2	0.7	0.8	88.2	1.2
3 mean	0.9	0.4	89.1	1.5
6 rep1	< LOQ	< LOQ	92.4	1.9
6 rep2	< LOQ	< LOQ	87.9	1.9
6 mean	< LOQ	< LOQ	90.2	1.9
9 rep1	< LOQ	< LOQ	90.5	1.2
9 rep2	< LOQ	< LOQ	91.3	1.2
9 mean	< LOQ	< LOQ	90.9	1.2
13 rep1	< LOQ	< LOQ	91.2	1.7
13 rep2	< LOQ	< LOQ	91.7	1.4
13 mean	< LOQ	< LOQ	91.5	1.6
15 rep1	< LOQ	< LOQ	93.6	1.4
15 rep2	< LOQ	< LOQ	94.9	1.4
15 mean	< LOQ	< LOQ	94.2	1.4

TAR = Total applied radioactivity
t_R = HPLC retention time
DAT = Days after treatment
LOQ = Limit of quantification (0.312% TAR)

Table 7.2.1.2-8: HPLC quantitation of ^{14}C-residues in the dark control test system for triazole-labelled BAS 750 F

	% TAR	
Compound t_R [min]	**~4.9**	**BAS 750 F ~31.2**
Days after treatment (DAT)		
0 rep1	< LOQ	99.8
0 rep2	< LOQ	100.2
0 mean	< LOQ	100.0
0.25 rep1	< LOQ	93.1
0.25 rep2	< LOQ	94.1
0.25 mean	< LOQ	93.6
1 rep1	< LOQ	93.4
1 rep2	< LOQ	90.2
1 mean	< LOQ	91.8
2 rep1	< LOQ	92.4
2 rep2	< LOQ	94.7
2 mean	< LOQ	93.5
3 rep1	< LOQ	90.6
3 rep2	< LOQ	89.4
3 mean	< LOQ	90.0
6 rep1	< LOQ	90.0
6 rep2	< LOQ	91.4
6 mean	< LOQ	90.7
9 rep1	1.5	89.7
9 rep2	< LOQ	92.6
9 mean	0.8	91.2
13 rep1	< LOQ	92.3
13 rep2	< LOQ	93.9
13 mean	< LOQ	93.1
15 rep1	< LOQ	99.4
15 rep2	< LOQ	95.6
15 mean	< LOQ	97.5

TAR = Total applied radioactivity
t_R = HPLC retention time
LOQ = Limit of quantification (0.312% TAR)

C. KINETIC MODELING RESULTS

The data generated in this study was analysed in accordance with FOCUS kinetic guidance to determine DT_{50}, and DT_{90} values for BAS 750 F and its photodegradation products. The kinetic endpoint values are presented in Table 7.2.1.2-9. A SFO (single first-order) model was used for the fittings.

Rapid degradation of BAS 750 F was observed in the irradiated samples following first order kinetics. The SFO DT_{50}, and DT_{90} values for BAS 750 F were estimated to be 2.3, 7.6 days, respectively.

Four metabolites (M750F005, M750F006, M750F007, M750F008) in the irradiated samples were observed in levels > 5% TAR. Two metabolites were not kinetically evaluated, as they were either increasing at the end of the study (M750F007), or there were too few sampling points beyond the maximum reported value (M750F008). The other two metabolites (M750F005, M750F006) were kinetically evaluated from their maximum reported values onward. Both were observed to degrade following first order kinetics.

The SFO DT_{50}, and DT_{90} values for metabolite M750F005 were estimated to be 34.8, and 116 days, respectively. The SFO DT_{50}, and DT_{90} values for metabolite M750F006 were estimated to be 12.4, and 41.3 days, respectively.

Table 7.2.1.2-9: Kinetic endpoints for the aqueous photolysis of BAS 750 F and two metabolites

Substance	System[a]	Kinetic Model	DT_{50} [days]
BAS 750 F	Irradiated, pH 7	SFO	2.3
M750F005	Irradiated, pH 7	SFO[b]	34.8
M750F006	Irradiated, pH 7	SFO[b]	12.4

[a] Systems were continuously irradiated for 15 days.
[b] Metabolites decline fit from peak reported value onward.

Based on these results a quantum yield was calculated for BAS 750 F. The quantum yield of BAS 750 F was determined to be 3.5×10^{-1}.

Additionally, chiral analysis was performed on the samples and no variation of the enantiomeric ratio of BAS 750 F was observed. The ratio remained approximately 1:1 throughout the study duration.

III. CONCLUSION

Rapid degradation of BAS 750 F was observed in the continuously irradiated samples with a half-life of 2.3 days. No degradation of ^{14}C-BAS 750 F was observed in the dark control samples. The quantum yield of BAS 750 F was determined to be 3.5×10^{-1}. It may be concluded that aqueous photolysis could play a significant role in the overall environmental degradation of BAS 750 F.

CA 7.2.1.3 Indirect photochemical degradation

Due to significant degradation under direct aquatic photolysis, investigations into indirect photolysis were not deemed necessary.

CA 7.2.2 Route and rate of biological degradation in aquatic systems

CA 7.2.2.1 "Ready biodegradability"

Report:	CA 7.2.2.1/1 Schwarz H., 2014 a BAS 750 F - Determination of the ready biodegradability in the CO_2-evolution test 2014/1239574
Guidelines:	OECD 301 B, ISO 9439, EPA 835.3110, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part C.4
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive summary

The objective of the study was to determine the ready biodegradability of BAS 750 F in aerobic aqueous medium by measurement of the formed carbon dioxide.

Test assays were prepared with mineral medium, comprising two blank control assays, two test substance assays, one inhibition control test assay, and one reference substance assay. After a pre-incubation period of 48 h, the test vessels for the test substance assays and the inhibition control test assay were treated with the test substance to reach a concentration of 20 mg L^{-1}. Further, the test vessels for the inhibition control test assay and the reference substance assay were treated with aniline at a concentration of 20 mg L^{-1}. The pH values in the test vessels were adjusted to 7.4 ± 0.2, if necessary. Aliquots of activated sludge suspension were added to all test vessels at 30 mg L^{-1} dry weight and the test vessels were incubated at 22 ± 2°C. Aeration was performed with CO_2-free air and adsorption of evolved CO_2 was performed using NaOH traps.

The measured amount of carbon dioxide in the traps was compared with the calculated maximal theoretical production ($ThCO_2$) and indicated as biodegradation degree in percent.

The degree of biodegradation of the test substance at the end of exposure (mean value) was < 10% CO_2/$ThCO_2$, while it was 91% for the reference substance and 39% in the inhibition control test.

Hence, the required pass level for ready biodegradability within a ten-day window was not reached. The test substance can be evaluated as poorly biodegradable in this test.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	(2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.8 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$
Batch No.:	COD-001880
Purity:	98.6%

2. Test system

Municipal activated sludge from the wastewater treatment plant of Mannheim, Germany was used to determine the "Ready Biodegradability" of BAS 750 F. The inoculum was collected on 18 August 2014 from the aeration tank of the plant.

A suitable aliquot of the activated sludge suspension was sieved by a finely woven mesh with a mesh size about 1 mm. To reduce the content of inorganic carbon in the blank controls the activated sludge was aerated with carbon dioxide free air for about 48 hours at 22 ± 2°C.

B. STUDY DESIGN

1. Experimental conditions

At the day of exposure the activated sludge suspension was washed one time with drinking water. Subsequently, the aeration was stopped and the sludge was allowed to settle. After settling, the supernatant was discarded and the remaining sludge suspension was filled up with drinking water and the concentration of the sludge was adjusted to 6.0 g L^{-1} dry weight.
Aliquots of 7.5 mL were added to the test vessels to obtain an activated sludge concentration of 30 mg L^{-1} dry weight.

Test assays were prepared, comprising two blank control assays, two test substance assays, one inhibition control test assay, and one reference substance assay.

A mineral medium was prepared consisting of the following four solutions:

Solution A:	8.5 g KH_2PO_4 + 21.75 g K_2HPO_4 + 33.4 g Na_2HPO_4 x 2 H_2O + 0.5 g NH_4Cl in 1 L deionized water (pH value was adjusted to 7.4)
Solution B:	36.4 g $CaCl_2$ x 2 H_2O in 1 L deionized water
Solution C:	22.5 g $MgSO_4$ x 7 H_2O in 1 L deionized water
Solution D:	0.25 g in $FeCl_3$ x 6 H_2O in 1 L deionized water

15 mL solution A, 1.5 mL solution B, 1.5 mL solution C and 1.5 mL solution D was used for the preparation of the test assays, which were performed in 2 L incubation bottles filled up to a volume of 1.5 L with deionized water.

The incubation bottles were connected to two serial scrubbing bottles, containing 0.05 M NaOH solution for the adsorption of CO_2. The incubation bottles were stirred on magnetic stirrers; the aeration was performed with CO_2-free air.

Test assays were prepared, comprising two blank control assays, two test substance assays, one inhibition control test assay, and one reference substance assay.

For preparation of the test vessels with test substance and the inhibition control, the required amounts of the test substance were added to reach a concentration of 20 mg L^{-1} TOC (total organic carbon). The reference substance assay was treated with the reference item (aniline) to reach 20 mg TOC L^{-1} and 20 mg TOC L^{-1} in the inhibition control.

The pH values in the test vessels were measured and adjusted to 7.4 ± 0.2, if necessary. Aliquots of activated sludge suspension were added to all test vessels, to adjust the concentration of activated sludge to 30 mg L^{-1} dry weight. The test assays were stirred using magnetic stirrers.

At the end of exposure, the pH values were measured in each test vessel. For stripping of CO_2 dissolved in the test medium, each test vessel was acidified by adding 2 mL of concentrated HCl. The concentration of dissolved organic carbon (DOC) in the blank controls and reference substance assays were determined. Since the test substance was insufficiently soluble in water, no DOC measurements could be performed from the test assay of the inhibition control and from the test substance test assays.

The aeration was continued for about 24 hours and the released CO_2 amounts in both traps of each test vessel were determined and added to the calculated amount of the previous day.

2. Sampling

Two to three times per week the total inorganic carbon (TIC) values of the adsorption solutions of the first trap were determined and used for the calculation of the produced CO_2. After each sampling, the second trap was moved forward and the new trap with fresh NaOH solution was placed into the second position. Each trap was analyzed separately.

Samples for measurement of dissolved inorganic carbon (DIC; validity criterion) from the blank control assays were taken. For determination of the decrease of dissolved organic carbon (DOC), samples were taken from the test vessels of the blank control and from the test vessel of the reference substance control and the DOC content was determined after centrifugation.

3. Description of analytical procedures

The TIC value of the freshly prepared NaOH solution was determined and considered by the calculation of biogenic produced CO_2 amount.

The TIC and DOC analyses were performed as repeat determination, using a TOC analyzer equipped with an auto sampler. The system worked with a combustion/non-disperse infrared gas analysis method. The samples for TIC analysis (absorption solution) were measured without further treatment. The samples for the DOC analysis were centrifuged and analyzed on the day of sampling.

The measured amount of CO_2 at the end of the test was compared with the calculated maximal theoretical production ($ThCO_2$) and indicated as biodegradation degree in percent. As the microorganisms oxidize only a part of the test substance and incorporate the rest into biomass, a degradation level > 60% is considered as sufficient biodegradation. The relation of formed CO_2 to $ThCO_2$ is evaluated in accordance to the OECD Test Guideline 301B.

II. RESULTS AND DISCUSSION

The degree of biodegradation of the test substance at the end of exposure (mean value) was < 10% $CO_2/ThCO_2$, while it was 91% for the reference substance and 39% in the inhibition control test. A summary of the degrees of biodegradation is presented in Table 7.2.2.1-1.

Mean concentrations of DOC decreased from 1.4 to 1.0 mg L^{-1} in the control assays and from 19.6 to 1.2 mg L^{-1} in the reference substance assays, representing a decrease of 99% for the latter.

Table 7.2.2.1-1: Degree of biodegradation [% $CO_2/ThCO_2$]

Test duration [days]	Test assays				
	Reference substance	Inhibition control	Test substance 1	Test substance 2	Test substance mean
0	0	0	0	0	0
2	2	0	0	0	0
5	38	11	1	0	1
7	52	17	1	0	1
12	68	23	0	-1	-1
14	73	29	0	-1	-1
19	82	33	-1	-1	-1
21	86	35	-1	-1	-1
23	88	35	-2	-2	-2
27	90	36	-2	-1	-2
28	91	39	-4	0	-2

III. CONCLUSION

BAS 750 F was not readily biodegradable in this carbon dioxide evolution test based on the quantitative determination of the formed carbon dioxide in the test substance assays by comparison with the calculated maximal theoretical CO_2 production.

CA 7.2.2.2 Aerobic mineralisation in surface water

Report: CA 7.2.2.2/1
Michel A., 2015 a
14C-BAS 750F: aerobic mineralization in surface water
2015/1186902
Guidelines: OECD 309 (April 2004)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive summary

The aerobic mineralization and degradation rate of the fungicidal active substance BAS 750 F in an aquatic system under dark conditions was investigated. The test system chosen for this study was used to mimic the pelagic zone in natural water bodies. In addition to monitoring the mineralization and degradation rate of BAS 750 F, the enantiomeric ratio was observed throughout the study to determine if any enantiomer specific effects could be observed under the study conditions.

The test was performed with two different BAS 750 F concentrations (10 µg L^{-1} and 100 µg L^{-1}). Triazol-3(5)-^{14}C-labelled-BAS 750 F was used as test item. The test vessels were attached to a flow-through system for continuous aeration and incubated at a temperature of 20 ± 1°C in the dark. Samples for the experiment were taken at 0, 3, 7, 14, 21, 35 and 63 days after treatment.

The amount and nature of radioactivity in the water samples was determined by liquid scintillation counting (LSC) and radio-HPLC. The enantiomeric ratio was determined by using a chiral HPLC method. Potential volatiles were trapped in appropriate trapping solutions and also analyzed by LSC. Parent substance and metabolite identification was done by comparison of retention times with the corresponding reference items.

The obtained results show that BAS 750 F is degraded only very slowly in a pure water environment as provided in the pelagic test. More than 90% TAR (total applied radioactivity) was still detectable as unchanged parent in the water samples 63 days after treatment. The results of the sterile vessels were comparable to the viable test vessels. Metabolites were formed only in trace amounts. M750F003 reached maximum of 1.3% TAR in the high concentration test and 4.1% TAR in the low concentration test after 63 days. M750F006 reached maximum of 1.9% TAR and 4.8% TAR after 21 days, in the high concentration test and the low concentration test, respectively. All other peaks never exceeded 0.7% TAR. The ratio of the two enantiomers of BAS 750 F was approximately 1:1 in all analyzed samples indicating that no enantiomeric shift took place during incubation.

Overall, the degradation of BAS 750 F was characterized by a low mineralization rate irrespective of test concentration. The amount of $^{14}CO_2$ never exceeded 3.0% TAR within 63 days.

No kinetic evaluation of BAS 750 F degradation rates was performed since no significant degradation was observed in the pelagic test.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	(2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

Labelled test item:

Label:	Triazole-3(5)-C14
Batch No.:	1062-2201
Specific radioactivity of a.s.:	5.47 MBq mg^{-1}
Radiochemical purity:	99.4%, see certificate of analysis
Purity:	98.2%

2. Test system

Water was collected on May 29th, 2015 from Ranschgraben, a small stream east of Schifferstadt surrounded by a forest. The site is located in Rhineland-Palatinate, in the south-western part of Germany.

The water was filtered through a 0.2 mm sieve, directly at the field sampling site. A small amount of surface sediment was also collected, passed through a 2 mm sieve and dried at room temperature. Water and sediment were stored at 4°C under dark conditions until filling into the test vessels.

The test system was characterized with respect to various hydrological characteristics. pH, O_2 content, redox potential and temperature of the water as well as redox potential of the sediment were measured directly at the site of sampling. The physico-chemical properties of the system are summarized in Table 7.2.2.2-1.

Table 7.2.2.2-1: River water and sediment characteristics

Designation Origin		Ranschgraben Rhineland-Palatinate, Germany	
Parameters measured at sampling site			
Temperature	[°C]	13.6	
pH (water)	-	7.20	
Oxygen concentration	[mg L^{-1}]	9.0	
Redox potential water	[mV]	265	
Redox potential sediment	[mV]	-220	
Parameters measured at 0 day sample			
Temperature	[°C]	20.9; 20.4; 20.7; 20.9 [a]	
pH (water)	-	8.06; 8.01; 8.01; 8.02 [a]	
Oxygen concentration	[mg L^{-1}]	8.8;8.8; 8.8; 8.8 [a]	
Redox potential water	[mV]	190; 196; 196; 167 [a]	
Redox potential sediment	[mV]	n.p.	
Water parameters			
TOC (total organic carbon)	[mg L^{-1}]	4.9	
DOC (dissolved organic carbon)	[mg L^{-1}]	3.8	
Hardness	[mmol L^{-1}]	0.09	
Carbonate hardness	[mmol L^{-1}]	0.63	
Total N	[mg L^{-1}]	0.49	
Total P	[mg L^{-1}]	0.227	
Microbial plate count	[colony forming units mL^{-1}]	Beginning [b]	End [c]
Bacteria		4720	5080
Fungi		48	26
Actinomycetes		136	66

[a] Four test vessels for the 0 day sampling (two replicates of the low concentration test and two replicates of the high concentration test)
[b] Water sample on the day of sampling
[c] Untreated water sample at the end of the incubation period
n.p. = not performed

B. STUDY DESIGN

1. Experimental conditions

A total number of 49 test vessels was prepared for incubation. Each flask was filled with 400 mL of water. In order to provide a minimum of mineral and nutrient source for the microbial population, a small concentration of suspended solids was added to the water. For this, the dried sediment was grinded in an analytical mill and 0.508 g were suspended in 6 mL of water. 50 µL of the obtained suspension were pipetted into each test vessel to reach a concentration of sediment solids of about 0.01 g L^{-1}. The test system can still be considered as pelagic. Table 7.2.2.2-2 illustrates the experimental setup. To provide sterile controls, six test vessels were sterilized in an autoclave (20 min at 120°C).

Table 7.2.2.2-2: Experimental setup

System	Test conc. [µg L^{-1}]	No. of test vessels
Test vessels		
triazole-3(5)-^{14}C-labelled-BAS 750 F	10	18
	100	18
Controls		
^{14}C-benzoic acid	10	4
untreated*	-	3
sterile - triazole-3(5)-^{14}C-labelled-BAS 750 F	100	6

* for water characterization

The test vessels were placed on multiple magnetic stirrers and incubated at 20 ± 1°C in a metabolism chamber providing the test vessels with a continuous flow of fresh air. A glass rod with an encapsulated small magnetic bar hanging from the test vessel screw cap slightly agitated the upper 1-2 cm water layer to keep the oxygen saturation at a sufficient level.

Application

The nominal application rates of the test item were 10 µg L^{-1} and 100 µg L^{-1} for the low and the high application rate, respectively. This was achieved by pipetting 25 µL of the corresponding application solutions into the upper water layer of the test vessels. The nominal application rate for viability control (^{14}C-benzoic acid treated vessels) was 10 µg L^{-1}. This rate was attained by treating four test vessels with 185µL of the ^{14}C-benzoic acid application solution. The sterilized test vessels were treated under sterile conditions to reach a nominal application rate of 100 µg L^{-1}.

After application of the test item, each test vessel was connected to the air stream leading to a trapping system of two gas washing bottles containing different trapping solutions for adsorption of potentially occurring ^{14}C-volatiles. The first flask was filled with about 25 mL ethylene glycol and the second flask with about 45 mL 1 M NaOH (amended with a colored pH indicator).

The sterilized test vessels were kept closed and were not connected to the air flow system. They were only opened for a short period to be treated with test item under sterile conditions.

The test vessels treated with ^{14}C-benzoic acid were connected to a trapping system consisting of two volatile traps filled with 1 M NaOH (45 mL). After each sampling, the volatile traps were replaced by new traps containing fresh solutions.

The untreated control vessels were only connected to one volatile trap filled with ethylene glycol. One untreated control sample was used for microbial plate count at the beginning of the experiment. Two untreated control samples were used for system characterization at the end of the experiment.

2. Sampling

Test vessels were sampled at 0, 3, 7, 14, 21, 35 and 63 days after treatment for both test variants (high and low concentration). The volatile traps collected at each sampling time were disconnected from the air stream and stored at room temperature until measurement. The sterile vessels (only high concentration test) were sampled after 21 and 63 days.

Two untreated control vessels for system characterization were sampled after 63 days. The parameters temperature, O_2 content, pH and redox potential of the water were recorded. The water from both vessels was combined and the sample was prepared for microbial plate counts.

Test vessels treated with ^{14}C-benzoic acid were sampled at 3, 8, 15, 22, 36 and 64 days after treatment. The NaOH traps of test vessels treated with ^{14}C-benzoic acid were sampled at 3, 8, 15, 22, 36 and 64 days after treatment.

3. Description of analytical procedures

At each sampling date, the respective flasks were removed from the incubator. The parameters temperature, O_2 content, pH and redox potential of the water were measured in all water sample types. The water was then transferred into graduated cylinders and the test vessels were rinsed with approx. 10 mL of ACN. The ACN was added to the corresponding sample and the total volume was determined. For determination of the ^{14}C-concentration, three 1 mL aliquots were measured by LSC. A 25 mL aliquot of water was then filtered through a 70 µm filter and an aliquot of the filtrate was analyzed by HPLC.

For water sampling of the test vessels treated with ^{14}C-benzoic acid, the test vessels were disconnected from the air flow system and the water volume in the test vessels were determined by weighing. Three 1 mL aliquots were taken per test vessel for LSC measurements. On day 15 and day 64, an aliquot of the water phase was subjected to HPLC analysis without further workup. The vessels were then reconnected to the air flow system.

Trapping solutions were transferred into 50 mL volumetric flasks filled to volume with distilled water. Three 1 mL aliquots were measured by LSC.

4. Kinetic modeling

No kinetic evaluation of the recorded data was performed because no significant degradation of BAS 750 F was observed in the pelagic test.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

The material balance for the pelagic test ranged from 93.6% TAR to 102.7% TAR. In the sterile vessels, the material balance ranged from 96.7 to 98.8% TAR. The material balance and the distribution of radioactivity in the pelagic test for high and low concentration tests are shown in Table 7.2.2.2-3.

Table 7.2.2.2-3: Material balance and distribution of radioactivity after application of triazole3(5)-^{14}C-labeled-BAS 750 F to the pelagic test system Ranschgraben and incubation under dark conditions [% TAR]

Days after treatment	Water	Volatiles Ethylene Glycol trap	Volatiles NaOH trap	Material balance
High concentration (100 µg L^{-1})				
0 I	99.8	n.p.	n.p.	99.8
0 II	100.2	n.p.	n.p.	100.2
0 mean	**100.0**	**n.p.**	**n.p.**	**100.0**
3 I	98.4	0.0	0.0	98.4
3 II	100.8	0.0	0.0	100.8
3 mean	**99.6**	**0.0**	**0.0**	**99.6**
7 I	97.1	0.0	0.0	97.1
7 II	98.3	0.0	0.0	98.3
7 mean	**97.7**	**0.0**	**0.0**	**97.7**
14 I	98.9	0.0	0.0	98.9
14 II	98.6	0.0	0.0	98.6
14 mean	**98.8**	**0.0**	**0.0**	**98.8**
21 I	98.7	0.0	0.0	98.7
21 II	96.2	0.0	0.0	96.2
21 mean	**97.4**	**0.0**	**0.0**	**97.5**
35 I	97.3	0.0	0.1	97.3
35 II	93.5	0.0	0.0	93.6
35 mean	**95.4**	**0.0**	**0.1**	**95.4**
63 I	97.6	0.0	0.1	97.7
63 II	94.0	0.0	0.1	94.1
63 mean	**95.8**	**0.0**	**0.1**	**95.9**
21 I (sterile)	96.7	n.p.	n.p.	96.7
21 II (sterile)	98.8	n.p.	n.p.	98.8
21 mean (sterile)	**97.8**	**n.p.**	**n.p.**	**97.8**
63 I (sterile)	98.4	n.p.	n.p.	98.4
63 II (sterile)	98.7	n.p.	n.p.	98.7
63 mean (sterile)	**98.6**	**n.p.**	**n.p.**	**98.6**

Table 7.2.2.2-3: Material balance and distribution of radioactivity after application of triazole3(5)-^{14}C-labeled-BAS 750 F to the pelagic test system Ranschgraben and incubation under dark conditions [% TAR]

Days after treatment	Water	Volatiles Ethylene Glycol trap	Volatiles NaOH trap	Material balance
Low concentration (10 µg L^{-1})				
0 I	100.2	n.p.	n.p.	100.2
0 II	99.8	n.p.	n.p.	99.8
0 mean	**100.0**	**n.p.**	**n.p.**	**100.0**
3 I	100.1	0.0	0.0	100.1
3 II	98.7	0.0	0.0	98.8
3 mean	**99.4**	**0.0**	**0.0**	**99.4**
7 I	98.6	0.0	0.0	98.6
7 II	95.1	0.0	0.0	95.2
7 mean	**96.9**	**0.0**	**0.0**	**96.9**
14 I	97.4	0.0	0.0	97.5
14 II	96.7	0.0	0.0	96.7
14 mean	**97.1**	**0.0**	**0.0**	**97.1**
21 I	97.8	0.0	0.0	97.8
21 II	95.0	0.0	0.0	95.0
21 mean	**96.4**	**0.0**	**0.0**	**96.4**
35 I	95.0	0.0	0.1	95.1
35 II	98.7	0.0	0.1	98.7
35 mean	**96.9**	**0.0**	**0.1**	**96.9**
63 I	99.8	0.1	2.9	102.7
63 II	93.7	0.0	0.1	93.8
63 mean	**96.7**	**0.1**	**1.5**	**98.3**

n.p. = not performed
TAR = total applied radioactivity

In general, no significant differences were found in BAS 750 F behavior between the high and the low test concentrations.

The amount of radioactivity in the water ranged from 93.7% TAR to 99.8% TAR after 63 days. For all pelagic test samples and sampling time points, the radioactivity in the volatile traps never exceeded 3.0% TAR indicating a low rate of mineralization.

The control vessels treated with ^{14}C-benzoic acid showed that the system Ranschgraben was microbially active. After 64 days, 60.5 - 90.1% TAR were evolved as $^{14}CO_2$. The average material balance over the four replicates ranged from 80.5% to and 90.2% TAR.

B. TRANSFORMATION OF PARENT COMPOUND

Physicochemical parameters of the test systems

During the incubation with ^{14}C-BAS 750 F, the O_2 saturation in the water of system was always > 65%.The redox potential in the water ranged from +146 to + 275 mV demonstrating overall aerobic conditions. The pH values were measured in the range 7.01 to 8.41.

Characterization and identification of residues in water

In the pelagic test, no significant degradation of BAS 750 F was observed. After 63 days, between 90.2% TAR and 94.8% TAR could still be recovered as unchanged parent for the different concentrations. An overview of active ingredient and metabolites for the water samples is presented in Table 7.2.2.2-4.

Two peaks were detected in very low amounts. They were assigned to the metabolites M750F003 and M750F006 by comparison of the retention times with those of reference substances. The metabolite M750F003 reached maxima of 1.3% TAR and 4.1% TAR after 63 days in the high concentration test and the low concentration test, respectively. M750F006 reached maxima of 1.9% TAR and 4.8% TAR after 21 days, in the high concentration test and the low concentration test, respectively. Additionally, two peaks were detected in the HPLC chromatograms in minor amounts. They never exceeded 0.7% TAR and were therefore not further investigated.

The sterile controls showed in principle the same results as the viable test vessels. After 63 days, 98.6% TAR could be attributed to BAS 750 F. No other metabolites were detected at the end of the incubation.

Enantiomer specific analyses

In addition to the quantification of the parent, enantiomer-specific analyses were performed. The obtained ratios for BAS 750 F (*R*) and BAS 750 F (*S*) was about 1:1 for all analyzed samples (high concentration). This near constant ratio demonstrates that under the test conditions no chiral inversion nor chiral differentiation in degradation was observed.

Degradation of the reference test item

No kinetic evaluation of BAS 750 F degradation rates was performed since no significant degradation was observed under the applied test conditions.

Table 7.2.2.2-4: Metabolite overview for water after application of triazole3(5)-^{14}C-labeled-BAS 750 F to the pelagic test system Ranschgraben and incubation under dark conditions [% TAR]

Days after treatment	Total t_R ~	M750F003 25.3'	M750F006 32.9'	BAS 750 F 35.3'	Others*
High concentration (100 µg L^{-1})					
0 I	99.8	n.d.	1.2	98.4	0.2
0 II	100.2	n.d.	1.2	98.9	0.2
0 mean	**100.0**	**n.d.**	**1.2**	**98.6**	0.2
3 I	98.4	n.d.	1.3	96.9	0.2
3 II	100.8	n.d.	1.3	99.5	n.d.
3 mean	**99.6**	**n.d.**	**1.3**	**98.2**	0.1
7 I	97.1	0.3	0.9	95.7	0.2
7 II	98.3	0.3	1.1	96.8	0.1
7 mean	**97.7**	**0.3**	**1.0**	**96.2**	0.1
14 I	98.9	0.4	1.0	97.2	0.2
14 II	98.6	0.8	1.7	96.2	n.d.
14 mean	**98.8**	**0.6**	**1.4**	**96.7**	0.1
21 I	98.7	0.7	1.5	96.3	0.2
21 II	96.2	0.9	2.3	93.0	n.d.
21 mean	**97.4**	**0.8**	**1.9**	**94.7**	0.1
35 I	97.3	1.0	1.2	94.9	0.3
35 II	93.5	1.4	1.4	90.4	0.3
35 mean	**95.4**	**1.2**	**1.3**	**92.7**	0.3
63 I	97.6	1.4	0.8	94.8	0.6
63 II	94.0	1.2	0.8	91.9	n.d.
63 mean	**95.8**	**1.3**	**0.8**	**93.3**	0.3
21 I (sterile)	96.7	n.d.	1.1	95.6	n.d.
21 II (sterile)	98.8	n.d.	0.9	97.9	n.d.
21 mean (sterile)	**97.8**	**n.d.**	**1.0**	**96.8**	**n.d.**
63 I (sterile)	98.4	n.d.	n.d.	98.4	n.d.
63 II (sterile)	98.7	n.d.	n.d.	98.7	n.d.
63 mean (sterile)	**98.6**	**n.d.**	**n.d.**	**98.6**	**n.d.**
Low concentration (10 µg L^{-1})					
0 I	100.2	n.d.	1.1	99.1	n.d.
0 II	99.8	n.d.	1.3	98.5	n.d.
0 mean	**100.0**	**n.d.**	**1.2**	**98.8**	**n.d.**
3 I	100.1	n.d.	n.d.	100.1	n.d.
3 II	98.7	n.d.	n.d.	98.7	n.d.
3 mean	**99.4**	**n.d.**	**n.d.**	**99.4**	**n.d.**
7 I	98.6	n.d.	1.8	96.2	0.5
7 II	95.1	n.d.	2.1	93.0	n.d.
7 mean	**96.9**	**n.d.**	**2.0**	**94.6**	**0.3**
14 I	97.4	1.2	1.6	94.6	n.d.
14 II	96.7	0.5	1.6	94.1	0.6
14 mean	**97.1**	**0.9**	**1.6**	**94.4**	**0.3**
21 I	97.8	4.3	3.7	89.8	n.d.
21 II	95.0	2.4	5.9	86.6	n.d.
21 mean	**96.4**	**3.4**	**4.8**	**88.2**	**n.d.**
35 I	95.0	3.9	3.0	87.3	0.8
35 II	98.7	3.5	2.2	93.0	n.d.
35 mean	**96.9**	**3.7**	**2.6**	**90.1**	**0.4**

Table 7.2.2.2-4: Metabolite overview for water after application of triazole3(5)-^{14}C-labeled-BAS 750 F to the pelagic test system Ranschgraben and incubation under dark conditions [% TAR]

Days after treatment	Total t_R ~	M750F003 25.3'	M750F006 32.9'	BAS 750 F 35.3'	Others*
63 I	99.8	4.7	2.2	92.8	n.d.
63 II	93.7	3.5	n.d.	90.2	n.d.
63 mean	**96.7**	**4.1**	**1.1**	**91.5**	**n.d.**

t_R = retention time
n.d. = not detected
* = sum of several peaks (each individual peak <0.7% TAR)

III. CONCLUSION

From the obtained results, it can be concluded that BAS 750 F is not significantly degraded in a pure water environment as provided in the pelagic test. After 63 days more than 90% TAR was recovered as unchanged active substance.

No kinetic evaluation of BAS 750 F degradation rates was performed since no significant degradation was observed under the applied test conditions.

CA 7.2.2.3 Water/sediment studies

Report: CA 7.2.2.3/1
Ebert D.,Dalkmann P., 2015 a
Aerobic aquatic metabolism of BAS 750 F (Reg.No. 5834378)
2015/1000941
Guidelines: OECD 308, EPA 835.4300
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive summary

The degradation of ^{14}C-BAS 750 F was investigated in two aerobic water/sediment systems under dark conditions. One system was taken from a pond like side-arm of a river (Berghäuser Altrhein), the second system was taken from a small stream (Ranschgraben) surrounded by a forest. Each of the systems was treated separately with chlorophenyl-U-^{14}C-, triazole-3(5)-^{14}C-, and trifluoromethylphenyl-ring-U-^{14}C-labelled BAS 750 F. About 11 µg of ^{14}C-BAS 750 F was applied to each of the test vessels containing about 300 mL water. This corresponds to a field application rate of about 300 g BAS 750 F per hectare when assuming overspray over a 1 m deep water body. The influence of microbial activity was tested during the experiment by applying the test substance to sterilised vessels.

The test vessels were attached to a flow-through system for continuous aeration and incubated at a temperature of 20 ± 1°C in the dark.

Samples for the chlorophenyl- and triazole-label experiments were taken at 0, 0.25, 1, 3, 7, 14, 30, 56, 78 and 100 days after treatment. The sterile vessels were worked up after 101 days. Samples treated with the trifluoromethylphenyl-labelled test items were worked up at 0, 3, 7, 14, 28, 56, 78 and 100 days after treatment. No sterile vessels were incubated for this label.

Water and sediment were worked up separately. Water samples and sediment extracts were analysed by radio-HPLC. The amount of non-extractable residues was determined by combustion and liquid scintillation counting (LSC). Volatiles were trapped in appropriate trapping solutions and also analysed by LSC. Metabolites were identified by comparison of retention times to those of reference items and/or mass spectrometry. Chiral analysis was applied to selected samples in order to check a potential enantiomeric shift during incubation.

The total radioactivity in the water decreased from initially 92.1% - 100.1% of the total applied radioactivity (TAR) to 3.2% - 16.8% TAR after 100 days. Correspondingly, the radioactivity in the sediment increased in both systems reaching 75.1% - 91.8% TAR at the end of the incubation. About 50 to 72% of the radioactivity in sediment was still extractable with acetonitrile and acetonitrile/water.

After 100 days, BAS 750 F was found in the water phase at levels of 0.7% - 3.2% TAR. Metabolite M750F001 (1,2,4-triazole) was detected in water samples treated with the triazole-labelled test item reaching a maximum concentration of 10.2% TAR at the end of incubation. Metabolite M750F003 was detected in water samples treated with the triazole- and trifluoromethylphenyl-labelled test item reaching a maximum of 3.8% and 3.1% TAR, respectively. All other metabolites never exceeded 2.2% TAR at any sampling time.

The analyses of sediment extracts showed that BAS 750 F reached its highest amount in sediment after 14 or 28/30 days with 67.6% - 75.7% TAR. It slowly declined to 45.6% - 67.3% TAR after 100 days. Besides several unknown metabolites (all ≤ 2.8% TAR at any sampling date), M750F001 was detected at a maximum amount of 4.9% TAR and M750F003 reached up to 5.4% TAR after 100 days. A shift in the BAS 750 F-enantiomer ratio over time could not be observed, neither in water samples nor in sediment extracts.

The non-extractable residues in sediment slowly increased to amounts of 17.0% - 26.6% TAR. They were further characterised in selected sediment samples by humic substance fractionation. About one-third to one-half of the bound radioactivity could be released by harsh NaOH extraction. The rest was still bound to soil matrix obviously tightly incorporated into the insoluble humins.

Differences in the distribution of radioactivity over the different humic fractions were more related to the test system than to the position of the radiolabel. After fractionation of the NaOH extract into fulvic acids and humic acids, about one-half to three quarters were found in the fulvic acids and one-half to one quarter in the humic acids of test system Berghäuser Altrhein. In test system Ranschgraben, about one-thirds to one-half were found in the fulvic acids and one-half to two-thirds in the humic acids.

The mineralisation rate was rather low in both systems and reached 5.1 to 9.6% TAR within 100 days with the chlorophenyl-labelled test item. For the triazole- and the trifluoromethylphenyl-labelled test item, almost no mineralisation was detected (≤ 1.5% TAR).

The sterilised test vessels showed slightly higher BAS 750 F-water concentrations (3.9% - 7.5% TAR) at the end of incubation than the viable vessels. Concentrations of BAS 750 F in the pooled sediment extracts were considerably higher for the sterilised samples (78.9% - 80.5% TAR) than for the viable samples (45.6% - 64.4% TAR). Nearly all radioactivity recovered in the water phases or sediment extracts consisted of unchanged parent. The non-extractable residues in the sterilised test vessels were significantly lower (8.5% - 10.1% TAR) than those of the biological active incubations (17.0% - 26.6% TAR) indicating that degradation of BAS 750 F in sediment by incorporation into the humic substance matrix is enhanced in the presence of an active microbial population.

Kinetic analysis and calculations of DT_{50} and DT_{90} values for BAS 750 F were performed following the recommendations of the FOCUS Kinetics workgroup in order to derive persistence and modelling aquatic degradation endpoints. The analysis was done by non-linear regression methods using the KinGUI software.

Metabolite M750F001 (1,2,4-triazole) exceeded 5% TAR in water samples. However, it increased until the end of incubation and no kinetic evaluation was attempted.

For the parent compound, best-fit DT_{50} values in the water phase were 1.7 and 1.3 days for the systems Berghäuser Altrhein and Ranschgraben, respectively. In the sediment, parent DT_{50} values were determined to be 225 and 396 days. For the total system, DT_{50} values of 122 and 213 days were calculated for the respective test system.

Overall, it can be concluded that BAS 750 F dissipates at a fast rate from the water phase and degrades at a moderate rate in the sediment when incubated in water/sediment systems under dark conditions.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material

The test item BAS 750 F was used in three ^{14}C-labeled forms.

Internal code:	BAS 750 F
Reg. No.:	5834378
CAS No.:	1417782-03-6
Chemical name (IUPAC):	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
Molecular mass:	397.78 g mol^{-1}
Molecular formula:	$C_{18}H_{15}ClF_3N_3O_2$

1. Triazole-3(5)-^{14}C-label

Batch No.:	1062-2001
Specific radioactivity of a.s.:	5.46 MBq mg^{-1}
Radiochemical purity:	98.8%, see certificate of analysis in the final report
Purity:	98.9%

2. Chlorophenyl ^{14}C-label

Batch No.:	CFQ41561
Specific radioactivity of a.s.:	7.878 MBq mg^{-1}
Radiochemical purity:	98.9%, see certificate of analysis in the final report
Purity:	99.1%

3. Trifluoromethylphenyl-ring-U-^{14}C-label

Batch No.:	CFQ42039
Specific radioactivity of a.s.:	8.288 MBq mg^{-1}
Radiochemical purity:	98.3%, see certificate of analysis in the final report
Purity:	96.3%

2. Test system

Two natural water/sediment systems were collected on November 15, 2013 (for the chlorophenyl- and triazole-label experiments), and May 21, 2014 (for the trifluoromethylphenyl-label experiment). Both sampling sites are located in the South-Western part of Germany. One system is designated as “Berghäuser Altrhein” (BA), a pond-like side arm of the river Rhine south of Speyer surrounded by a forest. The second system is designated as “Ranschgraben” (RG), a small stream east of Schifferstadt surrounded by a forest.

The sediment was passed through a 2 mm sieve, and the water was filtered through a 0.2 mm filter. Water and sediment were filled into the test vessels 1 – 3 days after collection from the field sites. The physico-chemical properties of the systems are summarized in Table 7.2.2.3-1.

Table 7.2.2.3-1: Characterization of the water/sediment systems

Water			**Berghäuser Altrhein**		**Ranschgraben**	
Field sampling			**Nov 15, 2013**	**May 21, 2014**	**Nov 15, 2013**	**May 21, 2014**
Temperature [a]	[°C]		9.1	22.9	6.7	16.6
pH [a]			7.4	8.40	7.30	7.10
Redox potential [a]	[mV]		281	263	273	219
O_2 content [a]	[mg L^{-1}]		7.4	21.7	9.9	8.2
Total N	[mg L^{-1}]	beginning	0.79	0.30	0.84	0.30
		end	1.02	0.90	1.82	1.72
Total P	[mg L^{-1}]	beginning	0.09	0.06	0.13	0.22
		end	1.07	0.29	0.73	0.27
TOC / org. C	[mg L^{-1}]	beginning	5.6	3.0	6.0	4.6
		end	10.0	6.7	6.1	4.7
Water hardness	[mmol L^{-1}]		1.62	2.72	1.44	0.90
Bacteria	[cfu mL^{-1}]	beginning	3.76×10^3	3.36×10^2	5.58×10^3	3.56×10^2
		end	5.76×10^3	1.78×10^3	6.24×10^3	3.0×10^2
Fungi	[cfu mL^{-1}]	beginning	14	0	8	0
		end	4	26	0	0
Actinomycetes	[cfu mL^{-1}]	beginning	0	4	10	0
		end	10	22	0	2

Sediment			**Berghäuser Altrhein**				**Ranschgraben**			
Batch No.			**13/1725/01 14/1725/01**		**14/1725/02 14/1725/03**		**13/1723/01 14/1723/01**		**14/1723/02 14/1723/03**	
Sampling depth*	[cm]		0 - 20		0 – 10		0 - 10		0 - 10	
pH	(H_2O)		7.9		7.2		5.5		6.4	
	($CaCl_2$)		7.1		7.0		5.2		6.0	
Redox potential*	[mV]		-134		-407		-402		-322	
Total N	[mg kg^{-1}]	beginning	1900		5000		2100		1300	
		end	1700		4600		1800		1300	
Total P	[mg kg^{-1}]	beginning	790		815		632		352	
		end	503		815		235		317	
TOC / org. C	[%]	beginning	1.80		6.27		2.94		2.00	
		end	1.79		6.10		2.79		1.72	
CEC	[$cmol^+ kg^{-1}$]		17.7		33.3		7.6		4.5	
Particle size distribution			USDA	DIN 4220	USDA	DIN 4220	USDA	DIN 4220	USDA	DIN 4220
Clay	[%]		27.7	22.7	31.8	31.8	4.6	4.6	4.1	4.1
Silt	[%]		55.0	57.0	54.7	55.0	6.5	7.7	6.0	6.7
Sand	[%]		22.3	20.3	13.5	13.2	88.9	87.8	89.9	89.2
Soil type			silty loam	Silt loam	silty clay loam	silty clay	fine sand	sand	fine sand	sand
Bacteria	[cfu g^{-1}]	beginning	4.46×10^6		4.26×10^6		9.6×10^5		4.58×10^5	
		end	9.1×10^5		3.98×10^5		5.02×10^5		5.48×10^5	
Fungi	[cfu g^{-1}]	beginning	3.1×10^4		3.94×10^4		2.22×10^3		7.2×10^2	
		end	4.32×10^3		1.6×10^3		6.2×10^2		2.8×10^4	
Actinomycetes	[cfu g^{-1}]	beginning	9.6×10^3		6.4×10^2		3.2×10^3		1.68×10^3	
		end	1.68×10^4		8.4×10^3		3.4×10^3		8.2×10^3	

CEC = Cation exchange capacity
TOC = Total organic carbon
[a] measured directly at sampling site

B. STUDY DESIGN

1. Experimental conditions

A total of 49 flasks were prepared for each water/sediment system and radiolabel: 13 flasks per radiolabel (10 sampling + 3 reserve samples) + 1 flask per radiolabel for incubation under sterile conditions. In addition, 7 untreated flasks were prepared for system characterization at the end of the incubation.

The flasks were filled with either about 185 g (BA) or about 140 g (RG) of wet sediment and 300 mL of the respective water. This corresponded to a sediment layer of about 2.5 cm and a water layer of about 7 cm. After being filled with sediment and water, the flasks were allowed to equilibrate for 15-19 days under dark conditions.

Appropriate amounts (20 µL) of the respective application solutions (prepared in acetonitrile) were pipetted to the water surface to achieve a nominal amount of about 11 µg test item per test vessel. This corresponded to a field application rate of about 300 g active substance per ha assuming overspray over a 1 m deep water body. The amount of test item per test vessel was calculated for a 300 mL water volume.

During incubation the test vessels were continuously aerated the upper water layer was slightly agitated to keep the oxygen saturation on sufficient high level. Each test vessel was connected to a volatile trapping system of two gas washing flasks containing different trapping solutions for potential ^{14}C-volatiles (ethylene glycol, 0.5 M NaOH).

Equilibration and subsequent incubation was carried out in an incubator at a temperature of 20 ± 1 °C in the dark.

The equilibration was monitored by measuring redox potential of water and sediment, temperature, O_2-content and pH of randomly selected flasks at intervals of a few days. After treatment, the same parameters were measured in each sample before workup.

2. Sampling

Samples were taken at 0, 0.25, 1, 3, 7, 14, 30, 56, 78 and 100 days after treatment (DAT) for the chlorophenyl- and triazole-label experiments and at 0, 3, 7, 14, 28, 56, 78, 100 days after treatment for the trifluoromethylphenyl-label experiment. After 101 days, the sterile vessels were worked up (chlorophenyl and triazole label only). The results obtained with the chlorophenyl- and triazole-labelled test item can be considered as duplicates for test item and common metabolites. As the experiment with the trifluoromethylphenyl-labelled test item was conducted at a later date, duplicate measurements were performed exemplarily at selected dates (0, 56 and 100 DAT).

3. Description of analytical procedures

Water
The water was decanted from the test vessels and its volume determined. The samples were homogenized by shaking in a round bottom flask, aliquots were measured by LSC and radio-HPLC.

Sediment
For extraction, the sediment samples of the incubation experiment were transferred into centrifuge tubes, the extraction solvent added and the centrifuge tubes placed on a rotary shaker. After each extraction step, the phases were separated by centrifugation. The extracts were collected in volumetric flasks and analyzed for radioactivity by LSC.

The sediment samples were extracted in a first step with acetonitrile, then with acetonitrile/water (50/50, v/v), and then twice with pure acetonitrile. Since there was still a considerable amount of water left in the sediments after water decantation, the first extraction with pure acetonitrile is considered as an acetonitrile/water extraction. In the case that the fourth extraction contained more than 3% of the total applied radioactivity (TAR), a fifth extraction with acetonitrile was added. For one test vessel (Ranschgraben, chlorophenyl-label, 100 days), a third extraction step with 95 mL of acetonitrile/water (50/50, v/v) was performed.

The four or five corresponding acetonitrile and acetonitrile/water extracts were combined and each volumetric flask was rinsed with acetonitrile or acetonitrile/water which was also added to the extracts. For HPLC analysis, aliquots of the pooled extracts were evaporated to dryness and redissolved in acetonitrile/water (80/20, v/v). After ultrasonication and centrifugation, aliquots were checked for recovery with LSC (sample recovery was always > 92%) and then analysed by radio-HPLC.

The extracted sediment was dried at room temperature and homogenized using an analytical mill. The amount of non-extractable radioactive residues (NER) in the extracted sediment was determined by combustion of aliquots in an oxidizer.

The limit of quantification (LOQ) was calculated and amounted to 0.218% TAR in the water phase and 0.073% TAR in the sediment phase. The corresponding limits of detection (LOD) were calculated as 0.145 and 0.048% TAR.

Characterization of bound residues
The non-extractable radioactivity in sediment was further characterized in selected samples (28 day sample of trifluoromethylphenyl label, 30 day sample of chlorophenyl- and triazole-label, and 100 day samples for all three labels) by separation into fulvic acids, humic acids and humins.

For each sample, the dried sediment (25 g) was extracted three times with 0.5 M NaOH. The radioactivity in the extracts of each extraction step was determined by LSC.

The corresponding extracts of a sample were then combined and acidified with conc. HCl to pH 1-2 to precipitate the acid-insoluble humic acids. For the precipitation phase, the samples were kept in a refrigerator. After centrifugation, the radioactivity in the supernatant (fulvic acids) was determined by LSC.

The precipitate (humic acids) was redissolved in 0.5 M NaOH and the solution measured for radioactivity. The remaining non-extractable radioactivity in the sediment (humins) was determined by combustion.

The fulvic acid fraction of four selected samples (100 days, both systems, chlorophenyl and triazole label) was further investigated by HPLC.

Volatiles
Radioactivity in the volatile trapping solutions (ethylene glycol and NaOH) was determined by LSC.

4. Kinetic evaluation

Kinetic evaluation was performed in order to derive degradation parameters as persistence endpoints as well as modeling endpoints. Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006*].

The software package KinGUI (version 2.2014.224.1704) was used for parameter fitting [*SCHÄFER et al. (2007); WITT et al. (2014)*]. The error tolerance and the number of iterations of the optimization tool were set to 0.00001 and 100, respectively.

Kinetic evaluation at Level P-I (one-compartment approach) was performed for BAS 750 F degradation in the total system as well as dissipation from the water and sediment phase of the test systems. At Level P-II (two-compartment approach: water and sediment), the kinetic analysis considered the degradation in water and sediment and the partitioning between both phases.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested in order to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO), the Gustafson-Holden model (FOMC), and bi-exponential (DFOP) kinetics are already implemented in KinGUI. At Level P-I, persistence endpoints were derived from the kinetic models that provided the best fit to the measured data. The Goodness-of-fit was evaluated by visual assessment, χ^2 minimum error, and type-I-error rate (t-test) [for details see Chapter 6.3 in *FOCUS (2006)*]. Modeling endpoints were derived preferably from the SFO model.

The results obtained with the three differently labeled test items were pooled and regarded as replicates for kinetic evaluation. At Level P-I and P-II of the analysis, the kinetic evaluation started on the day of treatment (i.e. 0 DAT). The initial concentration of the test substance in the total system or in water was set to the material balance recovered at 0 DAT as recommended by FOCUS [*FOCUS (2006)*]. Accordingly, the initial concentration in the sediment phase was assumed to be zero at Level P-II. The assessment of dissipation in sediment at Level P-I requires kinetics to be fitted to the corresponding decline data, starting from the maximum observed concentration in the compartment. The dissipation of the test item was thus evaluated starting at the day of maximum occurrence that was defined as 0 days after maximum concentration (0 DAMC). All later time points were adjusted accordingly as days after maximum concentrations (DAMC).

II. RESULTS AND DISCUSSION

A. MASS BALANCE

The material balance in the test vessels ranged from 90.6% to 98.5% TAR (chlorophenyl-label), from 88.5% to 99.8% TAR (triazole-label), and from 93.5% to 100.2% TAR (trifluoromethylphenyl-label). One test vessel of the trifluoromethylphenyl-label exhibited a lower material balance, amounting to 82.0% TAR. Results of the distribution of radioactivity are presented in Table 7.2.2.3-2 and Table 7.2.2.3-3.

Chlorophenyl label
Corresponding to the decline of radioactivity in the water phase the radioactivity in the sediment increased in both systems, reaching 81.9% and 88.2% TAR at 30 and 78 DAT in the systems BA and RG, respectively. At the end of incubation, the radioactivity in the sediment phase decreased to 75.1% and 84.4% TAR for the respective test system. 49.8% and 65.3% of the applied radioactivity (systems BA and RG, respectively) in the sediment was still extractable with acetonitrile and acetonitrile/water. The non-extractable radioactivity (NER) amounted to 25.3% and 19.1% TAR at the end of the incubation period, while 9.6% and 5.1% TAR were mineralized.

Triazole label
Similar to the chlorophenyl-labeled test item, the radioactivity in the water phase declined during the incubation period resulting in increasing amounts of radioactivity in the sediment phase reaching 81.5% and 87.3% TAR at 30 and 56 DAT for the test systems BA and RG, respectively. Until study end, the radioactivity related to the sediment phase decreased to 78.9% and 86.5% TAR for the respective test systems. After 100 days of incubation, 26.6% and 17.0% TAR were related to the fraction of NER, while only small amounts (0.8% and 0.5% TAR) were degraded to $^{14}CO_2$ in test system BA and RG, respectively.

Trifluoromethylphenyl label
Similar to the chlorophenyl-and triazole-labeled test item, the radioactivity in the water phase declined during the incubation period resulting in increasing amounts of radioactivity in the sediment phase reaching 92.9% and 91.8% TAR at 78 and 100 DAT for the test systems BA and RG, respectively. After 100 days of incubation, about 18.8% and 22.4% TAR were related to the fraction of NER, while only small amounts (1.5% and 0.5% TAR) were degraded to $^{14}CO_2$ in test system BA and RG, respectively.

Table 7.2.2.3-2: Material balance and distribution of radioactivity after application of ^{14}C-labeled BAS 750 F to water/sediment system Berghäuser Altrhein and incubation under dark conditions [% TAR]

days after treatment	vessel	[% TAR]												
		water	sediment									volatiles		material balance
			ACN/H_2O 1	ACN/H_2O 2	ACN/H_2O 3	ACN 1	CAN 2	CAN 3	total extractable	NER	total	ethylene-glycol	NaOH (CO_2)	
Berghäuser Altrhein; chlorophenyl-label														
0	VG01	92.1	2.5	0.8	n.p.	0.3	0.1	n.p.	3.8	0.1	3.9	n.p.	n.p.	96.1
0.25	VG02	77.3	12.1	3.5	n.p.	1.6	0.6	n.p.	17.8	1.1	19.0	0.0	0.0	96.3
1	VG03	55.8	24.8	6.0	n.p.	3.0	1.2	n.p.	35.0	1.9	36.9	0.0	0.0	92.7
3	VG04	39.7	35.6	8.3	n.p.	7.8	2.3	n.p.	53.9	3.1	57.1	0.0	0.1	96.9
7	VG05	24.6	44.4	12.6	n.p.	5.1	2.5	n.p.	64.7	5.9	70.6	0.0	0.5	95.8
14	VG06	15.5	47.4	12.3	n.p.	7.8	2.1	n.p.	69.6	7.4	77.0	0.0	1.3	93.8
30	VG07	10.5	45.1	12.9	n.p.	6.7	3.0	n.p.	67.8	14.1	81.9	0.0	3.6	96.1
56	VG08	8.4	37.6	11.9	n.p.	6.2	2.9	n.p.	58.6	19.7	78.3	0.0	6.1	92.8
78	VG09	6.1	31.6	11.8	n.p.	6.3	3.5	n.p.	53.2	23.4	76.7	0.0	9.1	91.9
100	VG10	6.1	29.0	13.4	n.p.	5.6	1.9	n.p.	49.8	25.3	75.1	0.1	9.6	90.8
101(s)	VG14	7.6	47.6	19.2	n.p.	9.6	4.2	n.p.	80.5	9.0	89.5	n.p.	n.p.	97.1
Berghäuser Altrhein; triazole-label														
0	VG15	95.9	0.4	0.1	n.p.	0.0	0.0	n.p.	0.6	1.1	1.6	n.p.	n.p.	97.5
0.25	VG16	81.1	12.2	3.2	n.p.	1.4	0.5	n.p.	17.3	1.3	18.6	0.0	0.0	99.8
1	VG17	60.1	24.7	5.6	n.p.	2.8	1.5	n.p.	34.6	2.3	36.9	0.0	0.0	97.0
3	VG18	47.0	32.6	8.0	n.p.	6.4	1.9	n.p.	48.9	2.6	51.5	0.0	0.0	98.6
7	VG19	25.7	44.3	13.5	n.p.	6.4	3.0	n.p.	67.1	4.7	71.8	0.0	0.1	97.6
14	VG20	20.2	47.7	11.3	n.p.	8.9	2.3	n.p.	70.1	6.2	76.3	0.0	0.2	96.7
30	VG21	16.1	46.4	13.2	n.p.	7.1	3.3	n.p.	70.0	11.4	81.5	0.0	0.1	97.6
56	VG22	15.1	40.8	12.9	n.p.	6.8	2.6	n.p.	63.1	18.1	81.3	0.1	0.2	96.7
78	VG23	16.1	32.9	15.5	n.p.	6.4	3.6	n.p.	58.4	21.1	79.5	0.0	0.4	96.0
100	VG25	16.8	32.7	11.8	n.p.	5.0	2.8	n.p.	52.2	26.6	78.9	0.0	0.8	96.6
101 (s)	VG28	7.9	49.4	19.4	n.p.	8.6	3.5	n.p.	80.8	8.5	89.4	n.p.	n.p.	97.3
Berghäuser Altrhein; trifluoromethylphenyl-label														
0	VG57	97.2	0.0	0.0	n.p.	0.0	0.0	n.p.	0.0	0.0	0.0	n.p.	n.p.	97.3
	VG58	98.9	0.0	0.0	n.p.	0.0	0.0	n.p.	0.0	0.0	0.0	n.p.	n.p.	98.9
	mean	**98.1**	**0.0**	**0.0**	**-**	**0.0**	**0.0**	**-**	**0.0**	**0.0**	**0.0**	**-**	**-**	**98.1**
3	VG59	33.8	9.9	17.4	n.p.	11.6	4.3	2.3	45.5	2.7	48.2	0.0	0.0	82.0
7	VG62	22.0	32.3	21.5	n.p.	12.0	3.6	n.p.	69.3	4.9	74.3	0.0	0.0	96.2
14	VG60	13.8	34.1	23.2	n.p.	14.2	4.5	1.8	77.8	4.5	82.3	0.0	0.1	96.2
28	VG61	9.6	29.7	27.2	n.p.	11.5	6.3	3.8	78.5	9.7	88.3	0.0	0.2	98.1
56	VG63	5.8	22.5	27.8	n.p.	16.5	6.7	3.2	76.7	14.9	91.7	0.0	0.3	97.7
	VG64	4.9	30.2	26.8	n.p.	13.8	5.0	2.5	78.4	12.1	90.6	0.0	0.5	95.9
	mean	**5.3**	**26.4**	**27.3**	**-**	**15.1**	**5.9**	**2.9**	**77.6**	**13.5**	**91.1**	**0.0**	**0.4**	**96.8**
78	VG66	3.5	38.4	20.7	n.p.	10.0	5.3	3.0	77.4	15.6	92.9	0.0	0.6	97.0
100	VG67	3.6	31.4	19.6	n.p.	12.1	6.2	3.1	72.4	18.6	91.1	0.0	1.2	95.9
	VG70	4.2	36.6	19.9	n.p.	8.2	4.8	2.1	71.6	19.0	90.6	0.1	1.7	96.6
	mean	**3.9**	**34.0**	**19.7**	**-**	**10.1**	**5.5**	**2.6**	**72.0**	**18.8**	**90.8**	**0.0**	**1.5**	**96.2**

ACN = Acetonitrile
NER = Non-extractable radioactive residues
n.p. = Not performed
(s) = Sterile vessels
TAR = Total applied radioactivity

Table 7.2.2.3-3: Material balance and distribution of radioactivity after application of ^{14}C-labeled BAS 750 F to water/sediment system Ranschgraben and incubation under dark conditions [% TAR]

days after treatment	vessel	[% TAR]												
			sediment									volatiles		material balance
		water	ACN/H_2O 1	ACN/H_2O 2	ACN/H_2O 3	ACN 1	CAN 2	CAN 3	total extractable	NER	total	ethylene-glycol	NaOH (CO_2)	
Ranschgraben; chlorophenyl-label														
0	VG29	97.7	0.4	0.1	n.p.	0.0	0.0	n.p.	0.5	0.2	0.8	n.p.	n.p.	98.5
0.25	VG30	75.2	13.6	4.7	n.p.	1.8	0.6	n.p.	20.6	0.8	21.4	0.0	0.0	96.6
1	VG31	56.9	21.1	8.1	n.p.	4.2	1.5	n.p.	35.0	2.2	37.2	0.0	0.1	94.2
3	VG32	35.5	33.0	12.0	n.p.	5.8	1.8	n.p.	52.7	2.1	54.8	0.0	0.3	90.6
7	VG33	23.8	43.3	15.6	n.p.	6.5	2.3	n.p.	67.7	4.2	71.9	0.0	0.5	96.2
14	VG35	13.6	44.2	17.5	n.p.	7.8	2.6	n.p.	72.2	5.7	77.9	0.0	1.1	92.7
30	VG36	7.8	48.5	16.6	n.p.	6.9	2.6	n.p.	74.5	10.1	84.7	0.0	2.2	94.7
56	VG37	4.1	44.1	17.0	n.p.	7.6	2.7	n.p.	71.4	16.1	87.5	0.0	2.8	94.4
78	VG39	3.7	40.6	17.2	n.p.	8.3	3.9	n.p.	69.9	18.3	88.2	0.0	4.7	96.6
100	VG40	3.2	38.9	14.7	6.3	4.0	1.5	n.p.	65.3	19.1	84.4	0.0	5.1	92.8
101 (s)	VG42	4.9	45.4	22.5	n.p.	10.9	3.9	n.p.	82.6	10.1	92.7	n.p.	n.p.	97.5
Ranschgraben; triazole-label														
0	VG43	95.0	1.1	0.4	n.p.	0.2	0.1	n.p.	1.8	0.3	2.1	n.p.	n.p.	97.1
0.25	VG44	74.4	13.8	5.2	n.p.	2.1	0.6	n.p.	21.7	0.7	22.4	0.0	0.0	96.9
1	VG45	51.6	21.7	8.1	n.p.	4.3	1.5	n.p.	35.5	1.4	36.9	0.0	0.0	88.5
3	VG46	42.5	33.1	11.4	n.p.	5.8	1.8	n.p.	52.1	1.8	53.9	0.0	0.1	96.5
7	VG47	23.0	43.9	16.4	n.p.	6.8	2.4	n.p.	69.4	3.2	72.6	0.0	0.1	95.7
14	VG48	14.8	45.3	17.9	n.p.	7.7	2.4	n.p.	73.4	4.0	77.4	0.0	0.2	92.3
30	VG49	9.7	48.8	18.4	n.p.	7.5	2.7	n.p.	77.4	7.6	85.0	0.0	0.0	94.7
56	VG50	8.1	45.4	17.8	n.p.	8.0	3.0	n.p.	74.2	13.1	87.3	0.0	0.3	95.7
78	VG51	8.2	40.6	18.1	n.p.	8.4	4.0	n.p.	71.1	15.6	86.6	0.1	0.2	95.2
100	VG52	7.9	40.4	16.8	n.p.	9.3	3.0	n.p.	69.5	17.0	86.5	0.0	0.5	94.8
101 (s)	VG58	4.9	45.0	20.9	n.p.	10.4	3.8	n.p.	80.1	10.0	90.1	n.p.	n.p.	95.0
Ranschgraben; trifluoromethylphenyl-label														
0	VG71	99.1	0.0	0.0	n.p.	0.0	0.0	n.p.	0.0	0.1	0.1	n.p.	n.p.	99.2
	VG72	101.1	0.0	0.0	n.p.	0.0	0.0	n.p.	0.0	0.0	0.0	n.p.	n.p.	101.1
	mean	**100.1**	**0.0**	**0.0**	**-**	**0.0**	**0.0**	**-**	**0.0**	**0.1**	**0.1**	**-**	**-**	**100.2**
3	VG73	37.8	33.5	12.4	n.p.	6.0	1.9	n.p.	53.9	1.8	55.7	0.0	0.0	93.5
7	VG74	22.8	43.5	16.8	n.p.	7.0	2.2	n.p.	69.4	2.8	72.3	0.0	0.0	95.1
14	VG75	16.2	49.0	16.1	n.p.	7.3	2.3	n.p.	74.7	3.8	78.6	0.0	0.1	94.9
28	VG76	10.9	46.6	19.8	n.p.	8.2	2.7	n.p.	77.3	8.4	85.7	0.0	0.2	96.8
56	VG77	7.7	45.8	19.7	n.p.	7.9	3.2	n.p.	76.6	12.3	88.9	0.0	0.3	96.9
	VG78	6.8	46.3	20.6	n.p.	7.9	3.7	n.p.	78.6	12.2	90.8	0.0	0.0	97.6
	mean	**7.3**	**46.1**	**20.1**	**-**	**7.9**	**3.5**	**-**	**77.6**	**12.3**	**89.9**	**0.0**	**0.1**	**97.3**
78	VG81	5.9	44.2	19.1	n.p.	8.7	3.3	n.p.	75.3	15.6	90.9	0.0	0.3	97.1
100	VG82	5.6	41.7	16.8	n.p.	7.8	3.2	n.p.	69.5	22.8	92.3	0.0	0.5	98.4
	VG84	5.6	39.2	18.2	n.p.	8.3	3.5	n.p.	69.2	22.0	91.2	0.0	0.5	97.3
	mean	**5.6**	**40.4**	**17.5**	**-**	**8.0**	**3.4**	**-**	**69.4**	**22.4**	**91.8**	**0.0**	**0.5**	**97.8**

ACN = Acetonitrile
NER = Non-extractable radioactive residues
n.p. = Not performed
(s) = Sterile vessels
TAR = Total applied radioactivity

B. TRANSFORMATION OF PARENT COMPOUND

Characterization and identification of residues in water and sediment extracts

An overview of active ingredient and metabolites for the water samples and sediment extracts is presented in Table 7.2.2.3-4 to Table 7.2.2.3-9.

In the water phase of the water/sediment system Berghäuser Altrhein (triazole label, 78 days), metabolite M750F001 (1,2,4-triazole) was identified by mass spectrometric analysis and confirmed by chromatographic comparison with the retention time of the reference item.

Metabolite M750F003 was identified in the pooled acetonitrile/water extract of system Berghäuser Altrhein (trifluoromethylphenyl label, 100 days) and in the water phase of the water/sediment system Berghäuser Altrhein (triazole label, 100 days) by mass spectrometric analysis.

In addition, by using the pooled acetonitrile/water extract of system Berghäuser Altrhein (trifluoromethylphenyl label, 100 days), a minor metabolite with a retention time of approx. 32.7 min could be identified by means of mass spectrometric analysis. This metabolite was designated as M750F032.

A proposed route of degradation of BAS 750 F in water/sediment systems is given in Figure 7.2.2.3-1.

Table 7.2.2.3-4: Metabolite overview for the water and sediment phase after application of chlorophenyl-U-^{14}C-BAS 750 F to the water/sediment system Berghäuser Altrhein [% TAR]

days after treatment	vessel No.	[% TAR]					
		^{14}C total	unknown	unknown	BAS 750 F	unknown	sum others[a]
		~t_{Ret}	7.3	8.1	35.6	38.8	
water							
0	VG01	92.1	-	-	90.8	-	1.3
0.25	VG02	77.3	-	-	75.2	-	2.1
1	VG03	55.8	-	-	55.4	-	0.4
3	VG04	39.7	0.3	-	39.4	-	-
7	VG05	24.6	0.4	0.7	22.8	0.2	0.5
14	VG06	15.5	0.6	1.0	13.6	-	0.4
30	VG07	10.5	1.9	1.4	6.7	0.1	0.5
56	VG08	8.4	2.1	1.6	3.2	-	1.4
78	VG09	6.1	1.5	1.7	2.5	-	0.5
100	VG10	6.1	2.2	1.2	1.9	-	0.7
101 (s)	VG14	7.6	-	-	6.7	0.1	0.9
sediment							
0	VG01	3.8	-	-	3.8	-	-
0.25	VG02	17.8	-	-	17.8	-	-
1	VG03	35.0	-	-	33.9	0.5	0.6
3	VG04	53.9	-	-	52.3	1.0	0.6
7	VG05	64.7	-	-	63.3	1.4	-
14	VG06	69.6	-	-	67.6	1.3	0.7
30	VG07	67.8	-	-	64.8	1.9	1.1
56	VG08	58.6	-	-	56.3	1.5	0.7
78	VG09	53.2	-	-	49.4	1.9	1.9
100	VG10	49.8	-	-	48.8	1.1	-
101 (s)	VG14	80.5	-	-	78.9	1.0	0.5

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Table 7.2.2.3-5: Metabolite overview for the water and sediment phase after application of triazole-3(5)-^{14}C-BAS 750 F to the water/sediment system Berghäuser Altrhein [% TAR]

days after treatment	vessel No.	[% TAR]				
		^{14}C total	M750F001 (1,2,4-triazole)	M750F003	BAS 750 F	sum others[a]
		~t_{Ret}	5.7	25.4	35.7	
water						
0	VG15	95.9	1.6	-	94.3	-
0.25	VG16	81.1	0.9	-	80.2	-
1	VG17	60.1	0.8	-	59.3	-
3	VG18	47.0	1.1	0.3	45.7	-
7	VG19	25.7	1.4	0.6	23.0	0.8
14	VG20	20.2	2.3	1.3	16.6	-
30	VG21	16.1	4.4	2.0	8.6	1.0
56	VG22	15.1	7.9	1.8	4.5	0.9
78	VG23	16.1	9.4	2.2	3.9	0.8
100	VG25	16.8	10.2	2.5	2.3	1.8
101 (s)	VG28	7.9	0.2	0.2	7.5	-
sediment						
0	VG15	0.6	n.a.	n.a.	n.a.	n.a.
0.25	VG16	17.3	-	-	17.3	-
1	VG17	34.6	-	-	34.6	-
3	VG18	48.9	-	-	48.9	-
7	VG19	67.1	-	-	67.1	-
14	VG20	70.1	0.6	-	68.5	1.0
30	VG21	70.0	1.6	1.0	67.5	-
56	VG22	63.1	3.6	1.3	58.3	-
78	VG23	58.4	3.5	2.0	52.9	-
100	VG25	52.2	4.9	1.7	45.6	-
101 (s)	VG28	80.8	-	-	80.5	0.3

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
n.a. = Not analysed
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Table 7.2.2.3-6: Metabolite overview for the water and sediment phase after application of trifluoromethylphenyl-ring-^{14}C-BAS 750 F to the water/sediment system Berghäuser Altrhein [% TAR]

days after treatment	vessel No.	[% TAR]						
		^{14}C total	M750F003	M750F032	BAS 750 F	unknown	unknown	sum others[a]
		~t_{Ret}	25.2	32.7	35.4	36.9	38.0	
water								
0	VG57	97.2	-	-	94.5	0.6	2.1	-
	VG58	98.9	-	-	96.6	0.4	1.9	-
	mean	**98.1**	**-**	**-**	**95.5**	**0.5**	**2.0**	**-**
3	VG59	33.8	-	-	33.6	-	0.3	-
7	VG62	22.0	0.6	0.3	20.4	-	0.6	-
14	VG60	13.8	0.6	0.3	12.5	-	0.3	0.1
28	VG61	9.6	2.5	0.3	6.6	-	-	0.2
56	VG63	5.8	3.4	0.1	1.6	-	0.1	0.5
	VG64	4.9	3.0	0.1	1.0	-	-	0.8
	mean	**5.3**	**3.2**	**0.1**	**1.3**	**-**	**0.0**	**0.6**
78	VG66	3.5	3.0	-	0.4	-	-	0.1
100	VG67	3.6	2.9	-	0.6	-	-	-
	VG70	4.2	3.3	0.1	0.7	-	-	-
	mean	**3.9**	**3.1**	**0.1**	**0.7**	**-**	**-**	**-**
sediment								
0	VG57	0.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	VG58	0.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	mean	**0.0**	**-**	**-**	**-**	**-**	**-**	**-**
3	VG59	45.5	-	-	45.1	-	-	0.4
7	VG62	69.3	0.7	0.7	66.8	1.1	-	-
14	VG60	77.8	1.2	1.1	73.5	1.0	-	0.9
28	VG61	78.5	0.7	2.1	75.7	-	-	-
56	VG63	76.7	3.8	1.8	69.9	-	0.7	0.5
	VG64	78.4	2.7	2.1	73.6	-	-	-
	mean	**77.6**	**3.3**	**1.9**	**71.8**	**-**	**0.4**	**0.3**
78	VG66	77.4	4.6	2.8	69.3	-	0.7	-
100	VG67	72.4	5.0	2.6	63.4	-	0.8	0.7
	VG70	71.6	5.9	3.1	60.8	0.8	1.1	-
	mean	**72.0**	**5.4**	**2.8**	**62.1**	**0.4**	**0.9**	**0.4**

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
n.a. = Not analysed
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Table 7.2.2.3-7: Metabolite overview for the water and sediment phase after application of chlorophenyl-U-^{14}C-BAS 750 F to the water/sediment system Ranschgraben [% TAR]

days after treatment	vessel No.	[% TAR]					
		^{14}C total	M750F032	BAS 750 F	unknown	unknown	sum others[a]
		~t_{Ret}	32.9	35.7	38.8	39.5	
water							
0	VG29	97.7	-	93.7	-	1.2	2.8
0.25	VG30	75.2	-	74.2	-	1.0	-
1	VG31	56.9	-	56.2	-	0.5	0.3
3	VG32	35.5	-	34.8	-	0.2	0.5
7	VG33	23.8	0.4	22.8	0.2	0.1	0.4
14	VG35	13.6	-	13.3	-	-	0.3
30	VG36	7.8	-	7.3	-	-	0.5
56	VG37	4.1	-	4.1	-	-	-
78	VG39	3.7	-	3.7	-	-	-
100	VG40	3.2	-	3.2	-	-	-
101 (s)	VG42	4.9	-	3.9	-	0.1	0.9
sediment							
0	VG29	0.5	n.a.	n.a.	n.a.	n.a.	n.a.
0.25	VG30	20.6	-	19.0	0.6	0.7	0.3
1	VG31	35.0	-	34.4	-	0.6	-
3	VG32	52.7	0.8	50.6	0.5	0.5	0.4
7	VG33	67.7	-	67.7	-	-	-
14	VG35	72.2	1.4	70.0	0.9	-	-
30	VG36	74.5	2.3	71.5	0.6	-	-
56	VG37	71.4	1.8	68.4	1.2	-	-
78	VG39	69.9	2.1	67.0	0.8	-	-
100	VG40	65.3	2.3	61.6	0.8	-	0.6
101 (s)	VG42	82.6	0.4	80.5	-	1.2	0.5

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
n.a. = Not analysed
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Table 7.2.2.3-8: Metabolite overview for the water and sediment phase after application of triazole-3(5)-^{14}C-BAS 750 F to the water/sediment system Ranschgraben [% TAR]

days after treatment	vessel No.	[% TAR]					
		^{14}C total	M750F001 (1,2,4-triazole)	M750F003	M750F032	BAS 750 F	sum others[a]
		~t_{Ret}	5.9	25.4	32.8	35.7	
water							
0	VG43	95.0	-	-	-	95.0	-
0.25	VG44	74.4	0.8	-	-	73.6	-
1	VG45	51.6	0.7	0.3	-	50.5	-
3	VG46	42.5	0.6	0.2	-	41.6	-
7	VG47	23.0	0.4	0.6	-	22.0	-
14	VG48	14.8	0.7	0.6	-	13.3	0.2
30	VG49	9.7	0.5	1.4	0.1	6.9	0.8
56	VG50	8.1	0.4	3.1	-	4.3	0.3
78	VG51	8.2	1.0	3.3	-	3.8	-
100	VG52	7.9	1.1	3.8	-	2.9	-
101 (s)	VG56	4.9	0.2	0.2	-	4.5	0.1
sediment							
0	VG43	1.8	n.a.	n.a.	n.a.	n.a.	n.a.
0.25	VG44	21.7	-	-	-	21.7	-
1	VG45	35.5	-	-	-	35.5	-
3	VG46	52.1	-	-	-	52.1	-
7	VG47	69.4	-	-	-	69.4	-
14	VG48	73.4	-	-	0.9	72.5	-
30	VG49	77.4	-	1.9	0.6	74.9	-
56	VG50	74.2	-	1.7	2.1	70.4	-
78	VG51	71.1	-	1.9	1.6	67.5	-
100	VG52	69.5	-	3.3	1.8	64.4	-
101 (s)	VG56	80.1	-	-	-	80.1	-

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
n.a. = Not analysed
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Table 7.2.2.3-9: Metabolite overview for the water and sediment phase after application of trifluoromethylphenyl-ring-^{14}C-BAS 750 F to the water/sediment system Ranschgraben [% TAR]

days after treatment	vessel No.	[% TAR]					
		^{14}C total	M750F003	BAS 750 F	unknown	unknown	sum others[a]
		~t_{Ret}	25.3	35.4	37.0	38.1	
water							
0	VG71	99.1	-	96.6	0.5	2.0	-
	VG72	101.1	-	97.5	1.0	1.3	1.2
	mean	**100.1**	**-**	**97.1**	**0.7**	**1.7**	**0.6**
3	VG73	37.8	0.2	36.0	0.6	1.1	-
7	VG74	22.8	0.4	21.8	0.2	0.2	0.2
14	VG75	16.2	0.5	14.8	0.1	0.3	0.6
28	VG76	10.9	2.0	8.7	-	-	0.2
56	VG77	7.7	2.1	5.7	-	-	-
	VG78	6.8	1.7	5.1	-	-	-
	mean	**7.3**	**1.9**	**5.4**	**-**	**-**	**-**
78	VG81	5.9	2.3	3.4	-	-	0.1
100	VG82	5.6	2.7	2.9	-	-	-
	VG84	5.6	2.7	2.9	-	-	-
	mean	**5.6**	**2.7**	**2.9**	**-**	**-**	**-**
Sediment							
0	VG71	0.0	n.a.	n.a.	n.a.	n.a.	n.a.
	VG72	0.0	n.a.	n.a.	n.a.	n.a.	n.a.
	mean	**0.0**	**-**	**-**	**-**	**-**	**-**
3	VG73	53.9	-	53.9	-	-	-
7	VG74	69.4	-	69.4	-	-	-
14	VG75	74.7	-	73.3	-	0.8	0.6
28	VG76	77.3	-	74.9	-	-	2.5
56	VG77	76.6	1.4	73.6	-	-	1.6
	VG78	78.6	0.8	75.3	1.6	0.9	-
	mean	**77.6**	**1.1**	**74.4**	**0.8**	**0.5**	**0.8**
78	VG81	75.3	1.6	73.7	-	-	-
100	VG82	69.5	1.6	67.1	-	-	0.8
	VG84	69.2	1.8	67.5	-	-	-
	mean	**69.4**	**1.7**	**67.3**	**-**	**-**	**0.4**

TAR = Total applied radioactivity
t_{Ret} = Retention time [min]
n.a. = Not analysed
(s) = Sterile vessels
[a] Sum of several peaks, each individual peak <1.0% TAR

Water

Chlorophenyl label
The chlorophenyl-labeled test item dissipated fast from the water phase, decreasing to 1.9% TAR in system BA and 3.2% TAR in system RG after 100 days.

Several unknown metabolites were detected, most of them occurred only sporadically (≤ 2.2% TAR).

Triazole label
Similar to the chlorophenyl-labeled test item, triazole-labeled BAS 750 F dissipated fast from the water phase, decreasing to 2.3% TAR in system BA and 2.9% TAR in system RG at 100 DAT.

Several metabolites were detected; most of them were unknown and occurred only sporadically. Two of them occurred continuously and could be assigned to known metabolites M750F001 (1,2,4-triazole) and M750F003 (cleavage product having lost chlorophenyl ring). For system BA, M750F001 was detected in increasing amounts, reaching a maximum of 10.2% TAR at the end of the incubation period. For system RG, detected amounts of M750F001 were low (≤ 1.1% TAR). Detected amounts of metabolite M750F003 reached a maximum of 2.5% and 3.8% TAR in system BA and RG, respectively. None of the other metabolites exceeded 0.3% TAR at any sampling date.

Trifluoromethylphenyl label
A fast dissipation of BAS 750 F from the water phase was also observed with the trifluoromethylphenyl-label. Detected amounts decreased to 0.7% TAR in system BA and 2.9% TAR in system RG after 100 days of incubation.

Several unknown metabolites were detected; however, most of them occurred only sporadically. One metabolite could be assigned to the known metabolite M750F003, reaching a maximum of 3.4% and 2.7% TAR in system BA and RG, respectively. None of the other metabolites exceeded 1.7% TAR at any sampling date.

Sediment

Chlorophenyl label
Detected amounts of chlorophenyl-labeled test item extracted from the sediment phase reached a maximum of 67.6% (14 DAT) and 71.5% TAR (30 DAT) in system BA and system RG, respectively. After 100 days, extractable amounts declined to 48.8% TAR in system BA and to 61.6% TAR in system RG.

Several unknown metabolites were detected, most of them occurred only sporadically. None of the metabolites exceeded 2.3% TAR at any sampling date.

Triazole label
Detected amounts of triazole-labelled BAS 750 F extracted from the sediment phase reached a maximum of 68.5% (14 DAT) and 74.9% TAR (30 DAT) in system BA and system RG, respectively. After 100 days, amounts of BAS 750 F declined to 45.6% TAR in system BA and to 64.4% TAR in system RG.

Four metabolites were found in the sediment extracts of both test systems. Two of them were assigned to the known metabolites M750F001 and M750F003. While M750F003 was measured in both test systems, M750F001 occurred only in sediment extracts of test system BA, reaching a maximum of 4.9% TAR after 100 days. M750F003 occurred in maximum amounts of 2.0% TAR (BA) and 3.3% TAR (RG). None of the other metabolites exceeded 2.1% TAR.

Trifluoromethylphenyl label
Detected amounts of trifluoromethylphenyl-labeled BAS 750 F extracted from the sediment phase reached a maximum of 75.7% (28 DAT) and 74.9% TAR (28 DAT) in system BA and system RG, respectively. After 100 days, extractable amounts declined to 62.1% TAR in system BA and to 67.3% TAR in system RG.

Several unknown metabolites were detected, most of them occurred only sporadically. One metabolite was assigned to the known metabolite M750F003. In test system BA, extracted amounts of M750F003 exceeded 5% TAR (5.4% TAR), however, only at 100 DAT. In test system RG, it reached a maximum of 1.7% TAR at the end of the incubation period. None of the other metabolites exceeded 2.8% TAR at any sampling date.

One of this minor metabolites (max. 2.8% TAR, retention time ~ 32.7 min) could by chance be further characterized by LC-MS/MS in a pooled and concentrated sediment extract sample. It was designated as M750F032. In cases where a peak with similar retention time could be detected also in samples treated with the other radiolabels, this peak was assigned to M750F032 and listed separately in the result tables.

Sterilised assays

All results of the analysis of the sterilised test vessels are presented in the same tables as the results of the viable test vessels. For the trifluoromethylphenyl-labelled test item, no sterilised incubations were performed.

The sterilised samples (101 days) showed somewhat higher concentrations of BAS 750 F in water and sediment as the 100 day samples of the viable vessels. Chlorophenyl-labelled BAS 750 F was detected in amounts of 6.7% (BA) and 3.9% TAR (RA) in the water samples, the triazole-labelled test item amounted to 7.5% and 4.5% TAR. 78.9% and 80.5% TAR (BA) and 80.5% and 80.1% TAR (RA) were detected in the pooled sediment extracts of the sterilised test vessels at 101 days. Nearly all radioactivity recovered in the water phases or sediment extracts consisted of unchanged parent.

The non-extractable residues in the sterilised test vessels were significantly lower (8.5% – 10.1% TAR) than those of the viable incubations (17.0% – 26.6% TAR) indicating that degradation of BAS 750 F in sediment by incorporation into the humic substance matrix is enhanced in the presence of an active microbial population.

Chiral HPLC

Results of the chiral radio-HPLC analyses are summarised in Table 7.2.2.3-10.

No shift in the enantiomeric ratio of BAS 750 F in sediment extracts (chlorophenyl- and triazole-label) was detected.

Table 7.2.2.3-10: Chiral radio-HPLC analysis of water samples and sediment extracts after incubation with ^{14}C-labelled BAS 750 F [% ROI]

Matrix	Days after treatment	total radioactivity in sample [% TAR]	Enantiomer 1 [% ROI] t_{Ret} 20.7 min	Enantiomer 2 [% ROI] t_{Ret} 21.9 min
chlorophenyl-label Berghäuser Altrhein				
water	30	10.5	52.1	47.9
sediment extract	30	67.8	51.2	48.8
sediment extract	100	49.8	50.1	49.9
chlorophenyl-label Ranschgraben				
water	30	7.8	49.4	50.6
sediment extract	30	74.5	52.2	47.8
sediment extract	100	65.3	51.0	49.0
triazole-label Berghäuser Altrhein				
water	30	16.1	50.6	49.4
sediment extract	30	70.0	51.2	48.8
sediment extract	100	52.2	50.9	49.1
triazole-label Ranschgraben				
water	30	9.7	50.9	49.1
sediment extract	30	77.4	50.4	49.6
sediment extract	100	69.5	51.8	48.2

TAR = total applied radioactivity
ROI = "region of interest" = peak area with regard to the total integrated peak area in a chromatogram

C. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

Results of the humic substance fractionation are summarized in Table 7.2.2.3-11.

About one-third to one-half of the bound radioactivity could be released by harsh NaOH extraction. The rest was still bound to soil matrix obviously tightly incorporated into the insoluble humins. After fractionation of the NaOH extract into fulvic acids and humic acids, about one-half to three quarters were found in the fulvic acids and one-half to one quarter in the humic acids of test system Berghäuser Altrhein. In test system Ranschgraben, about one-thirds to one-half were found in the fulvic acids and one-half to two-thirds in the humic acids.

Table 7.2.2.3-11: Distribution of radioactivity between fulvic acids, humic acids and humins after application of ^{14}C-BAS 750 F to water/sediment systems

sediment sample	DAT	vessel No.	[% TAR]					Recovery [%]
			total NER	fulvic acids	humic acids	humins	sum	
Berghäuser Altrhein (chlorophenyl-label)	30	VG07	14.1	2.7	1.6	8.7	13.0	91.8
	100	VG10	25.3	4.8	3.3	15.4	23.5	93.0
Berghäuser Altrhein (triazole-label)	30	VG21	11.4	3.5	0.9	6.8	11.2	98.4
	100	VG25	26.6	10.4	2.7	13.5	26.7	100.0
Berghäuser Altrhein (trifluoromethylphenyl-label)	28	VG61	9.7	1.3	1.5	6.3	9.0	92.7
	100	VG70	19.0	3.4	2.9	10.1	16.4	86.3
Ranschgraben (chlorophenyl-label)	30	VG36	10.1	1.3	2.2	4.9	8.4	82.7
	100	VG40	19.1	2.5	4.2	8.2	14.9	78.0
Ranschgraben (triazole-label)	30	VG49	7.6	1.7	1.8	4.3	7.8	103.1
	100	VG52	17.0	4.1	3.9	7.5	15.5	91.6
Ranschgraben (trifluoromethylphenyl-label)	28	VG76	8.4	1.4	2.2	4.7	8.2	98.0
	100	VG82	22.8	3.4	5.4	10.6	19.3	84.5

DAT = Days after treatment
NER = Non-extractable radioactive residues
TAR = Total applied radioactivity

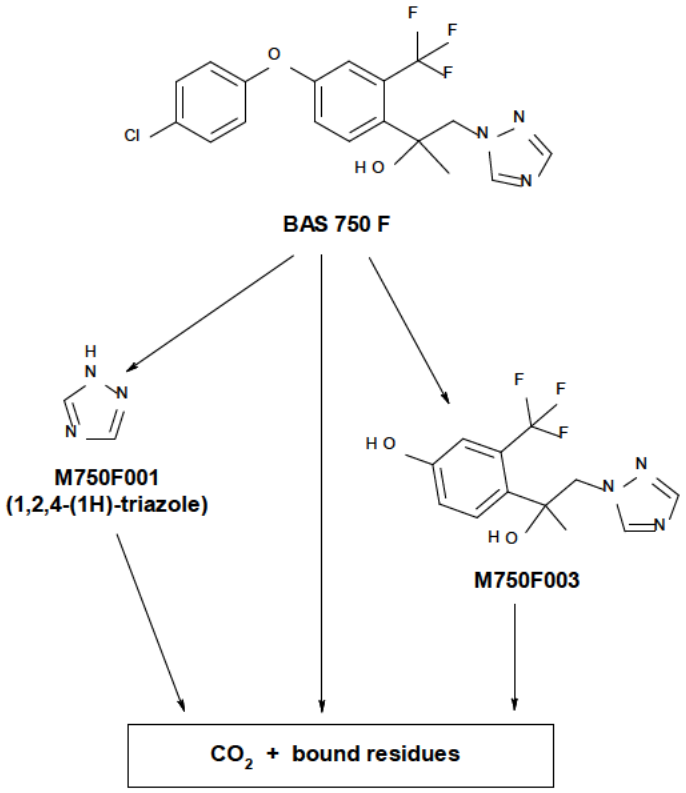


Figure 7.2.2.3-1: Proposed (major) route of degradation of BAS 750 F in water/sediment systems

D. KINETIC MODELING RESULTS

For the parent substance, the analysis at P-I level (one-compartment approach) was done for degradation in the total system as well as the respective dissipations from the water and sediment phases of the test systems. At the P-II level (two compartments approach); the kinetic analysis considered the degradation in water and sediment taking into account the partitioning between the two phases. However, the P-II evaluation could not be accepted according to FOCUS guidance because of the failure of the degradation parameter in water k_w and/or sediment k_{sed} regarding the t-test.

The following DT_{50}/DT_{90} values for BAS 750 F in water/sediment systems (P-I-level) were determined (Table 7.2.2.3-12):

Table 7.2.2.3-12: Persistence and modeling endpoints for BAS 750 F (Level P-I)

Compartment	System	Persistence endpoints				Modelling endpoints		
		Model	DT_{50} [d]	DT_{90} [d]	chi^2	Model	DT_{50} [d]	chi^2
Total system	Berghäuser Altrhein	DFOP	122.2	444.0	2.0	SFO	125.5	2.8
	Ranschgraben	HS	213.1	785.6	1.3	SFO	212.8	2.7
Water	Berghäuser Altrhein	FOMC	1.7	21.9	6.4	FOMC	6.6 [a]	6.4
	Ranschgraben	FOMC	1.3	26.2	6.7	FOMC	7.9 [a]	6.7
Sediment	Berghäuser Altrhein	SFO	224.8	746.7	4.0	SFO	224.8	4.0
	Ranschgraben	SFO	395.6	>1000	1.0	SFO	395.6	1.0

[a] Calculated as $DT_{50} = DT_{90}/3.32$ (less than 10% of maximum concentration at end of study)

III. CONCLUSION

Overall, it can be concluded that BAS 750 F dissipates at a fast rate from the water phase and degrades at a moderate rate in the sediment when incubated in water/sediment systems under dark conditions.

Metabolite M750F001 (1,2,4-triazole) is formed by split-off of the triazole ring from the parent molecule. It reached maximum amounts of 10.2% TAR in the water and 4.9% TAR in the sediment phase. M750F003 is formed after split-off of the chlorophenyl ring, which is then further degraded to CO_2 as shown with the chlorophenyl-labelled test item. M750F003 occurred in maximum amounts of 3.8% TAR in the water and 5.4% TAR in the sediment phase. All other metabolites never exceeded 2.8% TAR.

For the parent compound, best-fit DT_{50} values in the water phase were 1.7 and 1.3 days for the systems Berghäuser Altrhein and Ranschgraben, respectively. In the sediment, parent DT_{50} values were determined to be 225 and 396 days. For the total system, DT_{50} values of 122 and 213 days were calculated for the respective test system.

CA 7.2.2.4 Irradiated water/sediment study

Not required according to regulation 283/2013 and no data available.

CA 7.2.3 Degradation in the saturated zone

Due to its low leaching potential (geometric mean of $K_{f,oc}$ = 3456 mL g^{-1}), BAS 750 F is not expected to reach deeper soil layers or saturated zones. Therefore, investigations on the degradation in the saturated zone are considered to be not necessary.

Route and rate of degradation in aquatic systems - Overall conclusions

Chemical and photochemical degradation in aquatic systems

BAS 750 F is hydrolytically stable at environmentally relevant pH values (pH 4 - 9) at 25°C.

Under irradiated conditions, BAS 750 F is converted to M750F005, M750F006, M750F007, M750F008 and to some minor metabolites. The SFO DT_{50} for M750F005 and for M750F006 were estimated to be 34.8 and 12.4 days, respectively. DT_{50} for M750F007 and for M750F008 could not be derived based on available experimental data.

Route and rate of biological degradation in aquatic systems

BAS 750 F is poorly biodegradable.

The main degradation way of BAS 750 F in a dark aquatic system with natural water under dark was characterized by a low mineralization rate irrespective of test concentration. No major metabolites were observed other than M750F001 and M750F003 was in seen in < 5% TAR.

The following DT_{50}/DT_{90} values for test item BAS 750 F in water/sediment systems (P-I-level) were determined:

Table 7.2.3-1: Persistence and modeling endpoints for BAS 750 F (P-I)

Compartment	System	Persistence endpoints				Modelling endpoints		
		Model	DT_{50} [d]	DT_{90} [d]	chi^2	Model	DT_{50} [d]	chi^2
Total system	Berghäuser Altrhein	DFOP	122.2	444.0	2.0	SFO	125.5	2.8
	Ranschgraben	HS	213.1	785.6	1.3	SFO	212.8	2.7
Water	Berghäuser Altrhein	FOMC	1.7	21.9	6.4	FOMC	6.6 [a]	6.4
	Ranschgraben	FOMC	1.3	26.2	6.7	FOMC	7.9 [a]	6.7
Sediment	Berghäuser Altrhein	SFO	224.8	746.7	4.0	SFO	224.8	4.0
	Ranschgraben	SFO	395.6	>1000	1.0	SFO	395.6	1.0

[a] Calculated as DT_{50} = DT_{90}/3.32 (less than 10% of maximum concentration at end of study)

Potential effects of water treatment procedures on BAS 750 F and its metabolites

Currently there is neither a guideline for testing the effect of water treatment on pesticides (or other chemicals) nor is there a risk assessment procedure. Since conditions of water treatment are extremely variable across Europe (different treatment methods and intensities used in different sequences on different types of raw waters) it is currently not possible to comprehensively assess the potential formation of harmful by-products during drinking water production. An experimental guideline is essential because the effect of ozonolysis or chlorination strongly depends on treatment conditions (e.g. duration, applied concentration, properties of the raw water) which should be representative for real water treatment plants.

In the absence of such guidance documents an evaluation was made based on knowledge on the chemistry of BAS 750 F and its degradation products and applying chemical principles.

Experimental data on the aerobic degradation of BAS 750 F in soil demonstrate that the degradation pathway is mineralization, formation of the minor metabolite M750F001 (1,2,4-triazole), and formation of some other minor metabolites. The greatest sink observed for BAS 750 F in aerobic soil was formation of bound residues. Details are presented in chapter 7.1.1 on this study. BAS 750 F was found to be hydrolytically stable at all pH values tested (pH 4-9) at 25°C (see chapter 7.2.1.1). In an aerobic water/sediment study under dark conditions BAS 750 F was observed to quickly partition from the water phase to the sediment phase, as expected due to the high K_{OC} value. Moderate amounts of the metabolites M750F001 (1,2,4-triazole) and M750F003 as well as some minor metabolites were observed. Mineralization was low and the main sink was found to be bound residues. Details are presented in chapter 7.2.2.3. Under irradiated conditions in sterile buffer, BAS 750 F is converted to the metabolites M750F005, M750F006, M750F007, and M750F008 as well as some additional minor metabolites. Details are presented in chapter 7.2.1.2.

Neither BAS 750 F nor its metabolites contain any comparable aliphatic side chains as present in the chemical structure of tolylfluanid, which caused the problem of nitrosamine formation during water treatment for drinking water production. No N-nitrosamine formation is expected for BAS 750 F and its metabolites, since no secondary amine function is present. The only nitrogen-containing moiety is the electron-deficient heteroaromatic triazole ring, which has little propensity towards electrophilic attack of either ozone or NO^+.

With chlorine-based treatments, chlorination or hydroxylation and the possible loss of substituted chlorinated and hydroxylated structures is conceivable. However, when chlorine enters water it reacts chemically with any organic matter found in the water. There is always some organic matter in natural waters and by-products of this reaction include e.g. trihalomethanes. Any potential harmful degradation products resulting from any organic matter in the water treatment process will be eliminated in subsequent clean-up steps by using e.g. activated carbon filtration or sand filter beds.

It is therefore highly unlikely that water treatment processes such as ozonation or chlorination will result in the formation of by-products that would require a detailed health risk assessment. Consequently, further investigation into the effect of water treatment processes on the nature of residues present in surface water and groundwater is not considered necessary.

Assessement of persistence of BAS 750 F in water bodies and sediment

In the following it will be discussed if BAS 750 F fulfils the P criterion in water and sediment within the PBT (persistence, bioaccumulation and toxicity) and the vP criterion in the vPvB (very persistent very bioaccumulative) assessment, which are defined according to Section 3.7.2.1. and 3.7.3.1, respectively, of Annex II of EC Regulation 1107/2009 as follows:
An active substance, safener or synergist fulfils the persistence criterion where:

- *The half-life in marine water is higher than 60 days,*
- *The half-life in fresh or estuarine water is higher than 40 days,*
- *The half-life in marine sediment is higher than 180 days,*
- *The half-life in fresh or estuarine water sediment is higher than 120 days*

An active substance, safener or synergist fulfils the 'very persistent' criterion where:

- *the half-life in marine, fresh- or estuarine water is higher than 60 days,*
- *the half-life in marine, fresh- or estuarine water sediment is higher than 180 days*

The relevant endpoints for the persistence assessment were identified based on the DG SANCO working document on "Evidence Needed to Identify POP, PBT and vPvB Properties for Pesticides" [*SANCO 2012. DG SANCO Working Document on "Evidence Needed to Identify POP, PBT and vPvB Properties for Pesticides". Brussels: European Commission Health and Consumers Directorate-General. Report 25.09.2012 - rev. 3.*]. According to this document, degradation half-lives in the whole system of aerobic aquatic studies shall be compared with trigger values relevant for P and vP assessment for the degrading compartment. Additionally, data on aquatic photolysis should also be considered when relevant.

BAS 750 F was found to be hydrolytically stable at all pH values tested (pH 4-9) at 25°C (see chapter 7.3). Under irradiated conditions in sterile buffer, BAS 750 F was observed to degrade rather quickly to give a half-life of 2.3 days (see chapter 7.3.1.1).

In an aerobic water/sediment study under dark conditions BAS 750 F (see chapter 7.3.2.2.) was observed to quickly partition from the water phase to the sediment phase as expected due to the high K_{OC} value. The degradation half-life of BAS 750 F in the whole system was found to be 161 days as a geomean of both systems. The geomean dissipation half-life of BAS 750F from the water phase is 1.49 d, which is related to the sorption to sediment. Considering the high K_{oc} of BAS 750F and the rapid dissipation from water, the sediment compartment can be assumed to be the degrading compartment.

DT_{50} in the whole water/sediment system are appropriate for an initial assessment of persistence of BAS 750 F in sediment. Considering the geomean DT_{50} of 161 days BAS 750 F fulfils the criterion for P but not for vP. Due to the DT_{50} of 2.3 days observed in irradiated water, BAS 750 F does not fulfil either the P or the vP criterion for water.

It can, therefore be concluded that BAS 750 F is not P in water and is not vP in sediment.

CA 7.3 Fate and behaviour in air

Based on the physical-chemical properties, BAS 750 F has low potential for volatilization (vapor pressure, 3.2 x 10^{-6} Pa, 20°C; 6.5 x 10^{-6} Pa, 25°C). Further, due to the predicted facile degradation due to –OH radicals (atmospheric half-life: 1.666 d), there is no risk of long range transport.

CA 7.3.1 Route and rate of degradation in air

Report: CA 7.3.1/1
Hassink J., 2015 a
Photochemical oxidative degradation of BAS 750 F (QSAR estimates)
2015/1005046
Guidelines: EC 1107/2009 of the European Parliament
GLP: no

Executive Summary

The degradation rates for reactions of BAS 750 F with OH radicals and ozone in the atmosphere were calculated using the AOPWIN program based on ATKINSON's increment method.

Based on the resulting degradation rate of k_{OH} = 6.4193 x 10^{-12} cm^3 $molecule^{-1}$ s^{-1}, the atmospheric degradation half-life of the substance via this reaction route is $t_{1/2}$ = 1.666 d (12 h day).

Although O_3 is likely to react with BAS 750 F, the degradation rate resulting from ozone attack could not be estimated by the OECD method due to lack of increments.

I. MATERIAL AND METHODS

The degradation rate resulting from attack of OH-radicals was calculated with the AOPWIN Program (Atmospheric Oxidation Program for Microsoft Windows 3.1, Version 1.88, Syracuse Research Corp. 1997) based on ATKINSON's increment method [*Atkinson, R. (1987): A Structure-Activity Relationship for the Estimation of Rate Constants for the Gas-Phase Reactions of OH Radicals with Organic Compounds, Int.J. Chem. Kin. 19, 799*]. The degradation rate resulting from attack of ozone was calculated according to an OECD method [*Anonymous (1992): The rate of photochemical transformation of gaseous organic compounds in air under tropospheric conditions. OECD Environment Monographs No. 61, OECD, Paris*].

The degradation rate of BAS 750 F with OH-radicals was estimated based on the structural formula. The SMILE notation used for BAS 750 F in AOPWIN was:

c1cc(CL)ccc1Oc2cc(C(F)(F)(F))c(C(O)(C)Cn3ncnc3)cc2

II. RESULTS AND DISCUSSION

Assuming a pseudo-first order reaction, the degradation half-life was calculated by taking into account the diurnally and seasonally averaged concentration of hydroxyl-radicals in the troposphere. The total rate constant was estimated to be $k_{OH} = 6.4193 \times 10^{-12}$ cm^3 $molecule^{-1}$ s^{-1}.

Considering a weighted global average tropospheric hydroxyl-radical concentration of 1.5×10^6 mol cm^{-3}, the half-life for the degradation of BAS 750 F by OH-radicals was calculated according to Equation 7.3.1-1.

Equation 7.3.1-1 Estimation of the atmospheric degradation half-life (t1/2) of BAS 750 F

$$t_{1/2} = \ln 2 / (6.4193 \times 10^{-12} \times 1.5 \times 10^6)\ s$$
$$= 19.995\ h$$
$$= \underline{1.666\ d\ (12\ h\ day)}$$

Although BAS 750 F contains reactive sites for an ozone attack no increments are available and a reasonable approximation by AOPWIN is not possible. Therefore, although O_3 is likely to react with BAS 750 F no degradation estimation can be given.

III. CONCLUSION

Based on the results of the atmospheric degradation half-life of BAS 750 F ($t_{1/2}$ = 1.666 d), it can be concluded that the substance will be degraded by photochemical processes in the troposphere. Hence, due to its degradation in air, it can be concluded that there is low risk of long-range transport of BAS 750 F.

CA 7.3.2 Transport via air

Due to low vapor pressure (3.2×10^{-6} Pa, 20°C; 6.5×10^{-6} Pa, 25°C) and a calculated half-life of 1.67 days, long range transport is not expected to be a significant transport route for BAS 750 F.

CA 7.3.3 Local and global effect

No effects are expected since transport via air is highly unlikely (for details see above).

CA 7.4 Definition of the residue

CA 7.4.1 Definition of the residue for risk assessment

According to the results presented in M-CA 7.1-7.3 the following compounds are to be considered for the environmental risk assessment.

Soil:

BAS 750 F
M750F001 (1,2,4-triazole)

BAS 750 F and the minor metabolite M750F001 (1,2,4-triazole) (observed < 5% in all but a single soil in lab soil metabolism studies) are included in the residue definition for soil. Based on lab and field data it is not expected that 1,2,4-triazole will be substantially produced from BAS 750 F under field conditions. M750F001 is included in the residue definition because of its commonality among triazole fungicides. No risk was determined based upon the assessment of either compound.

Groundwater:

BAS 750 F
M750F001 (1,2,4-triazole)

BAS 750 F and the minor metabolite M750F001 (1,2,4-triazole) (observed < 5% in all but a single soil in lab soil metabolism studies) are included in the residue definition for groundwater. Based on lab and field data it is not expected that 1,2,4-triazole will be substantially produced from BAS 750 F under field conditions. M750F001 is included in the residue definition because of its commonality among triazole fungicides. Both compounds were assessed for groundwater concentrations and neither present a risk.

Surface Water and Sediment:

BAS 750 F
M750F001 (1,2,4-triazole)
M750F003
M750F005
M750F006
M750F007
M750F008

BAS 750 F and all relevant metabolites are considered in an aquatic risk assessment. Based on PEC_{sw} values (with conservative assumptions), no relevant risk could be derived.

Air:
BAS 750 F
No volatile metabolites were detected.

CA 7.4.2 Definition of the residue for monitoring

Soil: BAS 750 F (parent only)

Ground Water: BAS 750 F (parent only)

Surface Water: BAS 750 F (parent only)

Sediment: BAS 750 F (parent only)

Air: BAS 750 F (parent only)

CA 7.5 Monitoring data

Being a new A.I. there is no monitoring information available for BAS 750 F.

We create chemistry

BAS 750 F

Document M-CA, Section 8

ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Compiled by:

[redacted]

Telephone: [redacted]
E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 8 ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Introduction

BAS 750 F is a new fungicidally active substance intended for use in cereals.

This chapter provides the study summaries submitted in support of the first approval of the active substance BAS 750 F in Europe. Some of the studies are not required according to regulation 283/2013 but part of a global data package and provided for the sake of completeness.

Furthermore a literature search was performed on the parent molecule and the discovered metabolites from the soil and aquatic compartments. But, being a new active ingredient, no references in the literature were found for BAS 750 F. Literature search was also extended to metabolites and checked for relevance. A full set of the found references and assessment for relevance may be found in an Excel file attached to M-CA Section 9.

CA 8.1 Effects on birds and other terrestrial vertebrates

CA 8.1.1 Effect on birds

Table 8.1.1-1 provides information on the bird toxicity studies for the active ingredient BAS 750 F relevant for assessing the risk to birds.

Table 8.1.1-1: Summary of toxicity studies for the active substance BAS 750 F relevant for assessing the risk to birds

Test system	Test species	Reference [BASF DocID]
Acute oral toxicity	*Colinus virginianus*	2014/1095701
	Anas platyrhynchos	2014/1095700
	Serinus canaria	2015/1085493
Short term dietary toxicity	*Colinus virginianus*	2014/1127963 amendment 2015/1223324
	Anas platyrhynchos	2014/1117035
Sub-chronic toxicity and reproduction	*Colinus virginianus*	2013/1281276
	Anas platyrhynchos	2015/7005819

Table 8.1.1-2 lists the available toxicity studies in birds for the metabolites identified as relevant for the avian risk assessment. These studies were previously evaluated at the EU level as reviewed in the draft assessment report for epoxiconazole by the rapporteur member state Germany, Vol. 3, B.9 (March 2006). For details on these studies, consult this document.

Table 8.1.1-2: Summary of avian toxicity studies with metabolites of the active substance BAS 750 F

Test substance	Test system	Test species	Reference [Author, Year, BASF DocID]
Triazole alanine (M750F029)	Short-term dietary toxicity	*Colinus virginianus*	[redacted] 1983 1983/1000462
Triazole alanine (M750F029)	Short-term dietary toxicity	*Anas platyrhynchos*	[redacted], 1983 1983/1000463
Triazole acetic acid (M750F030)	Acute oral toxicity	*Colinus virginianus*	[redacted], 2003 2003/1004105

CA 8.1.1.1 Acute oral toxicity to birds

Report: CA 8.1.1.1/1
[redacted] 2014 a
BAS 750 F - Acute toxicity in the bobwhite quail (*Colinus virginianus*) after single administration (LD50)
2014/1095701
Guidelines: EPA 850.2100, EPA 850.2000
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The objective of the study was to determine a dose-response curve for avian mortality after oral dosing of the test substance and an observation period of at least 14 days to establish the acute LD_{50} (standard error and 95% confidence limits), as well as the slope of the dose response curve.

The test substance was administered via a single oral dose of 0 (control), 300, 480, 770, 1250 and 2000 mg active substance/kg body weight to groups of about 22 weeks old northern bobwhites. Ten birds (5 males and 5 females) were used in each test substance group. The test substance was applied undiluted directly into the crop. Birds have been fasted for about 17 to 19 hours. The birds were offered a commercial quail diet ad libitum throughout maintenance during the test with the exception of a fasting period prior to dosing and water ad libitum throughout bird maintenance and the test. The test was terminated after 14 days.

All groups were observed for mortality, clinical signs, impact on food consumption, and body weight for 14 consecutive days post dosing. Gross-pathological examinations were conducted on all birds that died during the study and all birds sacrificed at the termination of the observation period.

Highest dose tested causing no mortality: 300 mg a.s./kg b.w. for males and 480 a.s./kg b.w. for females. The following acute oral LD_{50} value of the test substance in bobwhite quail was determined at the end of the observation period to be LD_{50} = 816 mg a.s./kg b.w.. Mortality occurred on days 1 to 11 after dosing. No regurgitation was observed after dosing. Substance-related impairment of food uptake in comparison to the control was observed in all of the dose groups. Substance-related reduction of the body weights in the male and female birds was observed in the first week in all of the dose groups compared to the control. Overall, the body weight development was impaired by the test substance in the dose groups ≥ 480 mg a.s./kg b.w.. Toxic signs were observed in all dose groups.

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was 816 mg active substance / kg body weight. The "No Observed Effect Level" (NOEL) for mortality was 480 mg active substance / kg body weight.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No.5834378), CAS No.: 1417782-03-6, test substance number 11/0741-8, batch identification: COD-001740, purity 98.8% (tolerance ± 1.0%), homogeneous

B. STUDY DESIGN

Test species: Bobwhite quail (*Colinus virginianus*) before their first egg-laying season, visually indistinguishable from wild birds. Approx. 22 weeks at dosing, hatch date: 26 Apr 2013. Wachtelzucht Küberich GbR, Geesdorf-Wiesentheid, Germany.

Test design: Birds were administered single doses of 300, 480, 770, 1250 and 2000 mg a.s./kg b.w. of the test substance BAS 750 F. The test substance was applied undiluted in 2 gelatin capsules per animal directly into the crop, since this method is preferred by the test guideline. The control group was dosed with two empty capsules per animal. 5 males and 5 females per dose group were used. The birds were observed for regurgitation at least for 1 hour after dosing. An observation period of 14 days followed, during which mortalities and signs of toxicity were recorded, four times on the day of dosing and daily thereafter. Individual body weights were determined and group mean body weights calculated for male and female birds on the day of dosing and on days 7 and 14 after dosing. Mean food consumption (g/bird/day) was calculated from the weekly food consumption/cage separately for male and female birds for the first and second week after dosing. The cages were also checked for wasted food and excessive spills were recorded. A gross post-mortem examination was conducted for all birds that died during the study and all birds sacrificed by CO_2 asphyxia at the termination of the observation period. The examination included the GI tract, liver, kidney, heart, reproductive organs, spleen, subcutaneous fat, skeletal muscles and thyroid glands.

Endpoints: Mortality, clinical signs, feed consumption, body weight (b.w.), and gross-pathological examinations (conducted for all birds that died during the study and all birds sacrificed by CO_2 asphyxia at the termination of the study). Calculation of LD_{50} and NOEL.

Test concentrations: 0 (Control), 300, 480, 770, 1250 and 2000 mg a.s./kg body weight

Test conditions: Birds fasted for about 17 hours to 19 hours before administration of the test substance; temperature: 17.9 °C (minimum) and 21.8 °C (maximum); time below limits (21 °C ± 2 °C) was 13 hours; relative humidity: 44.9% and 100%; time below limits (45% - 70%) was 15 minutes. Time above limits was 15 days 12 hours and 30 minutes. The laboratory limits for relative humidity were exceeded during the daily cleaning. The deviations were considered to have no relevant influence on the test results, since the appearance and behaviour of the birds in the control group was normal and did not indicate any adverse effects; photoperiod: 8 hours light, 16 hours dark, warm-light fluorescent lamps. Light intensity (floor in the middle of the cages): 21 - 90 Lux.

Analytics: No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier. The purity of the test substance was analysed using HPLC.

Statistics: Statistical calculation of the LD_{50} was performed using probit-analysis according to Finney and a goodness-of-fit test (Pearson chi-square). The NOEL was calculated with Fisher´s exact test (one-sided).

II. RESULTS AND DISCUSSION

Analytical measurements:
No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier.

Biological results:
Highest dose tested causing no mortality was 300 mg a.s./kg b.w. for males and 480 a.s./kg b.w. for females. The following acute oral LD_{50} value of the test substance in bobwhite quail was determined at the end of the observation period: LD_{50} = 816 mg a.s./kg b.w. (95% confidence interval: 640 – 1038 mg active substance / kg body weight). Mortality occurred on days 1 to 11 after dosing. No regurgitation was observed during the first hour after dosing.

Toxic signs were observed in all dose groups. In the 300 mg a.s./kg b.w. dose group, diarrhea was observed until day 3 after dosing, most likely as a consequence of the reduced food uptake in the first week after dosing. In the 480 mg a.s./kg b.w. dose group, diarrhea was observed until day 5 after dosing for the same reason as in the lowest dose group. In one male, apathy was observed before it died and slight apathy was observed in 4 females. In the 770 mg a.s./kg b.w. dose group, diarrhea was observed until day 7 after dosing. Apathy was observed in all males and 3 females. In the 1250 mg a.s./kg b.w. dose group, diarrhea was observed until day 10 after dosing. Apathy was observed in all males and 3 females. One male bird was killed in extremis. In the 2000 mg a.s./kg b.w. dose group, diarrhea was observed until all birds died. Apathy was observed in all males and 3 females.

Substance-related impairment of food uptake in comparison to the control was observed in all of the dose groups. In the first week after dosing the food consumption was decreased in all dose groups. The decrease was clearly dose dependent with a reduction of 19% in comparison to the control group in the lowest dose group and a 91% reduction in the highest dose group. In the second week after dosing, the food consumption of the surviving birds returned to values which were similar to or higher than the food consumption of the control group, indicating a compensation effect, except for the surviving male animal of the dose group 1250 mg a.s./kg b.w.

Substance-related reduction of the body weights in the male and female birds was observed in the first week in all of the dose groups compared to the control. Corresponding to the decreased food consumption, the body weight development from day -1 to day 7 was negative in all dose groups with a dose-related trend. In the control group, the body weight development was close to 0. The deviation was statistically significantly different compared to the control group in dose groups ≥ 480 mg active substance/kg body weight. The evaluation of the body weight data for the females was potentially slightly affected by the higher mean body weight at dosing in the 300 mg a.s./kg b.w. group. However, taking into account the data for males, it is unlikely that the decrease in mean body weight for females would have been statistically significant without the incorrect recording of one female's weight in the 300 mg a.s./kg b.w. group on day -14. This resulted in a higher mean body weight for birds in this group at the time of dosing. In the second week after dosing, a statistically significantly increased body weight development in comparison to the control group was observed in dose groups 480 and 770 mg active substance / kg body weight. In conclusion, the body weight development was impaired by the test substance in the dose groups ≥ 480 mg active substance/kg body weight.

Several birds that died had liquid content in the gut (dose groups ≥ 480 mg active substance / kg body weight). In birds sacrificed at the end of the study, no abnormalities were observed. The relevant data and endpoints are summarized in the table below.

Table 8.1.1.1-1: Acute toxicity of BAS 750 F to the northern bobwhite (*Colinus virginianus*)

	Dose rate [mg a.s./kg b.w.]					
	0 (control)	300	480	770	1250	2000
Number of birds per dose group	10	10	10	10	10	10
Number of dead birds	0	0	1	6	7	10
Dead birds percentage [%]	0	0	10	60	70	100
Endpoints	Dose [mg a.s./kg b.w.]					
Highest dose causing no substance-related mortality	300					
LD_{50} (14 d)	816					
NOEL[1)]	480					

b.w. = body weight
1) NOEL is the highest tested concentration without significant substance related effects

III. CONCLUSION

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was 816 mg active substance / kg body weight (confidence interval: 640 – 1038 mg active substance / kg body weight). The "No Observed Effect Level" (NOEL) for mortality was 480 mg active substance / kg body weight.

Report:	CA 8.1.1.1/2 [redacted] 2014 b BAS 750 F - Acute toxicity in the mallard duck (Anas platyrhynchos) after single oral administration (LD50) 2014/1095700
Guidelines:	EPA 850.2100, EPA 850.2000
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The objective of the study was to provide evidence that the LD_{50} for the test substance is above the tested limit-dose.

The test substance was administered via a single oral dose of 2000 mg active substance / kg body weight to a group of about 24 week old mallard ducks. Ten birds (5 males and 5 females) were used in each test substance group. The test substance was applied undiluted in four gelatin capsules directly into the crop. The control group was dosed with four empty capsules per animal. Birds were fasted for about 17 to 18 hours. The birds were offered a commercial quail and duck diet ad libitum throughout maintenance during the test with the exception of a fasting period prior to dosing and water ad libitum throughout bird maintenance and the test. The test was terminated after 14 days.

The birds were observed for regurgitation for 1 hour after dosing. An observation period of 14 days followed, during which mortalities and signs of toxicity were recorded. Gross-pathological examinations were conducted on all birds that died during the study and all birds sacrificed at the termination of the observation period.

Highest dose tested causing no mortality: 2000 mg active substance / kg body weight for males and females. The following acute oral LD_{50} value of the test substance in mallard was determined at the end of the observation period to be LD_{50} > 2000 mg a.s./kg b.w. No regurgitation was observed after dosing, thus all birds received the full dose. No substance-related impairment of feed uptake in comparison to the control was observed in the tested dose group. The body weight was not statistically significantly reduced in the male and female birds at day 7 or at day 14 (sacrifice) in the tested dose group and the body weight development was not impaired in comparison to the control group. No toxic signs were observed in the control or in any dose group.

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was > 2000 mg a.s./kg b.w. The "No Observed Effect Level" (NOEL) for mortality was ≥ 2000 mg active substance / kg body weight.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No.5834378), CAS No.: 1417782-03-6, test substance number 11/0741-8, batch identification: COD-001740, purity 98.8% (tolerance ± 1.0%), homogeneous

B. STUDY DESIGN

Test species: Mallard duck (*Anas platyrhynchos*) before their first egg-laying season, visually indistinguishable from wild birds. Approx. 24 weeks at dosing, hatch date: 13 Apr 2013. Deindl GmbH & Co. KG, Geflügelzucht und –vertrieb, Rietberg, Germany.

Test design: Birds were administered a single dose of 2000 mg a.s./kg b.w of the test substance BAS 750 F. The test substance was applied undiluted in 4 gelatin capsules per animal directly into the crop, since this method is preferred by the test guideline. The control group was dosed with four empty capsules per animal. 5 males and 5 females per dose group were used. The birds were observed for regurgitation at least for 1 hour after dosing. An observation period of 14 days followed, during which mortalities and signs of toxicity were recorded, four times on day of dosing and daily thereafter. Individual body weights were determined and the group means calculated separately for male and female birds on the day of dosing and on days 7 and 14 after dosing. Mean food consumption (g/bird/day) was calculated from the weekly food consumption/cage separately for male and female birds for the first and second week after dosing. The cages were also checked for wasted food and excessive spills were recorded. A gross post-mortem examination was conducted for all birds that died during the study and all birds sacrificed by CO_2 asphyxia at the termination of the observation period. The examination included the GI tract, liver, kidney, heart, reproductive organs, spleen, subcutaneous fat, skeletal muscles and thyroid glands.

Endpoints: Mortality, clinical signs, feed consumption, body weight (b.w.), and gross-pathological examinations (conducted for all birds that died during the study and all birds sacrificed by CO_2 asphyxia at the termination of the study). Calculation of LD_{50} and NOEL.

Test concentrations: 0 (control) and 2000 mg a.s./kg body weight

Test conditions:	Birds fasted for about 17 hours to 18 hours before administration of the test substance; temperature: 16.4 °C (minimum) and 19.8 °C (maximum); time below limits (21 °C ± 2 °C) was 8 hours, no time above limits; relative humidity: 51.8% (minimum) and 96.2% (maximum), no time below limits (45% - 70%). Time above limits was 6 hours. The deviations were considered to have no relevant influence on the study results, since the birds were apparently healthy during the whole acclimation and observation period; photoperiod: 8 hours light, 16 hours dark, warm-light fluorescent lamps. Light intensity (floor in the middle of the cages): 59 - 66 Lux.
Analytics:	No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier. The purity of the test substance was analysed using HPLC.
Statistics:	No statistical calculation of the LD_{50} was performed since no mortality was observed in the tested dose. The NOEL was calculated with Fisher´s exact test.

II. RESULTS AND DISCUSSION

Analytical measurements:
No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier.

Biological results:
Highest dose tested causing no mortality was 2000 mg a.s./kg b.w.. The following acute oral LD_{50} value of the test substance in mallard duck was determined at the end of the observation period: LD_{50} > 2000 mg a.s./kg b.w. No regurgitation was observed after dosing.

No toxic signs were observed in the control or in the dose group. Highest dose tested without toxic signs: 2000 mg active substance / kg b.w.

The body weight was not statistically significantly reduced in the male and female birds at day 7 or at day 14 (sacrifice) in the dose group and the body weight development was not impaired in comparison to the control group.

No abnormalities were observed during gross post-mortem examinations.

Table 8.1.1.1-2: Acute toxicity of BAS 750 F to the mallard duck (*Anas platyrhynchos*)

	Dose rate [mg a.s./kg b.w.]	
	0 (control)	2000
Number of birds per dose group	10	10
Number of dead birds	0	0
Dead birds percentage [%]	0	0

Endpoints	Dose [mg a.s./kg b.w.]
Highest dose causing no substance-related mortality	2000
LD_{50} (14 d)	> 2000
NOEL[1)]	≥ 2000

b.w. = body weight
1) NOEL is the highest tested concentration without significant substance related effects

III. CONCLUSION

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was > 2000 mg active substance / kg body weight. The "No Observed Effect Level" (NOEL) for mortality was ≥ 2000 mg active substance / kg body weight.

Report: CA 8.1.1.1/3
[redacted] 2015a
BAS 750 F - Acute toxicity in the canary (Serinus canaria) after single oral administration (LD50)
2015/1085493
Guidelines: EPA 850.2100
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The objective of the study was to determine the LD_{50} in canary after a single oral administration of the test substance and a 14-day post-dosing observation period. For this reason three experiments (experiment 1 and 2 both limit tests, experiment 3 was the definitive test) were conducted. Only experiment 3 was considered as sufficient for risk assessment and is reported in detail in this summary (experiments 1 and 2 were unsuccessful limit tests).

The test substance was administered via a single oral dose of 0 (control), 1001, 1302, 1692, 2200 and 2860 mg a.s./ kg b.w. to a group of 18 week old canaries. Ten birds (5 males and 5 females) were used in each test substance group. The test substance was applied undiluted in three gelatin capsules directly into the crop. The control group was dosed with three empty capsules per animal. Birds were fasted for about 2 to 3 hours. The birds were offered a commercial diet for canaries ad libitum throughout maintenance including acclimation and observation period after dosing with the exception of a fasting period prior to dosing and water ad libitum throughout bird maintenance and the test. The test was terminated after 14 days.

The birds were observed for regurgitation for 1 to 2 hours after dosing. An observation period of 14 days followed, during which mortalities, signs of toxicity, food consumption and body weight were recorded. Gross-pathological examinations were conducted on all birds that died during the study and all birds sacrificed at the termination of the observation period.

The NOEL for mortality was: ≥ 2860 mg a.s./kg b.w. for males and females. The acute oral LD_{50} value of the test substance in canaries was determined at the end of the observation period to be LD_{50} > 2860 mg a.s./kg b.w.. No regurgitation was observed after dosing. Thus, all birds received the full dose. No substance-related impairment of feed uptake in comparison to the control was observed in the tested dose group. The body weight was not statistically significantly reduced in the male and female birds at day 7 or at day 14 (sacrifice) in the tested dose group and the body weight development was not impaired in comparison to the control group. No toxic signs were observed in the control group.

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was > 2860 mg a.s./kg b.w.. The "No Observed Effect Level" (NOEL) for mortality was ≥ 2860 mg active substance / kg body weight.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No.5834378), CAS No.: 1417782-03-6, test substance number 11/0741-8, batch identification: COD-001740, purity 98.8% (tolerance ± 1.0%), homogeneous

B. STUDY DESIGN

Test species: Canary (*Serinus canaria*) before their first egg-laying season, phenotypically indistinguishable from wild birds. Supplier: Zoowelt – Welt der Tiere, Bechtheim, Germany

Age of the test animals: 18 weeks at dosing, hatch date: 21 to 27 July 2014

Test design: Birds were administered doses of 0 (control), 1001, 1302, 1692, 2200 and 2860 mg a.s./kg b.w. of the test substance BAS 750 F. The test substance was applied undiluted in soluble hard gelatin capsules directly into the crop after a fastening time of 2 to 3 hours. 3 capsules per animal were used; 5 males and 5 females per dose group received the treatment. The control groups received the same amount of capsules like in the treatment groups. The birds were observed for regurgitation for 1 to 2 hours after dosing. An observation period of 14 days followed, during which mortalities and signs of toxicity were recorded. A check for moribund and dead animals was made before dosing, about 1, 2 and 4 hours after dosing, and daily thereafter. The check for clinical signs was following the same procedure like for mortality.

Mean food consumption (g/animals/day) was calculated for males and females separately every two days by cage. The measurement of the food consumption on day 0 started directly after dosing.

Determination of individual body weights and calculation of the group means separately for male and female animals on day -14 (for randomization), on the day before dosing and on days 7 and 14 after dosing. A gross post-mortem examination was conducted for all birds that died during the study and all animals sacrificed by CO_2 asphyxia at the termination of the observation period. The examination included the GI tract, liver, kidney, heart, reproductive organs, spleen, subcutaneous fat, skeletal muscles and thyroid glands.

Endpoints: Mortality, clinical observation, food consumption, body weight (b.w.), and gross-pathological examinations (for all birds that died during the study and all animals sacrificed by CO_2 asphyxia at the termination of the study). Calculation of LD_{50} and NOEL.

Test concentrations: 0 (control), 1001, 1302, 1692, 2200 and 2860 mg a.s./kg b.w.

Test conditions: The birds were fasted for 2 to 3 hours before administration of the test substance. Birds were observed for signs of regurgitation for 1 to 2 hours after dosing. Measured temperature: 21.0 °C (minimum) and 22.3 °C (maximum); no time above/below the limits; relative humidity: 41.8% (minimum) and 63.2% (maximum), no time above the limit, 2 days were below the limit. The deviations had no relevant influence on the study results, since the limits were exceeded only to a small extent within an acceptable range; photoperiod: 10 hours light, 14 hours dark, warm-light fluorescent lamps. Light intensity (floor in the middle of the cages): 89 - 98 Lux.

Analytics: No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier. The purity of the test substance was analysed using HPLC.

Statistics: No statistical calculation of the LD_{50} was performed since no dose response for mortality was observed in the tested doses. The NOEL was calculated using Fisher's exact test (one sided). For body weight and body weight change, Dunnett-test (two-sided) was used to compare dose groups with the control group. The food consumption was not examined statistically, since the food consumption was measured only per cage not per animal.

II. RESULTS AND DISCUSSION

Analytical measurements:
No analytical determinations of the test substance in the carrier were necessary since the test substance was applied without carrier.

Biological results:
Diarrhea on the day of dosing was considered to be a consequence of the fasting period and was observed in the dose groups as well as in the control group. Therefore, it was not considered to be a substance-related effect.

The highest dose tested causing no test substance-related mortality was 2860 mg a.s./kg b.w. The following acute oral LD_{50} value of the test substance in canary was determined at the end of the observation period: LD_{50} > 2860 mg a.s./kg b.w. No regurgitation was observed after dosing. One female animal of the lowest dose group (1001 mg a.s./kg b.w.) died one day after dosing, as well as one female animal in the highest dose group (2860 mg a.s./kg b.w). The mortality did not exceed 10% and no further mortality was observed in any other group. The mortality in the lowest dose group cannot be attributed to the test substance and was considered to be spontaneous. Therefore, there was no dose-response relationship in the findings. Hence, the LD_{50} calculation by Probit analysis was not performed.

Apathy was observed in all dose groups:

- 1001 mg a.s./kg b.w.: In one male and 3 female animals on the day of dosing.
- 1302 mg a.s./kg b.w.: In 5 female animals on the day of dosing.
- 1692 mg a.s./kg b.w.: In 5 female animals up to 1 day after dosing.
- 2200 mg a.s./kg b.w.: In 2 male animals up to 2 days after dosing and 5 female animals on the day of dosing.
- 2860 mg a.s./kg m.w.: In 5 female animals up to 1 day after dosing.

No toxic signs were observed in the control. Highest dose tested without toxic signs: < 1001 mg a.s./kg b.w..

No marked substance-related impairment of feed uptake in comparison to the control was observed in any of the tested dose groups.

The body weight was not statistically significantly reduced in the male and female birds at day 7 or at day 14 (sacrifice) in the tested dose group and the body weight development was not impaired in comparison to the control group.

No substance-related findings were observed during the gross post-mortem examination. In one female of the dose group 1001 mg a.s./kg b.w., a liquid content of the rectum was observed. See below for a table of summarized results:

Table 8.1.1.1-3: Acute toxicity of BAS 750 F to the canary (*Serinus canaria*)

	Dose rate [mg a.s./kg b.w.]					
	0 (control)	1001	1302	1692	2200	2860
Number of birds per dose group	10	10	10	10	10	10
Number of dead birds	0	1	0	0	0	1
Dead birds percentage [%]	0	10	0	0	0	10
Endpoints	**mg a.s./kg b.w.**					
Highest dose causing no substance-related mortality	2860					
LD_{50} (14 d)	> 2860					
NOEL[1)]	≥ 2860					

b.w. = body weight
1) NOEL is the highest tested concentration without significant substance related effects

III. CONCLUSION

The acute oral median lethal toxicity (LD_{50}) of BAS 750 F was > 2860 mg a.s./ kg b.w. The "No Observed Effect Level" (NOEL) for mortality was ≥ 2860 mg active substance / kg body weight.

CA 8.1.1.2 Short-term dietary toxicity to birds

Report:	CA 8.1.1.2/1 [redacted] 2014 c BAS 750 F - Avian dietary toxicity test in chicks of the bobwhite quail (Colinus virginianus) 2014/1127963
Guidelines:	EPA 850.2200, OECD 205, EPA 850.2000
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Report:	CA 8.1.1.2/2 [redacted] 2015 a Amendment No. 1 - BAS 750 F - Avian dietary toxicity test in chicks of the bobwhite quail (Colinus virginianus) 2015/1223324
Guidelines:	EPA 850.2200, OECD 205, EPA 850.2000, EPA 712-C-026, EPA 712-C-024
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

An avian acute dietary toxicity test with the active substance BAS 750 F was conducted. The objective of the study was the determination of the LC_{50} and the slope of the concentration effect curve in chicks of the bobwhite quail (*Colinus virginianus*) after dietary exposure of the test substance over 5 days plus a post exposure period of at least 3 days. Additionally, information on sublethal effects, an LDD_{50} and a no observable effect level (NOEL) were determined.

The test substance was administered via the diet for 5 days at concentrations of 1480, 2222, 3333, 5000 and 7500 mg active substance / kg diet to 13-day old northern bobwhite quails. Ten birds were used in each group.

All groups were observed for mortality, signs of clinical toxicity, impact on food consumption and body weight for 6 consecutive days post dosing. Birds of all groups received food and water ad libitum throughout the test.

Substance-related mortality was observed in test concentrations ≥ 3333 mg active substance / kg diet. The food consumption in the 2 highest groups was markedly decreased, so that starvation might have contributed to the observed mortality. The minimum concentration causing 100% mortality was > 7500 mg/kg diet. The LC_{50} was 6377 mg active substance/kg diet (95% confidence interval: 4909 – 12086 mg active substance/ kg diet). No clinical signs of toxicity were observed in the control group and concentration groups ≤ 5000 mg active substance / kg diet. During the 5-day exposure period the food consumption was decreased clearly in all tested concentration groups.

The body weights of the surviving chicks in all treated groups were statistically significantly decreased at the end of the exposure period on day 5. The body weight development during the exposure period was statistically significant decreased in comparison to the control group in all tested concentration groups. No substance-related effects were identified during the gross post-mortem examinations.

In an acute oral toxicity test with bobwhite quail chicks (*Colinus virginianus*), the LDD_{50} of BAS 750 F was found to be 858 mg a.s./kg b.w./day (LC_{50} = 6377 mg a.s./kg diet). The NOEL for mortality was 769 mg a.s./kg b.w./day.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Bobwhite Quail (*Colinus virginianus*), chicks, hatched from eggs of animals indistinguishable from wild birds. Age: 13 days old at start of substance feeding; Source: Geflügelzucht Küberich, 97353 Geesdorf/Wiesentheid, Germany.

Test design: The test substance was administered via the diet for 5 days at concentrations of 1480, 2222, 3333, 5000 and 7500 mg active substance / kg diet to 13-day old northern bobwhite quails. The birds were not sexed since sex determination is very uncertain and difficult at that age. 10 animal per dose group were used; dietary exposure period of 5 days plus a post exposure observation period of 6 days; assessment of mortality and signs of clinical toxicity was carried out four times on day of dosing and daily thereafter; assessment of body weight was carried out on days 0, 5, 8 and 11; The food consumption was determined per cage by weighing the food hopper at the start of feeding and before and after each refill. The weighing was performed daily. A gross post-mortem examination was conducted for all birds that died during the study and all animals sacrificed by CO_2 asphyxia at the termination of the study. The examination included the GI tract, liver, kidney, heart, reproductive organs, spleen, subcutaneous fat, and skeletal muscles.

Endpoints: Mortality, clinical signs, feed consumption, body weight (b.w.), and gross-pathological examinations (conducted for all birds that died during the study and all animals sacrificed by CO_2 asphyxia at the termination of the study). Calculation of LC_{50}, LDD_{50} and NOEL.

Test concentrations: 0 (Control), 1480, 2222, 3333, 5000 and 7500 mg a.s./kg body weight (nominal concentration based on active substance/kg diet).

Test conditions: Chicks were administered treated feed for 5 consecutive days followed by a post-exposure period of six days basal diet ad libitum without test substance; temperature: 19.7 °C – 23.6 °C; relative humidity: 34.2 - 100%; photoperiod: 14 hours light, 10 hours dark, light intensity 51 - 58 Lux in the middle of the cages on the floor level, warm light fluorescent lamps.

Analytics: The test substance concentrations were analysed using HPLC.

Statistics: LC_{50} and LDD_{50} Calculation: Probit-analysis according to Finney and Goodness-of-Fit - Test (Pearson Chi-Square); NOEL calculation: Fisher's exact test (one-sided); comparison of b.w.: Dunnett test (two-sided). The daily dose for each concentration group was calculated according to the following formula:

$$\text{Daily dose (mg/kg b.w./day)} = \frac{\text{Uptake test substance (mg/bird/day) * 1000 (g/kg)}}{\text{Mean body weight of day 0 and day 5 (g/bird)}}$$

II. RESULTS AND DISCUSSION

Analytical results:
The concentration control analyses in the feed yielded concentrations in a range of 96 - 114% of the nominal concentrations. The values indicated that the measured concentrations were in good agreement with the nominal concentrations and that the concentration of the test substance in the diet mix did not decrease under study conditions during the exposure period.

Biological results:
Substance-related mortality was observed in test concentrations ≥ 3333 mg active substance / kg diet. The food consumption in the 2 highest groups was markedly decreased, so that starvation might have contributed to the observed mortality. The minimum concentration causing 100% mortality was > 7500 mg/kg diet. The LC_{50} was 6377 mg active substance/kg diet (95% confidence interval: 4909 – 12086 mg active substance/kg diet). The LDD_{50} calculated on the basis of daily doses was 858 mg a.s./kg b.w./day. The mortality rate was not correlated with the daily dose. Possible explanations: The effect of starvation might have influenced the result more than the toxicity of the ingested test substance. No clinical signs of toxicity were observed in the control group and concentration groups ≤ 5000 mg a.s./kg diet.
During the 5-day exposure period, the food consumption was decreased clearly in all tested concentration groups. The decrease in the 3 lower concentration groups was similar and not correlated to the concentration of the test substance. In the two highest concentrations, the reduction was very pronounced. During the 6-day post-exposure period the food consumption of the surviving chicks in concentration groups ≤ 5000 mg a.s./kg diet was still lower than the food consumption of the control group (62 - 79% of the value from the control group). In the highest concentration group (7500 mg a.s/kg diet), the food consumption of the surviving birds indicated a compensation for the low food uptake during exposure with a food consumption of 112% of the value of the control group.

The body weights of the surviving chicks in all treated groups were statistically significantly decreased at the end of the exposure period on day 5. This is obviously an effect of the reduced food uptake. However, different to the food uptake, the body weight reduction clearly followed a concentration related trend also in the 3 lowest concentration groups. At the end of the 6 day post-exposure period the body weights of the groups exposed to 3333 and 5000 mg a.s./kg diet were still statistically significantly different from the control.
The body weight development during the exposure period was statistically significantly decreased in comparison to the control group in all tested concentration groups. During the post exposure period, the body weight development in the surviving chicks was similar to the control group and even statistically significantly increased during days 8 and 11 in the highest concentration group receiving 7500 mg a.s./kg diet. In all tested concentration groups the body weight development indicated a recovery after the end of exposure. No substance-related macroscopic abnormalities were detected in the gross-pathological post-mortem examination. The relevant endpoints are summarized in the table below.

Table 8.1.1.2-1: Avian dietary toxicity of BAS 750 F to the bobwhite quail (*Colinus virginianus*)

Parameter	Dose groups [mg a.s./kg diet]					
	Control	**1480**	**2222**	**3333**	**5000**	**7500**
Mortality [dead/survivor]	0/10	0/10	0/10	3/10	1/10[1)]	7/10
Daily dose [mg a.s./kg b.w./d] [4)]	not applicable	221	439	650	769	653
Mean feed consumption during (exposure) days 1 to 5 [g feed/bird/day]	6.8	3.8	4.7	4.4	3.2	2.0
Mean body weight on day 0 and day 5 [g/bird] [2)3)]	24.8/33.6	23.5/28.1**	25.1/27.3**	24.0/23.1**	24.3/21.4**	24.9/21.8**
Clinical signs	n.d.	n.d.	n.d.	n.d.	n.d.	a, t
Endpoints [mg a.s./kg diet]						
LC_{50}	6377					
NOEL (mortality)	5000					
Endpoints [mg a.s./kg b.w./day]						
LDD_{50}	858					
NOEL (mortality)	769					

a.s. = active substance
b.w. = body weight
n.d. = no symptoms detected
a = apathy
t = tumbling

1) One chick died in the post-exposure period.
2) Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01
3) Statistical analysis did not reveal significant differences
4) Based on measured concentration of active substance in the diet

III. CONCLUSION

Under the conditions of this study the LC_{50} for chicks of the bobwhite quail (*Colinus virginianus*) was 6377 mg active substance/kg diet. The LDD_{50} calculated on the basis of daily doses was 858 mg a.s. / kg b.w. / day. The NOEL for mortality calculated on the basis of daily dose was 769 mg / kg b.w. /day.

Report:	CA 8.1.1.2/3 [redacted] 2014 d BAS 750 F - Avian dietary toxicity test in ducklings of the mallard duck (Anas platyrhynchos) 2014/1117035
Guidelines:	EPA 850.2200, OECD 205, EPA 850.2000
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The objective of the study was the determination of the LC_{50} and the slope of the concentration effect curve in chicks of the mallard duck after dietary exposure of the test substance over 5 days plus a post exposure period of at least 3 days. Additionally, information on sublethal effects, an LDD_{50} and no observable effect level (NOEL) were determined.

The test substance was administered at concentrations of 0 (control), 1480, 2222, 3333, 5000 and 7500 mg a.s./kg diet. Ten 5-days old mallard ducklings were used in each substance group and 10 in the control group. All groups were observed for mortality, signs of clinical toxicity, impact on food consumption and body weight during the exposure period of 5 days and for a post-exposure period of 3 days. Birds of all substance groups and the control group received feed and water ad libitum throughout the test.

Biological results:
No mortality was observed in the control group. The highest concentration causing no test substance related mortality was 3333 mg a.s./kg diet, corresponding to a daily dose of 506 mg a.s./kg b.w. In the substance group receiving 5000 mg a.s./kg diet, 40% mortality was observed. In the substance group receiving 7500 mg a.s./kg diet, 30% mortality was observed. The mortality in the two higher concentrations was most likely caused by starvation. No clinical signs of toxicity related to the test substance were observed in any of the treatment groups. During the 5-day exposure period the food consumption was decreased markedly in all tested concentration groups to 18.5 - 55.1% of the food consumption of the control group. The decrease followed generally a concentration-related trend, however with some inconsistency. The food consumption was low enough to expect mortality from starvation in all tested groups. Mortality was observed, however, only in the two highest test concentrations. During the 3-day post-exposure period the food consumption of the surviving ducklings in all concentration groups returned to values between 64 and 104% of the food consumption in the control group without a clear concentration-related trend.

The body weights of the surviving ducklings on days 5 and 8 and the body weight development were statistically significantly decreased in all groups that were exposed to the test substance. The decrease followed a concentration-related trend. In the two highest concentrations, the body weight development was negative during the exposure period. In many birds of the treatment groups that were sacrificed on day 8, a discoloration of the liver was detected. The histological examination revealed a multifocal coagulation necrosis and fatty degeneration. The finding was often seen in combination with reduced body size and is most likely a consequence of starvation.

The LC_{50} of BAS 750 F for juvenile mallards (*Anas platyrhynchos*) was determined to be 8347 mg a.s./kg diet and the NOEL for mortality was 3333 mg a.s./kg diet, corresponding to a daily dose of 506 mg a.s./kg b.w.. Calculated on the basis of daily doses, the LDD_{50} was 1213 mg a.s./kg b.w./day.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5834378), batch no. COD-001740, purity: 98.8% (tolerance ± 1%).

B. STUDY DESIGN

Test species: Mallard duck (*Anas platyrhynchos*), ducklings; chicks, age: 5 days; hatched from eggs of animals visually indistinguishable from wild birds; source: Deindl GmbH & Co. KG Geflügelzucht und –vertrieb, Rietberg, Germany.

Test design: The test substance was administered via the diet for 5 days at concentrations of 0 (control), 1480, 2222, 3333, 5000 and 7500 mg a.s./kg diet to mallard ducklings, with a post-exposure period of 3 days; 10 birds per test substance concentration and 10 for the control group were used; assessment for mortality and clinical signs was carried out four times on the first day of exposure and daily thereafter; Birds were weighed individually on days 0, 5 and 8. The mean body weight was calculated for each of these days for each pen (test group); Mean food consumption per bird and day for the substance feeding and the post-exposure period was calculated from the daily mean food consumption of each group. Gross post-mortem examination was conducted for all birds that died during the test period and all birds sacrificed by CO_2 asphyxia at the end of the post-exposure period.

Endpoints: Mortality, clinical signs, feed consumption, body weight (b.w.) and gross-pathological examinations (conducted for all birds that died during the test period and all birds sacrificed by CO_2 asphyxia at the end of the post-exposure period). Calculation of LDD_{50}, LC_{50} and NOEL.

Test concentrations: 0 (Control), 1480, 2222, 3333, 5000 and 7500 mg a.s./kg diet.

Test conditions: Temperature: 17.1 °C - 21.6 °C (21±2 °C limits with time below limits of 1 d 7 h and 30 minutes); relative humidity: 49.6 - 100% (45% - 70% limits with time above limit of 7 d, 7 h and 15 minutes); photoperiod: 14 hours light: 10 hours dark, light intensity: 60 - 95 Lux in the middle of the cages on the floor level, warm light fluorescent lamps.

Analytics: The test substance concentrations were analyzed using HPLC.

Statistics: LC_{50} and LDD_{50} calculation (mortality): Probit-analysis according to Finney and Goodness-of-Fit - Test (Pearson Chi-Square); NOEL calculation: Fisher's exact test (one-sided); comparison of b.w.: Dunnett test (two-sided). The daily dose for each concentration group was calculated according to the following formula:

$$\text{Daily dose (mg/kg b.w./day)} = \frac{\text{Uptake test substance (mg/bird/day) * 1000 (g/kg)}}{\text{Mean body weight of day 0 and day 5 (g/bird)}}$$

II. RESULTS AND DISCUSSION

Analytical measurements:
The results of the analytical verification of the test substance concentration in the diet were within a range of 93% to 110% of the nominal concentrations during the test. The biological results are therefore based on the nominal values.

Biological results:
No mortality was observed in the control group. The highest concentration tested causing no substance-related mortality was 3333 mg active substance/kg diet, corresponding to a daily dose of 506 mg a.s./kg b.w. Substance-related mortality was observed in the test concentrations: ≥ 5000 mg active substance / kg diet. The mortality in the two higher concentrations was most likely caused by starvation. No other toxic signs were observed up to and the highest test concentration (7500 mg a.s./kg diet). The ducklings that died during and shortly after exposure had a markedly reduced body weight. Thus, the concentration effect relationship in the high concentrations is very flat and did not correlate with daily doses, since avoidance is highly variable for different individuals. The calculated LC_{50} was 8347 mg active substance/kg diet (95% confidence interval: 6068 – 64610 mg active substance/kg diet; extrapolated value), exceeding the highest tested concentration group of 7500 mg active substance / kg diet. The LDD_{50} calculated on the basis of daily doses was 1213 mg active substance / kg bodyweight / day. No clinical signs of toxicity, related to the test substance, were observed in any treatment group. During the 5-day exposure period, the food consumption was decreased markedly in all tested concentration groups to 18.5 - 55.1% of the food consumption of the control group. The decrease followed generally a concentration-related trend, with some inconsistency. The food consumption was low enough to expect mortality from starvation in all tested groups. Mortality was observed, however, only in the two highest test concentrations.

During the 3-day post-exposure period, the food consumption of the surviving ducklings in all concentration groups returned to values between 64 and 104 % of the food consumption in the control group without a clear concentration-related trend. No excessive spill of food was observed for any of the test groups over the whole observation period. The body weights of the surviving ducklings on days 5 and 8 and the body weight development were statistically significantly decreased in all groups that were exposed to the test substance. The decrease followed a concentration-related trend. In the two highest concentrations, the body weight development was negative during the exposure period. In many birds of the treatment groups that were sacrificed on day 8, a discoloration of the liver was detected during the gross post mortem examination. The histological examination revealed a multifocal coagulation necrosis and fatty degeneration. The finding was often seen in combination with reduced body size and is most likely a consequence of starvation. No abnormalities were observed in the control group. Results are presented in the table below.

Table 8.1.1.2-2: Avian dietary toxicity of BAS 750 F to the mallard duck (*Anas platyrhynchos*)

Parameter	Group [mg a.s./kg diet]					
	Control	**1480**	**2222**	**3333**	**5000**	**7500**
Daily dose [mg a.s./kg b.w.] 1)	not applicable	244	475	506	446	763
Mortality [%] (n=10)	0.0	0.0	0.0	0.0	40.0	30.0
Mean feed consumption during days 1 to 5 [g feed/bird/day]	31	13.6	17.1	11.2	5.7	6.6
Mean body weight on day 0 and day 5 [g/bird]	79.4 / 165.3	64* / 94.4**	78.5 / 94.9**	77.6 / 81.2**	71.1 / 64.9**	73.7 / 60.3**
Clinical signs	None	None	None	None	None	None
	Endpoints [mg a.s./kg diet]					
LC_{50}	8347					
NOEL	3333					
	Endpoint [mg a.s./kg b.w./day]					
LDD_{50}	1213					
NOEL	506					

a.s. = active substance
b.w. = body weight
Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01,

1) Based on measured concentration of active substance in the diet
2) The summary body weights are based on the mean body weights per duckling. Since the data recording system has no option to provide the data correctly, the summary body weights are provide as data for males.

III. CONCLUSION

Under the conditions of this study, the LC_{50} for mallard ducklings (*Anas platyrhynchos)* was 8347 mg a.s./kg diet (95% confidence interval: 6068 – 64610 mg a.s./kg diet; extrapolated value). The LC_{50} exceeded the highest tested dietary concentration group of 7500 mg a.s./ kg diet. Calculated on the basis of daily doses, the LDD_{50} was 1213 mg a.s./kg b.w./day. The "No Observed Effect Level" (NOEL) for mortality was 3333 mg a.s./kg diet. The NOEL for mortality calculated on the basis of daily dose was 506 mg a.s./kg b.w./day.

CA 8.1.1.3 Sub-chronic and reproductive toxicity to birds

Report:	CA 8.1.1.3/1 [redacted] 2014a BAS 750 F: A reproduction study with the northern bobwhite 2013/1281276
Guidelines:	EPA 850.2300, OECD 206
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The objective of this study was to evaluate the effects upon the adult northern bobwhite quails (*Colinus virginianus*) of dietary exposure to BAS 750 F over a five-month period. Effects on adult health, body weight, body weight change and feed consumption were evaluated. In addition, the effects of adult exposure to BAS 750 F on the number of eggs laid, normal development of eggs, embryo viability, hatchability, offspring survival and egg shell thickness were evaluated.

Northern bobwhite quails (72 males and 72 females) were randomly distributed into one control group and three treatment groups. Each treatment and control group contained 18 pairs of birds with one male and one female per pen. Three treatment groups were fed diets containing 150, 285 or 543 mg a.s./kg diet of BAS 750 F for 21 weeks. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

Each treatment and control group contained a total of 18 pairs of birds per concentration. All adult birds were observed daily throughout the test for signs of toxicity or abnormal behaviour. Adult body weights were measured at test initiation, at the end of week 2, 4, 6, 8, and at termination of the adults. Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production. Feed consumption was measured weekly throughout the test. All surviving adults were subjected to gross necropsy following termination of the adults. All findings observed were considered unrelated to treatment.

Reproductive parameters included eggs laid/hen/day, eggs cracked of eggs laid, fertile eggs of eggs set, viable embryos from eggs set, live three weeks embryos of viable embryos, hatchlings of 3 week embryos, 14-day old survivors of hatchlings, hatchlings of eggs set, hatchling of fertile eggs, 14-day old survivors of eggs set, hatchling/pen/day, 14-day old survivor/pen/day, offspring body weights and egg shell thickness.

Analysis of the control samples did not show any indication of the presence of the test substance or of the presence of a co-eluting substance at the characteristic retention time of the test substance.

Dietary concentrations were adjusted for purity of the test substance. Nominal preparation was based on a previously reported purity of 94.54% and prepared respectively.

No treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight were found at the 150, 285 or 543 mg a.s./kg diet test concentrations. There were no treatment-related effects upon feed consumption at the 150 or 285 mg a.s./kg diet test concentrations. At the 543 mg a.s./kg diet test concentration, there was a slight reduction in feed consumption during week 1 that may have been related to treatment.

There were no treatment related effects upon any of the reproductive parameters measured at the 150 or 285 mg a.s./kg diet test concentration. However, at the 543 mg a.s./kg diet test concentration there was a marked, statistically significant ($p \leq 0.01$) reduction in egg production and a reduction in offspring survival (not statistically significant). The no-observed-effect concentration for northern bobwhite quails exposed to BAS 750 F in the diet during the study was 285 mg a.s./kg diet (25.3 mg a.s./kg b.w./day).

The no-observed-effect concentration (NOEC) for northern bobwhite quails exposed to BAS 750 F in the diet during the study was 285 mg a.s./kg diet (25.3 mg a.s./kg b.w./day).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F, Reg. No.: 5834378, Batch No.: COD-001740, purity: 98.8%

B. STUDY DESIGN

Test species: Northern bobwhite quails (*Colinus virginianus*), phenotypically indistinguishable from wild type; adults, age: 23 weeks of age at the initiation of the test (before beginning of first egg-laying period); supplier: Trace Pheasantry, 288 Levengood Road, Douglassville, PA 19518.

Test design: Northern bobwhite quails approaching their first breeding season were kept in a group of 1 male and 1 female in a pen per replicate. 18 pens were allocated to the control and each treatment group. All adult birds and their offspring were given feed and water *ad libitum* during acclimation and testing. The study period was divided into five phases: 1. Acclimation to laboratory conditions – 6 weeks; 2. Pre-photostimulation – 8 weeks; 3. Pre-egg laying (with photostimulation) – 3 weeks; 4. Egg laying period – 10 weeks; 5. Post-adult termination (final incubation, hatching and 14-day offspring rearing period) – 6 weeks. Eggs were collected daily from all pens and stored in a cold room. At the end of a weekly interval, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement.

	Cracked or abnormal eggs were recorded and discarded. All eggs not discarded or used for egg shell thickness measurements were placed in an incubator. On day 21 of incubation, eggs were moved to a hatcher. Young birds were maintained for 14 days. Adult birds were sacrificed after the egg-laying period, young birds after 14 days.
Endpoints:	Adult birds: mortalities, clinical observations, gross necropsy, adult body weight and adult feed consumption Reproductive parameters: Eggs laid/hen/day, eggs cracked of eggs laid, fertile eggs of eggs set, viable embryos of eggs set, live 3-week embryos of viable embryos, hatchlings of 3 week-Embryos, body weight hatchlings, 14-day old survivors of hatchlings, hatchlings of eggs set, hatchlings of fertile eggs, hatchlings/pen/day, 14-day old survivors/pen/day, 14-day old survivors of eggs set, hatchling body weight and egg shell thickness
Test concentrations:	0 (Control), 150, 285 and 543 mg a.s./kg diet BAS 750 F (nominal).
Test conditions:	Adult bobwhite study room: Temperature 19.3 ± 1.6 °C (SD); relative humidity: 38 ± 14% (SD); ventilation: 15 times the room air /h; photoperiod: 7 hours light (week 1 – 8) approx. 331 Lux, lengthened photoperiod to 17 hours light (week 9 to the end of study) approx. 344 lux and during egg-laying approx. 242 Lux. Egg collection and storage: Collected daily, stored in cold room: temperature: 13.7 ± 0.3 °C (SD), relative humidity: 67 ± 8% (SD). Eggs set for incubation: temperature: 37.5 ± 0.0 °C (SD), relative humidity 55 ± 0.0 °C (SD); the eggs were transferred to the hatcher on day 21: temperature: 37.3 ± 0.0 °C (SD), relative humidity approximately 57 ± 1% (SD) Hatchlings: Brooding compartment temperature approximately 38°C from hatching until the birds were 14 days of age; average ambient room temperature 26.5 ± 2.7 °C (SD), relative humidity: 20 ± 8%; photoperiod: 16 hours light per day
Analytics:	The test substance concentrations were analysed using HPLC with UV detection
Statistics:	Analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Comparison of control and treatment groups: Dunnett´s multiple comparison procedure. Percentage data: Dunnett´s method following arcsine square root transformation

II. RESULTS AND DISCUSSION

Analytical measurements:
The result of the analytical verification of the test substance was 98.8 %. Concentrations of the test substance in the diet were adjusted to 100% active substance. Mean concentrations and standard deviations for the three test concentrations were 156 ± 4.18, 293 ± 4.59, and 569 ± 7.66 mg a.s./kg diet for the nominally 150, 285, and 543 mg a.s./kg diet concentrations, respectively.

Biological results:
Parental generation
While no adult mortalities occurred in the control group or the 285 mg a.s./kg diet treatment group, one incidental mortality occurred in the 150 mg a.s./kg diet treatment group and one incidental mortality occurred in the 543 mg a.s./kg diet treatment group. In the 150 mg a.s./kg diet treatment group, a hen was found dead on day 6 of week 12 without having exhibited any prior clinical signs. At necropsy the hen weighed 294 grams and no external lesions were noted. Internally, the spleen was noticeably pale and the liver had an obvious amount of blood surrounding the surface of the right lobe. A gelatinous portion of blood was found in the medial left lobe at approximately 2 cm in diameter. The liver was off-white in colour and the kidneys were noticeably pale. Necropsy of the pen mate was unremarkable. In the 543 mg a.s./kg diet treatment group, a male was found dead on day 5 of week 7 without having exhibited any prior clinical signs. At necropsy, the male weighed 231 grams. Upon necropsy, it was determined that there was a mid-level cervical break with attendant haemorrhage. Additionally, there was intracranial haemorrhaging over the cerebellum. Necropsy of the pen mate was unremarkable.
No other mortalities occurred during the course of the study. Due to the nature of the lesions observed, the mortalities that occurred were considered to be unrelated to treatment.

No overt signs of toxicity were observed at any of the concentrations tested. Clinical signs observed included a ruffled appearance, a thin appearance and slight loss of coordination. However, these signs were limited to few birds and only for one day. Other observations have included incidental injuries associated with pen wear such as foot lesions, feather loss, rump lesions, lameness and a slightly irritated eye. Otherwise, all birds have been normal in appearance and behaviour.

There were no apparent treatment-related effects upon adult body weight at any of the concentrations tested. There were no statistically significant differences in body weight between the control group and the 150, 285, and 543 mg a.s./kg diet treatment groups at any of the body weight intervals. Summarized results see Table 8.1.1.3-1.

There were no apparent treatment-related effects upon feed consumption at the 150 or 285 ppm a.s. test concentration. However, at the 543 mg a.s./kg diet test concentration there was a slight reduction in feed consumption during week 1 that may have been related to treatment.

No statistically significant differences between the control group and the 150 mg a.s./kg diet treatment group were observed at any of the feed consumption intervals. In the 285 mg a.s./kg diet treatment group, there were slight, but statistically significant ($p < 0.05$) increases in feed consumption during week 19 and 20. These differences were not considered treatment related since they were neither consistent over time nor concentration responsive. At the 543 mg a.s./kg diet test concentration, there was a slight reduction in mean feed consumption during week 1 that was statistically significant ($p < 0.05$). This slight reduction may have been due to avoidance and therefore may be considered treatment related.

Reproductive results
There were no apparent treatment related effects upon egg shell thickness at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in egg shell thickness in the 150, 285, or 543 mg a.s./kg diet treatment groups.

There were no treatment-related effects upon reproductive performance at the 150 and 285 mg a.s./kg diet test concentrations. When compared to the control group, there were no statistically significant differences in any of the reproductive parameters at these test concentrations. At the 543 ppm a.s. test concentration, there was a marked, statistically significant ($p \leq 0.01$) reduction in egg production. While not statistically significant, the reduction in egg production was also reflected in reductions in the number of hatchlings and 14-day old survivors per hen per day. Additionally, while not statistically significant, at the 543 mg a.s./kg diet test concentration there was a reduction in offspring survival.

There were no apparent treatment related effects upon offspring body weight at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in the body weight of hatchlings from any of the treatment groups and no statistically significant difference in the body weight of 14-day old survivors at the 150 and 543 mg a.s./kg diet test concentrations. However, when compared to the control group, there was a slight increase in 14-day old survivor body weights at the 285 mg a.s./kg diet treatment group that was statistically significant ($p \leq 0.01$). Since the difference was slight, represented an improvement in performance and was not concentration responsive, it was not considered to be treatment related. Results are summarized in Table 8.1.1.3-2 and Table 8.1.1.3-3.

Table 8.1.1.3-1: Effects of BAS 750 F on the parental generation of the northern bobwhite quail (*Colinus virginianus*)

Parameter	Treatment group [mg a.s./kg diet]			
	Control	150	285	543
No. of replicates (1 male and 1 female per replicate/pen)	18	18	18	18
No. of substance-related mortalities of adult birds	0	0	0	0
Adult body weight [g] at the end of study (male/female)	223/244	219/242	224/241	219/244
Gain of adult body weight [g] at the end of study (male/female)[1)]	18/42	16/38	18/41	16/40

1) Values from appendix VIII, table 1-8.

Table 8.1.1.3-2: Effects of BAS 750 F on the reproduction of the northern bobwhite quail (*Colinus virginianus*)

Parameter	Treatment group [mg a.s./kg diet]			
	Control	**150**	**285**	**543**
Number of surviving replicates	18	17	18	17
Total eggs laid	815	729	739	473
Eggs laid/hen	45	43	41	28
Eggs laid/hen/day	0.49	0.47	0.45	0.30**
Eggs cracked	22	12	27	4
Mean egg shell thickness (mm)	0.229 ± 0.014	0.239 ± 0.016	0.233 ± 0.014	0.231 ± 0.015
Eggs set	691	639	628	390
Viable Embryos	613	614	548	360
Mean body weight (g) of hatchlings per group	5.9 ± 0.7	6.0 ± 0.4	6.1 ± 0.4	5.5 ± 0.6
Live 3-week embryos	605	611	541	350
Mean bodyweight (g) of 14-day old survivors	26 ± 3	26 ± 3	29** ± 2	24 ± 3
Hatchlings	553	582	500	335
14-day old survivors	501	530	450	256
14-day old survivors/hen	28	31	25	15

** Significantly different from the control at $p < 0.01$ (Dunnett´s t-test).

Table 8.1.1.3-3: Effects of BAS 750 F on the reproduction of the northern bobwhite quail (*Colinus virginianus*) expressed as percentages

Parameter	Treatment group [mg a.s./kg diet]			
	Control	**150**	**285**	**543**
% viable embryos/eggs set	88	96	87	92
% live 3-week embryos/viable embryos	99	100	99	97
% hatchlings/live 3-week old embryos	89	96	93	95
% hatchlings/eggs set	80	92	80	84
% 14-day old survivors/eggs set	71	82	72	64
% 14-day survivors of hatchlings	83	89	91	75
% cracked eggs of eggs laid	3	2	3	1

III. CONCLUSION

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight at the 150, 285 or 543 mg a.s./kg diet test concentrations. There were no treatment-related effects upon feed consumption at the 150 or 285 mg a.s./kg diet test concentrations. At the 543 mg a.s./kg diet test concentration, there was a slight reduction in feed consumption during Week 1 that may have been related to treatment. There were no treatment related effects upon any of the reproductive parameters measured at the 150 and 285 mg a.s./kg diet test concentration. However, at the 543 mg a.s./kg diet test concentration, there was a marked, statistically significant ($p \leq 0.01$) reduction in egg production and a reduction in offspring survival (not statistically significant). The no-observed-effect concentration (NOEC) for northern bobwhite quails exposed to BAS 750 F in the diet during the study was 285 mg a.s./kg diet (25.3 mg a.s./kg b.w./day).

Report:	CA 8.1.1.3/2 [redacted] 2015 a BAS 750 F: A reproduction study with the mallard 2015/7005819
Guidelines:	EPA 850.2300, OECD 206
GLP:	yes (certified by United States Environmental Protection Agency)

The objective of this study was to evaluate the effects upon the adult mallard (*Anas platyrhynchos)* of dietary exposure to BAS 750 F over a five-month period. Effects on adult health, body weight and feed consumption were evaluated. In addition, the effects of adult exposure to BAS 750 F on the number of eggs laid, fertility of the eggs, normal development of eggs including viability and survival of the embryos, hatchability, offspring survival and egg shell thickness were evaluated.

Mallards (72 males and 72 females) were randomly distributed into one control group and three treatment groups. Each treatment and control group contained 18 pairs of birds with one male and one female per pen. Three treatment groups were fed diets containing 150, 300 or 600 ppm a.s. of BAS 750 F for 20 weeks. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

Each treatment and control group contained a total of 18 pairs of birds per concentration. All adult birds were observed daily throughout the test for signs of toxicity or abnormal behaviour. Adult body weights were measured at test initiation, at the end of week 2, 4, 6, 8, and at termination of the adults. Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production. Feed consumption was measured weekly throughout the test. All surviving adults were subjected to gross necropsy following termination of the adults. All findings observed were considered unrelated to treatment or biologically not meaningful.

Reproductive parameters included eggs laid/hen/day, eggs cracked of eggs laid, fertile eggs of eggs set, viable embryos from eggs set, live three weeks embryos of viable embryos, hatchlings of 3 week embryos, 14-day old survivors of hatchlings, hatchlings of eggs incubated, hatchling of fertile eggs, 14-day old survivors of eggs incubated, hatchling/pen/day, 14-day old survivor/pen/day, offspring`s body weights and egg shell thickness.

Analysis of the control samples did not show any indication of the presence of the test substance or of the presence of a co-eluting substance at the characteristic retention time of the test substance.

Dietary concentrations were adjusted for purity of the test substance. Nominal preparation was based on a previously reported purity of 98.8% and prepared respectively.

There were no treatment-related mortalities, overt signs of toxicity, or treatment related effects upon body weight or feed consumption at any of the test concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured at the 150, 300, or 600 ppm a.s. test concentrations. The no-observed-effect concentration for mallard exposed to BAS 750 F in the diet during the study was therefore considered to be 600 mg a.s./kg diet (80.5 mg a.s./kg b.w./day), the highest concentration tested.

The no-observed-effect concentration for mallard exposed to BAS 750 F in the diet during the study was therefore considered to be 600 mg a.s./kg diet (80.5 mg a.s./kg b.w./day), the highest concentration tested.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F, Reg. No.: 5834378, Batch No.: COD-001740, purity: 98.8% (tolerance ± 1%)

B. STUDY DESIGN

Test species: Mallard (*Anas platyrhynchos)*, phenotypically indistinguishable from wild type; adults, age: 26 weeks of age at the initiation of the test (before beginning of first egg-laying period); supplier: Whistling Wings 113 Washington Street, P.O. Box 1A, Hanover, IL 61041.

Test design: Mallards approaching their first breeding season were kept in a group of 1 male and 1 female in a pen per replicate. 18 pens were allocated to the control and each treatment group. The animals were acclimated to the laboratory conditions for 6 weeks. All adult birds and their offspring were given feed and water *ad libitum* during acclimation and testing. The study period was divided into five phases: 1. Acclimation – 6 weeks; 2. Pre-photostimulation – 10 weeks; 3. Pre-egg laying (with photostimulation) – 0 weeks. Egg production was considered to have begun at photostimulation; 4. Egg laying period – 10 weeks; 5. Post-adult termination (final incubation, hatching and 14-day offspring rearing period – 6 weeks. Eggs were collected daily from all pens and stored in a cold room. At the end of a weekly interval, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement. Cracked or abnormal eggs were recorded and discarded. All eggs not discarded or used for egg shell thickness measurements were placed in a NatureForm Incubator (Model No. NMC 4000). On day 24 of incubation, eggs were transferred to a hatcher. Young birds were maintained for 14 days. Adult birds were sacrificed after the egg-laying period, young birds after 14 days.

Endpoints:	Adult birds: mortalities, clinical observations, gross necropsy, adult body weight and adult feed consumption Reproductive parameters: Eggs laid/hen/day, eggs cracked of eggs laid, fertile eggs of eggs set, viable embryos of eggs incubated, live 3-week embryos of viable embryos, hatchlings of 3 week-embryos, 14-day old survivors of hatchlings, hatchlings of eggs incubated, hatchlings of fertile eggs, hatchlings/pen/day, 14-day old survivors/pen/day, 14-day old survivors of eggs incubated, hatchling´s body weight and egg shell thickness.
Test concentrations:	0 (Control), 150, 300 and 600 mg a.s./kg diet BAS 750 F in the diet (nominal).
Test conditions:	Adult mallard study room: Temperature 21.3 ± 1.6 °C (SD); relative humidity: 51 ± 15% (SD); ventilation: 15 times the room air/h; photoperiod: 8 hours light or less per day (week 1 – 10) approx. 226 Lux, lengthened photoperiod to 17 hours light (week 11 to the end of study) approx. 234 Lux and during egg-laying approx. 248 Lux (fluorescent lights). Egg collection and storage: Collected daily, stored in cold room: temperature: 12.3 ± 1.2 °C (SD), relative humidity: 84 ± 4% (SD). Eggs set for incubation: temperature: 37.4 ± 0.0 °C (SD), relative humidity approximately 55 ± 0.0 °C (SD); the eggs were transferred to the hatcher on day 24: temperature: 37.3 ± 0.0 °C (SD), relative humidity approximately 60 ± 1% (SD) Hatchlings: Brooding compartment temperature approximately 38°C from hatching to the birds were 6 days of age; average ambient room temperature 25.5 ± 0.0 °C (SD), relative humidity: 74% ± 8%; photoperiod: 16 hours light per day
Analytics:	The test substance concentrations were analysed using HPLC with UV detection
Statistics:	Analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Comparison of control and treatment groups: Dunnett´s multiple comparison procedure. Percentage data: Dunnett´s method following arcsine square root transformation

II. RESULTS AND DISCUSSION

Analytical measurements:
The result of the analytical verification of the test substance was 98.8 %. Concentrations of the test substance in the diet were adjusted to 100% active substance. Mean concentrations and standard deviations for the three test concentrations were 158 ± 6.06, 314 ± 8.37, and 615 ± 22.0 mg a.s./kg diet for the nominally 150, 300, and 600 mg a.s./kg diet concentrations, respectively.

Biological results:
Parental generation
A single incidental mortality occurred in this study. The female from Pen 1361 of the 600 ppm a.s. treatment group was found dead on Day 2 of Week 17. Prior to being found dead the female was noted as normal in appearance and behaviour. At necropsy the hen weighed 863 grams, her spleen and pancreas were pale, kidneys slightly pale, and there were extensive lesions of egg yolk peritonitis in the abdominal cavity. The hen's ovary was regressing. Necropsy of the female's penmate was unremarkable.

At the Week 2 body weight measurements, the plumage on the nominal hen from Pen 1335 of the 150 mg a.s./kg diet treatment group was cryptic but indicated that the bird was a male. The pair was euthanized and data from this pen were excluded from the test. Necropsy confirmed the sex of the nominal female as male. Otherwise, necropsy of both birds was unremarkable. No other mortalities occurred during the course of the study. Due to the nature of the lesions observed, the mortalities that occurred were considered to be unrelated to treatment.

No overt signs of toxicity were observed at any of the test concentrations. Observations included incidental injuries associated with pen wear and included foot lesions, feather loss, and an unkempt or thin appearance. Except for incidental findings, all birds appeared normal throughout the study. Otherwise, all birds have been normal in appearance and behaviour.

All surviving adults were subjected to gross necropsy following adult termination. Findings from birds in the 150, 300, and 600 mg a.s./kg diet test concentrations were considered to be incidental to treatment.

There were no apparent treatment-related effects upon adult body weight at any of the concentrations tested. There were no statistically significant differences in body weight between the control group and the 150 mg a.s./kg diet treatment groups at any of the body weight intervals. At the 300 mg a.s./kg diet test concentration, the mean body weight of hens at Week 8 of the test was slightly higher than the control group ($p < 0.05$). However, the increase was slight and represented an improvement and therefore it was considered to be unrelated to treatment. At the 600 mg a.s./kg diet test concentration, there was a slight reduction in weight gain between Week 8 and adult term, and overall, and mean weight of hens at adult term that were statistically significant ($p < 0.05$). However, the differences were slight and primarily due to the hen in Pen 1365 that was noted as thin and weighing 729 grams at adult termination. For summarized results, see Table 8.1.1.3-4.

There were no apparent treatment-related effects upon feed consumption at the 150, 300, or 600 mg a.s./kg diet test concentrations and no statistically significant differences between the control group and the 600 mg a.s./kg diet treatment group were observed at any of the feed consumption intervals. At the 150 test concentration there was a slight increase in mean feed consumption during Week 5 of the test that was statistically significant ($p < 0.05$). At the 300 mg a.s./kg diet test concentration there were slight increases in mean feed consumption that were statistically significant at $p < 0.05$ during Weeks 2, 3, and 6, and statistically significant at $p < 0.01$ during Weeks 1 and 5 of the test. The increases in mean feed consumption were slight, not concentration responsive, and appeared to have been related to feed wastage by individual pens and were therefore not considered to be biologically meaningful.

Reproductive results
There were no treatment-related effects upon reproductive performance measured at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in any of the reproductive parameters measured in the 150, 300, or 600 mg a.s./kg diet treatment groups.

There were no apparent treatment-related effects upon offspring body weights at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in weights of hatchlings for any of the treatment groups or for 14-day old survivors at the 300 and 600 mg a.s./kg diet test concentrations. At the 150 mg a.s./kg diet test concentration, there was a slight but statistically significant ($p < 0.05$) increase in weights of 14-day old survivors. However, the increase in 14-day old body weights was slight, not concentration responsive, and represented an improvement, and therefore was not considered to have been related to treatment. Results are summarized in Table 8.1.1.3-5 and Table 8.1.1.3-6.

Table 8.1.1.3-4: Effects of BAS 750 F on the parental generation of the mallard (*Anas platyrhynchos*)

Parameter	Treatment group [mg a.s./kg diet]			
	Control	150	300	600
No. of replicates (1 male and 1 female per replicate/pen)	18	18	18	18
No. of substance-related mortalities of adult birds	0	0	0	0
Adult body weight [g] at the end of study (male/female)[1)]	1090/1127	1100/1138	1090/1149	1088/1025*
Gain of adult body weight [g] at the end of study (male/female)[1)]	22/168	37/174	21/185	18/75*

* Significantly different from control at $p \leq 0.05$ (Dunnett's t-test).
1) Values table 4.

Table 8.1.1.3-5: Effects of BAS 750 F on the reproduction of the mallard (*Anas platyrhynchos*)

Parameter	Treatment group [mg a.s./kg diet]			
	Control	150	300	600
Number of surviving replicates	18	17	18	17
Total eggs laid	779	728	813	684
Eggs laid/hen[1)]	43	43	45	40
Eggs laid/hen/day[2)]	0.62	0.61	0.65	0.57
Eggs cracked	0	3	3	1
Mean egg shell thickness (mm)	0.391 ± 0.022	0.395 ± 0.020	0.380 ± 0.031	0.383 ± 0.027
Eggs set	698	644	716	614
Viable Embryos	559	602	686	577
Mean body weight (g) of hatchlings per group	36 ± 2.0	35 ± 1.9	35 ± 3.9	36 ± 3.3
Live 3-week embryos	555	593	683	569
Mean bodyweight (g) of 14-day old survivors	288 ± 17	308* ± 22	297 ± 15	289 ± 26
Hatchlings	492	520	645	526
14-day old survivors	488	513	643	522
14-day old survivors/pen	27	30	36	31

[1] The total number of eggs laid in each group.
[2] Based on 70 days of egg production.
* Significantly different from control at $p \leq 0.05$ (Dunnett's t-test).

Table 8.1.1.3-6: Effects of BAS 750 F on the reproduction of the mallard (*Anas platyrhynchos*) expressed as percentages

Parameter	Treatment group [mg a.s./kg diet]			
	Control	150	300	600
% viable embryos/eggs set	83	94	90	94
% live 3-week embryos/viable embryos	99	99	100	99
% hatchlings/live 3-week old embryos	89	88	94	93
% hatchlings/eggs set	73	81	84	87
% 14-day old survivors/eggs set	72	81	84	86
% 14-day survivors of hatchlings	99	99	100	99
% cracked eggs of eggs laid	0	0	1	0

Percent values represent replicate means for each experimental group. Differences between control group and each of the treatment groups were not significant ($p > 0.05$, Dunnett's t-test).

III. CONCLUSION

There were no treatment-related mortalities, overt signs of toxicity, or treatment related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured at the 150, 300 or 600 mg a.s./kg diet test concentrations. The no-observed-effect concentration for mallard exposed to BAS 750 F in the diet during the study was therefore considered to be 600 mg a.s./kg diet (80.5 mg a.s./kg b.w./day), the highest concentration tested.

CA 8.1.2 Effects on terrestrial vertebrates other than birds

Table 8.1.2-1 provides information on the mammal toxicity studies for the active ingredient BAS 750 F relevant for assessing the risk to mammals. Note that only studies relevant for the risk assessment in chapter M-CP 10.1 are listed.

Table 8.1.2-1: Summary of toxicity studies for the active substance BAS 750 F relevant for assessing the risk to mammals

Test system	Test species	Reference [BASF DocID]
Acute oral toxicity	Rat	2013/1149656
		2014/1170774
2-Generation reproductive toxicity	Rat	2014/1170754
Development toxicity	Rat	2014/1170755
	Rabbit	2014/1170757

Table 8.1.2-2 lists the relevant toxicity studies in mammals for the metabolites identified as relevant for the mammalian risk assessment. These studies were previously evaluated on the EU level. Endpoints for all studies except [redacted] (2010, BASF DocID 2010/1225534) were agreed during the PRAPeR 14 expert meeting in January 2007. [redacted] (2010) was reviewed in the UK's confirmatory data addendum for triazole derivative metabolites (2015). For details on these studies, consult the stated documents.

Table 8.1.2-2: Summary of mammalian toxicity studies with metabolites of the active substance BAS 750 F

Test substance	Test system	Test species	Reference [Author, year, BASF DocID]
Triazole alanine (M750F029)	Acute oral toxicity	rat	[redacted] 1980 1980/1000167
Triazole alanine (M750F029)	Acute oral toxicity	rat and mouse	[redacted] 1982 1986/1000485
Triazole alanine (M750F029)	2-generation reproduction toxicity	rat	[redacted], 1986 1986/1000486
Triazole acetic acid (M750F030)	Acute oral toxicity	rat	[redacted] 1984 1984/1000342
Triazole acetic acid (M750F030)	1-generation reproduction toxicity	rat	[redacted] 2010 2010/1225534

CA 8.1.2.1 Acute oral toxicity to mammals

Acute oral toxicity to mammals is addressed in M-CA 5.2.1.

CA 8.1.2.2 Long-term and reproductive toxicity to mammals

Long-term and reproductive toxicity to mammals is addressed in M-CA 5.6.

CA 8.1.3 Effects of active substance bioconcentration in prey of birds and mammals

The potential effects of active substance bioconcentration in prey of birds and mammals are addressed according to the guidance document EFSA/2009/1438 with the secondary poisoning risk assessments presented in the documents M-CP 10.1.1 and M-CP 10.1.2. All TER values exceed the trigger of 5 set by Commission regulation (EU) 546/2011 for acceptability of effects with considerable margins of safety. Consequently, no additional study is considered to be necessary.

CA 8.1.4 Effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

According to the revised data requirements under regulation 1107/2009 (Commission Regulations (EU) 283/2013[1] and 284/2013[2] for the active ingredient and the plant protection products, respectively), the risk to amphibians and reptiles shall be addressed. Nevertheless, unlike birds and mammals, toxicity tests for amphibian and reptile species are not requested. In the EU there is no guidance or validated regulatory protocols yet available either on the type of regulatory testing necessary or how to conduct a risk assessment for amphibian and reptiles.

According to the new aquatic guidance document (EFSA, 2013) amphibians should be included in the aquatic and terrestrial risk assessment. In the absence of GLP studies, the assessment should be based on any existing relevant information (testing of amphibian is not recommended at first instance due to animal welfare reasons and the absence of standard guidelines for amphibian testing).

[1] Commission Regulation (EU) No 283/2013 setting out data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union: 1st March 2013.

[2] Commission Regulation (EU) No 284/2013: setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union: 1st March 2013.

With regard to the aquatic risk assessment, several data analyses indicate that the risk assessment for aquatic organisms (and fish in particular) covers the risk assessment for aquatic phases of amphibians (Fryday and Thompson, 2009, 2012[3]; Weltje et al., 2013[4]). Based on these extensive data reviews, it can be concluded that the acute and chronic risk to aquatic life stages of amphibians is covered by the currently requested and conducted risk assessment for aquatic organisms (CP 10.2).
Compared to aquatic studies, regulatory ecotoxicological information on amphibians based on dosing studies (LD_{50}) is rather scarce. However, in the few cases where terrestrial stages of amphibians were tested in the same kind of study as birds and mammals, the general pattern is that amphibians are less sensitive than the latter two taxa (see Table 12 and 13 in Fryday and Thompson, 2012).

In the case of reptiles there is even less information available than for amphibians (see the revision by Fryday and Thompson, 2009). The risk from dietary exposure can be assumed to be lower for reptiles than for birds and mammals (Fryday and Thompson 2009). This is because reptiles are poikilotherms (*i.e.* do not maintain a constant body temperature) and as a result, feeding activity will peak on warm days and will be zero during hibernation or on cold days. In contrast, birds and mammals will have to maintain a constant body temperature and, hence, will need to feed every day (Fryday & Thompson 2009). Uncertainties remain on the contribution of dermal exposure to the overall exposure to reptiles. However, in contrast to amphibians, the skin of reptiles is much less permeable; its function is in general protection and as a barrier, and it is not an organ used for respiration or water/mineral exchange with the environment. Accordingly, reptiles are considered less vulnerable to dermal exposure compared to amphibians. Nevertheless, some uncertainty with respect to the risk to reptiles, i.e. whether they are sufficiently covered by other (more standard) ecotoxicological data will remain and further research is needed.

[3] Fryday S and Thompson H (2009): Literature reviews on ecotoxicology of chemicals with a special focus on plant protection products. Lot 1. Exposure of reptiles to plant protection products. EFSA (CFT/EFSA/PPR/2008/01).

Fryday S and Thompson, H (2012): Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural; Food and Environment research agency, UK.

[4] Weltje L., Simpson P., Gross M., Crane M., Wheeler J.R. (2013): Comparative acute and chronic sensitivity of fish and amphibians: a critical review of data. Environmental Toxicology and Chemistry, Vol. 32, No. 5, pp. 984-994.

CA 8.1.5 Endocrine disrupting properties

Mammals

For mammals, the endocrine disrupting potential of BAS 750 F is discussed in detail in chapter M-CA 5.8.3.

Birds

Up to this point, there are no formal criteria available in the EU for what constitutes an endocrine disruptor under Regulation 1107/2009. For birds, there is also no internationally validated regulatory testing guideline available. Effects on avian reproduction are covered by the avian reproduction study, which is part of the standard data package for an active ingredient.

For BAS 750 F, two avian reproduction studies are available, one in bobwhite quail and one in mallard duck (see chapter M-CA 8.1.1.3 above). As guideline regulatory studies, the focus of these studies was on the general and reproductive toxicity of BAS 750 F to birds, but they do include reproductive endpoints that are under endocrine control. With their long-term exposure (≥ 20 weeks) and detailed assessment of fitness and reproductive parameters, and gross necropsy assessment, the studies provide adequate information on the overall effect pattern of the active substance BAS 750 F in birds. Hence, the studies are considered suitable to allow for a full evaluation of the reproductive toxicity, including any endocrine potential of BAS 750 F that might impact the reproductive performance in birds. This is in line with a recent conclusion of the US EPA in its Endocrine Disruptor Screening Program (EDSP)[5]. In June 2015, US EPA released its review[6] of the Tier 1 screening assay results for the list 1 chemicals (total of 52 chemicals) in the EDSP. EPA clearly states that data obtained from the "avian reproduction studies (OCSPP 850.2300) are considered sufficient for evaluating potential reproductive effects to birds" and "additional testing is not recommended" (e.g. propiconazole, p. 33[7]).

Many plant protection products were among the chemicals screened in the EDSP, including four triazoles, namely myclobutanil, propiconazole, tebuconazole and triadimefon. All these compounds belong to the same chemical group as BAS 750 F, i.e. triazoles in the fungicidal group of demethylase inhibitors (DMI) (FRAC group G1). For all evaluated triazoles, the US EPA concluded that further bird testing is not recommended and the avian reproduction studies were considered sufficient for the evaluation of potential reproductive effects on birds.

[5] The Endocrine Disruptor Screening Program (EDSP) of US EPA is a program to screen chemicals for their potential to affect the estrogen, androgen and thyroid hormone systems using a two-tiered screening and testing process. The results of the screening are evaluated in a weight of evidence (WoE) approach by EPA to determine whether a chemical has the potential to interact with the endocrine system and whether more thorough testing is required. The WoE conclusion on the tier 1 screening assays for list 1 chemicals was published in June 2015.

[6] United States Environmental Protection Agency Washington, D.C. 20460, Memorandum, June 29, 2015, EDSP Weight of Evidence Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals.

[7] United States Environmental Protection Agency. EDSP: Weight of Evidence Analysis of Potential Interaction with Estrogen, Androgen or Thyroid Pathways. Chemical: Propiconazole. Washington, D.C. 20460, Memorandum, June 29, 2015.

The standard data requirements for registration in the EU for reproductive testing is with one bird species. However, for BAS 750 F, avian reproduction studies are available for two species, hence reducing the risk linked to potential variations in species sensitivity. Under the conservative assumptions in both the study design (constant long-term exposure to high concentrations) and the long-term bird risk assessment (which demonstrated that even under the very conservative assumptions of tier 1, the long-term TER values are above the trigger value of 5), sufficient data is available to conclude that the risk to birds from the proposed uses of BAS 750 F is acceptable.

In summary, based on i) the availability of two avian reproduction studies for BAS 750 F and ii) the margin of safety despite the conservative nature of the avian reproductive risk assessment, it can be reasoned for the case of BAS 750 F that the standard endpoints from the avian reproduction studies are sufficiently protective to address the potential endocrine activity and are suitable for use in the avian reproductive risk assessment for BAS 750 F. This also supported by the conclusions of the US EPA regarding the sufficiency of the avian reproduction studies for evaluating potential endocrine effects on birds.

CA 8.2 Effects on aquatic organisms

Toxicity studies on the active substance BAS 750 F (Reg. No. 5834378) and its major metabolites have been performed and the endpoints are used in the aquatic risk assessment. Some of the studies are not required according to regulation 283/2013 but part of a global data package and provided for the sake of completeness. Study summaries are given below.

Studies of the metabolite 1,2,4-triazole have already been evaluated and EU agreed within the Annex I inclusion of the active substance epoxiconazole (BAS 480 F); for details see EU Review Documents for epoxiconazole.

The results of all studies are summarized in Table 8.2-1 and Table 8.2-2. Full references to cited literature used within the following chapters are given at the end of MCA 8.2. Document N3 contains structures and synonyms for the metabolites of BAS 750 F.

Table 8.2-1: List of studies and endpoints for aquatic organisms exposed to the active substance BAS 750 F

Organism	Endpoint	Value [mg/L] (except BCF & sediment endpoint of spiked sediment study)	Reference (BASF DocID)
Active substance: BAS 750 F			
Acute toxicity to fish			
Oncorhynchus mykiss	96 h LC_{50}	**0.532**	2014/1036951
Danio rerio, (Syn. *Brachydanio rerio*)	96 h LC_{50}	0.906	2015/1001581
Cyprinodon variegatus	96 h LC_{50}	0.761	2014/7002810
Cyprinus carpio	96 h LC_{50}	1.13	2015/1249071
Chronic toxicity to fish			
Danio rerio (ELS)	36 d NOEC	**0.027**	2014/1262160
Danio rerio (FSDT)	69 d NOEC	≥ 0.045	2015/1099093
Cyprinodon variegatus (ELS)	35 d NOEC	0.16	2015/7000619
Bioconcentration			
Oncorhynchus mykiss (BCF; 14d uptake, 7 d depuration)	BCF_{KLg} (whole fish)	385	2015/1122811
Acute toxicity to aquatic invertebrates			
Daphnia magna	48 h EC_{50}	**0.944**	2013/1250866
Americamysis bahia #	48 h LC_{50}	1.53	2014/7002845
Crassostrea virginica	96 h EC_{50}	0.947	2015/7000021
Chronic toxicity to aquatic invertebrates			
Daphnia magna	21 d NOEC	**0.010**	2014/1098028
Americamysis bahia #	28 d NOEC	≥ 0.0132	2016/7001293
Daphnia pulex	21 d NOEC	0.0282	2015/1003913

Organism	Endpoint	Value [mg/L] (except BCF & sediment endpoint of spiked sediment study)	Reference (BASF DocID)
Daphnia longispina	21 d NOEC	0.0338	2015/1003912
Acute/ sub-chronic toxicity to sediment dwelling aquatic invertebrates			
Chironomus dilutes (spiked sediment)	10 d EC_{50}	> 97 mg /kg dry sediment	2015/7000621
	10 d NOEC	7.2 mg /kg dry sediment	
Hyalella azteca (spiked sediment)	10 d NOEC	100 mg /kg dry sediment	2015/7000622
	10 d EC_{50}	> 100 mg /kg dry sediment	
Leptocheirus plumulosus (spiked sediment)	10 d NOEC	95 mg /kg dry sediment	2015/7000623
	10 d LC_{50}	> 95 mg /kg dry sediment	
Chronic toxicity to sediment dwelling aquatic invertebrates			
Chironomus riparius (spiked sediment)	28 d NOEC	≥ 1.158 mg/kg dry sediment	2014/1243181
Algae [1)]			
Pseudokirchneriella subcapitata	72 h E_rC_{50}	1.352	2013/1250865
Skeletonema costatum [#]	72 h E_rC_{50}	**0.723**	2015/7000620
Navicula pelliculosa	72 h E_rC_{50}	1.570	2015/7000618
Anabaena flos-aquae	72 h E_rC_{50}	> 3.20	2015/7000617
Macrophytes [1)]			
Lemna gibba	7 d E_rC_{50}	> 1.894	2014/1001322

Bold figures: Where several endpoints are available for the same group or where several endpoints are available for one study based on different effect parameters (*e.g.* for algae and macrophytes), only the relevant endpoint(s) is used in the (tier 1) risk assessment presented in chapter 10.2 of the MCP dossier.

Abbreviations: ELS = early life stage; FSDT = fish sexual development test; BCF_{KLg} = growth corrected kinetic bioconcentration factor normalized to 5% lipid content;

[#] Estuarine/Marine species.

[1)] In accordance to the EFSA Aquatic Guidance Document (EFSA 2013), only the EC_{50} values determined for the more relevant endpoint 'growth rate' (E_rC_{50}) are considered for the risk assessment for aquatic primary producers if both “growth rate” and “yield / biomass” endpoints are available.

Table 8.2-2: List of studies and endpoints for aquatic organisms exposed to the metabolites of the active substance BAS 750 F

Organism	Endpoint	Value [mg/L] (except sediment endpoint of spiked sediment study)	Reference (BASF DocID)
1,2,4-triazole (Reg. No. 87084; M750F001)*			
Fish			
Oncorhynchus mykiss	96 h LC_{50}	760	1983/1000494
	28 d NOEC	3.2	2002/1007850
Aquatic invertebrates			
Daphnia magna	48 h EC_{50}	> 100	1995/1001851
Algae			
Pseudokirchneriella subcapitata [1)]	72 h E_rC_{50}	> 31	2001/1022266
M750F003 (Reg. No. 5924326)			
Sediment-dwelling aquatic organisms			
Chironomus riparius (spiked sediment study)	28 d NOEC	≥ 1.944 mg/kg dry sediment	2015/1003916
M750F005 (Reg. No. 6003433)			
Aquatic invertebrates			
Daphnia magna	48 h EC_{50}	> 8.58	2015/1001490
Algae			
Pseudokirchneriella subcapitata [1)]	72 h E_rC_{50}	> 8.57	2015/1184816
M750F006 (Reg. No. 5863469)			
Aquatic invertebrates			
Daphnia magna	48 h EC_{50}	4.42	2015/1001492
Algae			
Pseudokirchneriella subcapitata [1)]	72 h E_rC_{50}	1.42	2015/1184815
M750F007 (Reg. No. 6003432)			
Fish			
Oncorhynchus mykiss	96 h LC_{50}	> 7.20	2015/1001489
Aquatic invertebrates			
Daphnia magna	48 h EC_{50}	> 10	2015/1003915
Algae			
Pseudokirchneriella subcapitata [1)]	72 h E_rC_{50}	> 10	2015/1003914

Organism	Endpoint	Value [mg/L] (except sediment endpoint of spiked sediment study)	Reference (BASF DocID)
M750F008 (Reg. No. 6010286)			
Aquatic invertebrates			
Daphnia magna	48 h EC_{50}	> 8.07	2015/1001493
Algae			
Pseudokirchneriella subcapitata [1)]	72 h E_rC_{50}	4.08	2015/1001491

[1)] In accordance to the EFSA Aquatic Guidance Document (EFSA 2013), only the EC_{50} values determined for the more relevant endpoint 'growth rate' (E_rC_{50}) are considered for the risk assessment for aquatic primary producers.

* Studies of the metabolite 1,2,4-triazole have already been evaluated and EU agreed within the approval of the active substance epoxiconazole (BAS 480 F); for details see EFSA Scientific Report (2008) 138, 1-80, Conclusion on the peer review of epoxiconazole.

CA 8.2.1 Acute toxicity to fish

Several fish species were acutely tested due to different data requirements in other regions than EU. The respective study reports are summarized below.

Report:	CA 8.2.1/1 [redacted] 2014a BAS 750 F - Acute toxicity study in the rainbow trout (Oncorhynchus mykiss) 2014/1036951
Guidelines:	EC 440/2008 C.1, EPA 72-1, EPA 850.1075, OECD 203 (1992), EPA 540/9-82-024
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 96-hour flow-through acute toxicity laboratory study, juvenile rainbow trout were exposed to a dilution water control and to nominal concentrations of 4.6, 10, 22, 46 and 100% of a saturated solution of BAS 750 F (corresponding to mean measured concentrations of < LOQ (limit of quantification), 0.069, 0.142, 0.380, 0.826 and 1.55 mg a.s./L) in groups of 10 animals in glass aquaria containing 9 L water. Fish were observed for survival and symptoms of toxicity within 1 hour after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations of the test item. After 96 hours of exposure, no mortality was observed in the dilution water control and at test item concentrations of up to and including 0.142 mg a.s./L, whereas 5% mortality was observed at 0.380 mg a.s./L. At the two highest tested concentrations, all fish were dead after 96 hours of exposure. Sub-lethal effects (*i.e.* swimming at the bottom) were found at 0.380 mg a.s./L after 96 hours.

In a flow-through acute toxicity study with rainbow trout the LC_{50} (96 h) of BAS 750 F was 0.532 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% ± 1.0%.

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss*), age: approx. 4 months; mean body length of control fish: 6.0 cm (5.5 cm - 6.6 cm); mean wet weight of control fish: 1.68 g (1.17 g - 2.43 g); supplied by Forellenzucht Trostadt GbR, Trostadt, Germany.

Test design: Flow through system (96 h); 5 test item concentrations plus a dilution water control, 2 replicates per treatment; 10 fish per aquarium (loading 0.37 g fish/L/day); assessment of mortality and sub-lethal effects within 1 hour after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure.
Test item preparation: Because the test item is poorly water soluble a saturated stock solution was prepared by using saturation columns. Two columns were prepared and used in parallel to generate the quantity of saturated solution required for this study.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), 4.6, 10, 22, 46 and 100% of a saturated solution of BAS 750 F corresponding to mean measured concentrations of < LOQ, 0.069, 0.142, 0.380, 0.826 and 1.55 mg a.s./L.

Test conditions: 9 L glass aquaria, test volume: 9 L; dilution water: charcoal-filtered drinking water mixed with deionised water; flow rate: 31.3 mL/min; hardness: approx. 100 $CaCO_3$ mg/L; temperature: 13 - 14°C; pH 7.8 - 8.1; oxygen content: 7.5 mg/L - 8.9 mg/L; conductivity: 197 µS/cm; photoperiod 16 h light : 8 h dark; light intensity: approx. 60 - 585 lux; no aeration; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a HPLC-method with MS detection.

Statistics: Descriptive statistics; probit analysis for calculation of LC_{50}.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of BAS 750 F concentrations was conducted in each test item concentration at the beginning of the test, after 48 h and at the end of the test. The mean measured concentrations of the test item were < LOQ, 0.069, 0.142, 0.380, 0.826 and 1.55 mg a.s./L. The analyzed contents of BAS 750 F ranged from 95% to 101% of mean measured concentrations at test initiation, from 100% to 104% after 48 h and from 95% to 103% of mean measured concentrations at test termination. Thus, the measured concentrations of the test item in the test solutions during the exposure period were within ± 20% of the overall mean measured value. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality was observed in the dilution water control and at test item concentrations of up to and including 0.142 mg a.s./L, whereas 5% mortality was observed at 0.380 mg a.s./L. At the two highest tested concentrations, all fish were dead after 96 hours of exposure. Sub-lethal effects (*i.e.* swimming at the bottom) were found at 0.380 mg a.s./L after 96 hours. The results are summarized in Table 8.2.1-1.

Table 8.2.1-1: Acute toxicity (96 h) of BAS 750 F to rainbow trout (*Oncorhynchus mykiss*)

Concentration [% saturation] (nominal)	**Control**	**4.6**	**10.0**	**22.0**	**46.0**	**100.0**
Concentration [mg a.s./L] (mean measured)	**--**	**0.069**	**0.142**	**0.380**	**0.826**	**1.55**
Mortality [%] (96 h)	0	0	0	5	100	100
Symptoms (after 96 h) *	none	none	none	D	n.d.	n.d.
Endpoints [mg BAS 750 F/L] (mean measured)						
LC_{50} (96 h)	0.532 (95% confidence limits: 0.47 - 0.61)					
NOEC (96 h)	0.142					

n.d. = not determined; all fish dead
* Symptoms after 96 h: D = swimming at the bottom.

III. CONCLUSION

In a flow-through acute toxicity study with rainbow trout the LC_{50} (96 h) of BAS 750 F was 0.532 mg a.s./L based on mean measured concentrations.

Report: CA 8.2.1/2
[redacted] 2015 b
Reg.No. 6003432 (metabolite of BAS 750 F, M750F007) - Rainbow trout, acute toxicity test
2015/1001489
Guidelines: OECD 203 (1992), EPA 850.1075
GLP: yes
(certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a static acute toxicity laboratory study, the effect of M750F007 (metabolite of BAS 750 F) on juvenile rainbow trout was investigated. Due to the expected low toxicity, a limit test design was chosen, *i.e.* only one test concentration. Fish were exposed to a filtrate of a loading of 10 mg M750F007/L (corresponding to a geometric mean measured test item concentration of 7.2 mg/L). Additionally a water control and a solvent control were set up. Fish were exposed in two replicates per treatment with 10 animals in each aquaria containing 30 L water. Fish were observed for survival and symptoms of toxicity within 3 hours after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on the geometric mean measured concentration of the test item. No mortality or other symptoms of toxicity were observed after 96 hours of exposure in the control groups and at a geometric mean measured test item concentration of 7.2 mg M750F007/L.

In a static acute toxicity study (limit test) with rainbow trout, the LC_{50} (96 h) of M750F007 (metabolite of BAS 750 F) was determined to be > 7.2 mg/L based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F007 (metabolite of BAS 750 F, Reg. No. 6003432), batch no. L87-32-1, purity: 97.0%.

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss* Walb.), approx. 7 months old; body length 5.7 ± 0.3 cm; body weight 2.25 g ± 0.32; supplied by 'The Culture of Salmonidae Fish in Zawoja', Poland.

Test design: Static system (96 hours); limit test; two replicates with 10 fish per aquarium (loading 0.75 g fish/L); assessment of mortality and symptoms of toxicity within 3 hour after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control, solvent control (DMF), filtrate of a loading of 10 mg M750F007/L, corresponding to a geometric mean measured concentration of 7.2 mg M750F007/L.

Test conditions: Glass aquaria, test volume: 30 L; reconstituted water; temperature: 13.4 °C -14.3 °C; pH 7.18 - 7.80; oxygen saturation: 91% - 99%; total hardness: 246.71 - 247.37 mg $CaCO_3$/L; conductivity: 635 - 659 µS/cm; photoperiod 16 h light : 8 h dark with 30 min transition; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with DAD detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each replicate at test initiation, after 48 h of exposure and at test termination. At exposure initiation the determined concentrations of test item in the filtrate of a loading of 10 mg/L were 7.38 mg/L and 7.43 mg/L in replicates A and B, respectively. After 48 h of exposure the determined concentrations of test item were 7.37 mg/L and 7.19 mg/L, respectively. At exposure termination determined concentrations of test item were 7.24 mg/L (98.10% of initial concentration) and 6.86 mg/L (92.33% of initial concentration), respectively. The geometric mean concentration of the test item in the filtrate of a loading of 10 mg/L was 7.20 mg/L. The following biological results are based on geometric mean measured concentrations.

Biological results: No mortality or other symptoms of toxicity were observed after 96 hours of exposure in the control groups and at 7.2 mg M750F007/L. The results are summarized in Table 8.2.2.1-2.

Table 8.2.2.1-2: Acute toxicity (96 h) of M750F007 (metabolite of BAS 750 F) on rainbow trout (*Oncorhynchus mykiss*)

Concentration [mg/L] (geometric mean measured)	**Control**	**Solvent control**	**7.2**
Mortality [%]	0	0	0
Symptoms	none	none	none
Endpoints [mg M750F007/L] (geometric mean measured)			
LC_{50} (96 h)	> 7.2		
NOEC (96 h)	≥ 7.2		

III. CONCLUSION

In a static acute toxicity study (limit test) with rainbow trout, the LC_{50} (96 h) of M750F007 (metabolite of BAS 750 F) was determined to be > 7.2 mg/L based on geometric mean measured concentrations.

Report:	CA 8.2.1/3 [redacted] 2015a BAS 750 F (Reg.No. 5834378) Zebra fish acute toxicity test 2015/1001581
Guidelines:	OECD 203 (1992), EPA 850.1075
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 96-hour static acute toxicity laboratory study, juvenile zebrafish were exposed to a reconstituted water control, the filtrate of a loading of 10 mg BAS 750 F/L and to its 1.5, 2.25, 3.38, 5.06-fold dilutions (corresponding to geometric mean concentrations of 1.110, 0.913, 0.735, 0.593 and 0.475 mg a.s./L, respectively.) in groups of 10 animals in 10 L glass aquaria. Fish were observed for survival and symptoms of toxicity 3, 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on geometric mean concentrations of the test item. After 96 hours of exposure, no mortality was observed in the control and at test item concentrations of up to and including 0.593 mg a.s./L, whereas 5% and 45% mortality was observed at 0.735 and 0.913 mg a.s./L, respectively. At the highest tested concentration, all fish were dead after 96 hours of exposure. Sub-lethal effects (i.e. unbalanced swimming behavior and faulty respiratory function) were found at 0.735 and 0.913 mg a.s./L after 96 hours.

In a static acute toxicity study with zebrafish the LC_{50} (96 h) of BAS 750 F was 0.906 mg a.s./L based on geometric mean concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% ± 1.0%.

B. STUDY DESIGN

Test species: Zebrafish (*Brachydanio rerio*, syn. *Danio rerio*), age: approx. 6 months; mean body length of fish: 2.77 cm ± 0.14; mean wet weight of fish: 0.29 g ± 0.04; in house culture, originally obtained from "The culture of fish in Goczalkowice", Poland.

Test design: Static system (96 h); 5 test item concentrations plus a reconstituted water control, 2 replicates per treatment; 10 fish per aquarium (loading 0.29 g fish/L); assessment of mortality and sub-lethal effects 3, 6, 24, 48, 72 and 96 hours after start of exposure.
Test item preparation: Because the test item is poorly water soluble a saturated stock solution was prepared by using saturation columns. Two columns were prepared and used in parallel to generate the quantity of saturated solution required for this study.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (reconstituted water), the filtrate of a loading of 10 mg BAS 750 F /L, its 1.5, 2.25, 3.38, 5.06-fold dilution corresponding to geometric mean concentrations of 1.110, 0.913, 0.735, 0.593 and 0.475 mg a.s./L, respectively.

Test conditions: 10 L glass aquaria; dilution water: reconstituted water; hardness: 242.04 - 264.44 mg $CaCO_3$/L; temperature: 24.0 - 24.4 °C; pH 7.60 - 7.76; oxygen content: 80.7 - 96.2%; conductivity: 630 - 648 µS/cm; photoperiod 16 h light : 8 h dark with 30 min transition; no aeration; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with DAD detection.

Statistics: Descriptive statistics; probit analysis for calculation of LC_{50}.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of BAS 750 F concentrations was conducted in each test item concentration at the beginning of the test, after 48 h and at the end of the test. The concentrations determined in samples collected at exposure initiation were 1.566 and 1.511 mg/L in the filtrate of a loading of 10 mg/L, 1.094 and 1.097 mg/L in its 1.5-fold dilution, 0.713 and 0.676 mg/L in its 2.25-fold dilution, 0.492 and 0.461 mg/L in its 3.38-fold dilution, 0.331 and 0.320 mg/L in its 5.06-fold dilution. The concentrations of test item determined in samples collected after 48 h of exposure were in the range of 60.73 - 79.59% of initial concentrations. The concentrations of test item determined in samples collected at exposure termination were in the range of 51.25 - 69.16% of initial concentrations. The following biological results are based on geometric mean concentrations.

Biological results: After 96 hours of exposure, no mortality was observed in the control and at test item concentrations of up to and including 0.593 mg a.s./L, whereas 5% and 45% mortality was observed at 0.735 and 0.913 mg a.s./L, respectively. At the highest tested concentration, all fish were dead after 96 hours of exposure. Sub-lethal effects (i.e. unbalanced swimming behavior and faulty respiratory function) were found at 0.0.735 and 0.913 mg a.s./L after 96 hours. The results are summarized in Table 8.2.1-3.

Table 8.2.1-3: Acute toxicity (96 h) of BAS 750 F to zebrafish (*Brachydanio rerio*)

Concentration [mg a.s./L] (geometric mean)	**Control**	**0.475**	**0.593**	**0.735**	**0.913**	**1.110**
Mortality [%] (96 h)	0	0	0	5	45	100
Symptoms (after 96 h) *	none	none	none	U	U, F	n.d.
Endpoints [mg BAS 750 F/L] (geometric mean)						
LC_{50} (96 h)	0.906 (95% confidence limits: 0.857 - 0.954)					
NOEC (96 h)	0.735					

n.d. = not determined; all fish dead
* Symptoms after 96 h: U = unbalanced swimming behavior; F = faulty respiratory function.

III. CONCLUSION

In a static acute toxicity study with zebrafish the LC_{50} (96 h) of BAS 750 F was 0.906 mg a.s./L based on geometric mean concentrations.

Report: CA 8.2.1/4
[redacted] 2014 a
BAS 750 F: Acute toxicity to the sheepshead minnow, Cyprinodon variegatus, determined under static-renewal test conditions
2014/7002810
Guidelines: EPA 850.1075
GLP: yes
(certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 96-hour semi-static acute toxicity laboratory study, juvenile sheepshead minnow (*Cyprinodon variegatus*) were exposed to a water control, a solvent control and to nominal concentrations of 0.18, 0.35, 0.70, 1.4 and 2.7 mg BAS 750 F/L (corresponding to mean measured concentrations of 0.131, 0.258, 0.517, 1.12 and 2.05 mg a.s./L) in groups of 10 animals in glass aquaria containing 8 L water. Fish were observed for survival and symptoms of toxicity 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations of the test item. After 96 hours of exposure, no mortality was observed in the water control and the solvent control, whereas 5% mortality occurred at 0.131 mg a.s./L, however, this mortality was not considered biologically significant, as no mortality was observed in the two higher test item concentrations of 0.258 and 0.517 mg a.s./L. All fish were dead at the two highest test item concentrations after 96 hours of exposure. Sublethal effects (*i.e.* fish laying on bottom of the test chamber, discoloration, loss of equilibrium and fish breaking the surface of the test solution (surfacing)) were observed at 0.258 and 0.517 mg a.s./L after 96 hours.

In a semi-static acute toxicity study with sheepshead minnow the LC_{50} (96 h) of BAS 750 F was 0.761 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Sheepshead minnow (*Cyprinodon variegatus*), juveniles; mean body length of control fish: 17 ± 2.4 mm; mean wet weight of control fish: 0.1388 ± 0.0579 g; supplied by "Aquatic BioSystems", Fort Collins, Colorado, USA.

Test design: Semi-static system (96 h): renewal of test solutions 48 hours after test initiation, 5 test item concentrations plus a dilution water control and a solvent control; 2 replicates per treatment; 10 fish per aquarium (loading 0.1735 g fish/L); assessment of mortality and sublethal effects 6, 24, 48, 72 and 96 hours after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.18, 0.35, 0.70, 1.4 and 2.7 mg BAS 750 F/L (nominal), corresponding to mean measured concentrations of 0.131, 0.258, 0.517, 1.12 and 2.05 mg a.s./L.

Test conditions: 10 L glass aquaria, test volume: 8 L; dilution water: laboratory saltwater (prepared by mixing a commercial sea salt mix to laboratory freshwater), salinity: 19.7 - 19.9 ‰; temperature: 21.8 °C - 22.6 °C; pH 8.0 - 8.1; oxygen content: 7.4 mg/L - 7.6 mg/L (100 - 103% saturation) in fresh solutions, 6.0 mg/L - 7.2 mg/L (79 - 95% saturation) in spent solutions; photoperiod 16 h light : 8 h dark; light intensity: approx. 956 lux; no aeration; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with MS/MS-detection.

Statistics: Descriptive statistics; probit analysis for determination of the LC_{50} value, Dunnett's one-tailed t-test and one-tailed Fisher`s t-test with Hochberg's family-wise correction for determination of the NOEC

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of BAS 750 F concentrations in fresh and old solutions was conducted in all concentrations at test initiation, after 48 hours and at test termination. Samples of the highest test item concentration were not analyzed beyond the samples of the old solution after 48 hours due to 100% mortality. The analyzed contents of BAS 750 F in new solutions ranged from 69% to 81% of nominal and the measured concentrations in old solution were between 69% and 81% of nominal. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality was observed in the water control and the solvent control, whereas 5% mortality occurred at 0.131 mg a.s./L, however, this mortality was not considered biologically significant, as no mortality was observed in the two higher test item concentrations of 0.258 and 0.517 mg a.s./L. All fish were dead at the two highest test item concentrations after 96 hours of exposure. Sublethal effects (*i.e.* fish laying on bottom of the test chamber, discoloration, loss of equilibrium and fish breaking the surface of the test solution (surfacing)) were observed at 0.258 and 0.517 mg a.s./L after 96 hours. The results are summarized in Table 8.2.1-4.

Table 8.2.1-4: Acute toxicity (96 h) of BAS 750 F to sheepshead minnow (*Cyprinodon variegatus*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.18**	**0.35**	**0.70**	**1.4**	**2.7**
Concentration [mg a.s./L] (mean measured)	**Control**	**Solvent control**	**0.131**	**0.258**	**0.517**	**1.12**	**2.05**
Mortality [%] (96 h)	0	0	5 [a)]	0	0	100	100
Symptoms (after 96 h) [#]	none	none	none	S, LE, DC	DC, B, LE,	n.d.	n.d.
Endpoints [mg BAS 750 F/L] (mean measured)							
LC_{50} (96 h)	0.761 (95% confidence limits: n.c.)						
NOEC (96 h)	0.131						

[a)] This mortality was not considered biologically significant as compared to the control.
[#] Symptoms after 96 h: B = on bottom, DC = discoloration, LE = loss of equilibrium, S = surfacing.
n.d. = not determined; all animals dead
n.c. = not calculated

III. CONCLUSION

In a semi-static acute toxicity study with sheepshead minnow the LC_{50} (96 h) of BAS 750 F was 0.761 mg a.s./L based on mean measured concentrations.

Report:	CA 8.2.1/5 [redacted] 2015 c BAS 750 F - Acute toxicity study in the common carp (Cyprinus carpio) 2015/1249071
Guidelines:	EC 440/2008 C.1 Acute Toxicity for Fish, OECD 203, EPA 72-1, EPA 850.1075
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 96-hour flow-through acute toxicity laboratory study, common carp were exposed to a dilution water control and to nominal concentrations of 4.6, 10, 22, 46 and 100% of a saturated solution of BAS 750 F (corresponding to mean measured concentrations of 0.082, 0.171, 0.414, 0.812 and 1.57 mg a.s./L) in groups of 10 animals in glass aquaria containing 9 L water. Fish were observed for survival and symptoms of toxicity within 1 hour after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations of the test item. After 96 hours of exposure, no mortality was observed in the control and at test item concentrations of up to and including 0.812 mg a.s./L, whereas 100% mortality was observed at 1.57 mg a.s./L. No sub-lethal effects were found in the control and at test item concentrations of up to and including 0.812 mg a.s./L.

In a flow-through acute toxicity study with common carp the LC_{50} (96 h) of BAS 750 F was 1.126 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% ± 1.0%.

B. STUDY DESIGN

Test species: Common carp (*Cyprinus carpio*), age: approx. 5 months; body length of control fish: 3.9 cm (3.6 cm - 4.0 cm); body weight of control fish: 0.81 g (0.56 g - 1.11 g); supplied by Osage Catfisheries, Inc., Osage Beach, MO, USA.

Test design: Flow through system (96 h); 5 test item concentrations plus a dilution water control, 2 replicates per treatment; 10 fish per aquarium (loading 0.18 g fish/L/day); assessment of mortality and sub-lethal effects within 1 hour after start of exposure and 6, 24, 48, 72 and 96 hours after start of exposure. Test solution preparation: Because the test item is poorly water soluble a saturated stock solution was prepared by using saturation columns. Two columns were prepared and used in parallel to generate the quantity of saturated solution required for this study.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), 4.6, 10, 22, 46 and 100% of a saturated solution of BAS 750 F corresponding to mean measured concentrations of 0.082, 0.171, 0.414, 0.812 and 1.57 mg a.s./L.

Test conditions: Stainless steel aquaria (29 x 21 x 22 cm), test volume: 9 L; test water: non chlorinated charcoal-filtered drinking water mixed with deionised water; flow rate: min. 1.88 L/h (min. 5 volume exchanges per day); hardness: 1.11 mmol/L; temperature: 23°C; pH 8.0 - 8.1; oxygen content: 7.5 mg/L - 8.0 mg/L; conductivity: 248 µS/cm; photoperiod 16 h light : 8 h dark; light intensity: approx. 80 - 328 lux; no aeration; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a HPLC-method with MS detection.

Statistics: Descriptive statistics; probit analysis for calculation of LC_{50}.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of BAS 750 F concentrations was conducted in each test item concentration at the beginning of the test, after 48 h and at the end of the test. The mean measured concentrations in the test item treatments were 0.082, 0.171, 0.414, 0.812 and 1.57 mg BAS 750 F/L. The analyzed contents of BAS 750 F ranged from 107% to 116% of mean measured concentrations at test initiation, from 89% to 102% after 48 h and from 91% to 103% of mean measured concentrations at test termination. Thus, the measured concentrations of the test item in the test solutions during the exposure period were within ± 20% of the overall mean measured values. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality was observed in the control and at test item concentrations of up to and including 0.812 mg a.s./L, whereas 100% mortality was observed at 1.57 mg a.s./L. No sub-lethal effects were found in the control and at test item concentrations of up to and including 0.812 mg a.s./L. The results are summarized in Table 8.2.1-5.

Table 8.2.1-5: Acute toxicity (96 h) of BAS 750 F to common carp (*Cyprinus carpio*)

Concentration [% saturation] (nominal)	**Control**	**4.6**	**10**	**22**	**46**	**100**
Concentration [mg a.s./L] (mean measured)	**--**	**0.082**	**0.171**	**0.414**	**0.812**	**1.57**
Mortality [%] (96 h)	0	0	0	0	0	100
Symptoms (after 96 h)	none	none	none	none	none	n.d.
Endpoints [mg BAS 750 F/L] (mean measured)						
LC_{50} (96 h)	1.126 (95% confidence limits: n.c.)					
NOEC (96 h)	0.812					

n.d. = not determined; all fish dead; n.c. = not calculated due to mathematical reasons / inappropriate data

III. CONCLUSION

In a flow-through acute toxicity study with common carp the LC_{50} (96 h) of BAS 750 F was 1.126 mg a.s./L based on mean measured concentrations.

CA 8.2.2 Long-term and chronic toxicity to fish

CA 8.2.2.1 Fish early life stage toxicity test

Report: CA 8.2.2.1/1
[redacted] 2015 a
BAS 750 F: Early life-stage toxicity test with the sheepshead minnow, Cyprinodon variegatus, under flow-through conditions
2015/7000619
Guidelines: EPA 850.1400
GLP: yes
(certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The chronic toxicity of BAS 750 F to sheepshead minnow (*Cyprinodon variegatus*) embryos and fry was investigated in a 35-day early life-stage test under flow-through conditions. Embryos were exposed to a dilution water control, a vehicle control (0.05 mL DMF/L) and to BAS 750 F at nominal concentrations of 0.010, 0.020, 0.040, 0.080, and 0.16 mg a.s./L (corresponding to mean measured concentrations of 0.00861, 0.0172, 0.0356, 0.0725 and 0.147 mg a.s./L, respectively). Hatchability, survival rate and behavior of sheepshead minnow embryos and fry were assessed throughout the study. Individual fish lengths and weights were measured at test termination.

The biological results are based on nominal concentrations. Egg hatch began on day 5 and ended between study days 6 and 11 in the control and all test item treatments. 95% hatch was reached in all treatments by study day 9 (day 0 post-hatch) and the overall hatching success in the control and vehicle control was 91 and 93%, respectively. Post-hatch survival was 97 and 93% in the control and vehicle control, respectively and between 94 and 97% in all test item treatments. No statistically significant effects on the hatching success, post-hatch survival, time to start of hatch and time to end of hatch was observed for any of the test item treatments as compared to the control (Dunnett`s test, $\alpha = 0.05$).
The mean standard fish lengths in the test item treatments ranged from 14.0 mm to 14.7 mm compared to 14.1 mm in the control and 14.2 mm in the vehicle control treatments. Mean blotted wet weight in the control and vehicle control was 0.0836 g and 0.0887 g, respectively. Mean blotted wet weight in the test substance treatments ranged from 0.0826 g to 0.0969 g. There was no statistically significant ($\alpha = 0.05$) reduction in length and blotted wet weight in any of the test substance treatments as compared to the control.
The only morphological or behavioral abnormalities observed during the exposure were fish on the bottom of the test chamber and spinal curvature. Fish on the bottom of the test chamber was observed for one fish in the 0.147 mg a.s./L test treatment on study days 12 and 13. Spinal curvature was observed for one fish in the vehicle control on study days 18 and 19. These abnormality were judged not to be test substance related because no concentration-response relationship was evident. No other morphological or behavioral abnormalities were noted.

The NOEC is given as primary endpoint, since no dose-response relationship was derived from the study which could be used for EC_x calculations.

In an early life stage study with sheepshead minnow (*Cyprinodon variegatus*), the overall NOEC (35 d) for BAS 750 F was determined to be 0.16 mg a.s./L, based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no. 5834378); batch no. COD-001750; purity: 98.8%.

B. STUDY DESIGN

Test species: Sheepshead Minnow (*Cyprinodon variegatus*); newly fertilized eggs (< 24 hours post-fertilization) obtained from in-house culture.

Test design: Flow-through system (35 d); 5 test item concentrations plus a dilution water control and a vehicle control; 4 replicate test chambers per treatment with 20 fertilized eggs in each; a proportional diluter system was used for the preparation of test solutions and intermittent introduction of the solutions to the test chambers. During the embryo stage, the developing embryos were incubated in glass cups. On a daily basis during incubation, the embryos were counted and dead embryos were removed and discarded. On study day 11 (*i.e.*, day 4 post-hatch), all live fry were counted and released into their respective replicate growth chamber. Survival of the fry was monitored daily following hatch by visually inspecting each test chamber, and any behavioral or physical changes were recorded, including abnormalities. On day 35 (28 days post hatch) surviving animals were sacrificed and measured for length and weight.

Endpoints: NOEC values based on hatchability, survival, toxic signs and growth.

Test concentrations: Control (dilution water), vehicle control (0.05 mL DMF/L), 0.010, 0.020, 0.040, 0.080, and 0.16 mg a.s./L (nominal); corresponding to mean measured concentrations of <MQL, <MQL, 0.00861, 0.0172, 0.0356, 0.0725 and 0.147 mg a.s./L, respectively.

Test conditions:	Test vessels: glass aquaria (15 x 21.5 x 24 cm) with a test volume of approx. 5.0 L; 4 replicate test chambers; glass incubation cups (used during embryo stage) with 9 cm diameter and Nitex® screen replacing the bottom; one incubation cup per test chamber; test medium: commercial sea salt mix (Crystal Sea Marine Mix, Marine Enterprise International, Inc. Baltimore, Maryland) added to demineralized, filtered and sterilized laboratory freshwater; water temperature 24.6 °C - 25.9 °C; pH 7.9 - 8.2; dissolved oxygen: 5.6 mg/L - 7.7 mg/L (78 - 110% saturation); salinity: 19.6 - 20.1‰; light intensity: ranged from 483 to 602 lux; photoperiod: 16 h light : 8 h dark (30-minute simulated dawn and dusk transition period); flow rate: approx. 3.0 cycles per hour with 0.5 L per diluter cycle in each replicate test chamber (resulting in 7.2 volume additions per test chamber over a 24 h period); feeding: fish larvae were fed three times daily *ad libitum* brine shrimp nauplii (*Artemia*), standard commercial fish food was added to the daily food beginning on day 21; fish were fed *ad libitum* at least three times daily.
Analytics:	Analytical verification of BAS 750 F concentrations was conducted using an HPLC-method with MS/MS detection.
Statistics:	Descriptive statistics; Fisher's exact test and t-test for comparison of controls; Fisher's exact test and/or ANOVA followed by one-tailed Dunnett's test (α = 0.05) for determination of NOEC.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical measurements of BAS 750 F concentrations were conducted in samples collected three days prior to initiation and in the control and in all test item concentrations at test initiation, on study days 0, 7, 11, 14, 21, 28 and at test termination after 35 d of exposure. Measured concentrations of BAS 750 F in the test item treatments prior to initiation of the definitive test ranged from 81% to 84% of the nominal concentrations. Measured concentrations of BAS 750 F in the test item treatments on day 0 of the exposure ranged from 84% to 89% of the nominal concentrations. On days 7 through 35, measured values were between 82% and 96% of nominal. The mean measured concentrations of BAS 750 F in the test-substance treatments for the 35-day exposure ranged from 86% to 92% of the nominal concentrations. The concentration of BAS 750 F in all test substance treatments throughout the exposure was maintained within 20% of the mean measured values. The following biological results are based on nominal concentrations.

Biological results: Egg hatch began on day 5 and ended between study days 6 and 11 in the control and all test item treatments. 95% hatch was reached in all treatments by study day 9 (day 0 post-hatch) and the overall hatching success in the control and vehicle control was 91 and 93%, respectively. Post-hatch survival was 97 and 93% in the control and vehicle control, respectively and between 94 and 97% in all test item treatments. No statistically significant effects on the hatching success, post-hatch survival, time to start of hatch and time to end of hatch was observed for any of the test item treatments as compared to the control (Dunnett`s test, α = 0.05).

The mean standard fish lengths in the test item treatments ranged from 14.0 mm to 14.7 mm compared to 14.1 mm in the control and 14.2 mm in the vehicle control treatments. Mean blotted wet weight in the control and vehicle control was 0.0836 g and 0.0887 g, respectively. Mean blotted wet weight in the test substance treatments ranged from 0.0826 g to 0.0969 g. There was no statistically significant ($\alpha = 0.05$) reduction in length and blotted wet weight in any of the test substance treatments as compared to the control.
The only morphological or behavioral abnormalities observed during the exposure were fish on the bottom of the test chamber and spinal curvature. Fish on the bottom of the test chamber was observed for one fish in the 0.147 mg a.s./L test treatment on study days 12 and 13. Spinal curvature was observed for one fish in the vehicle control on study days 18 and 19. These abnormality were judged not to be test substance related because no concentration-response relationship was evident. No other morphological or behavioral abnormalities were noted. The results are summarized in Table 8.2.2.1-1.

Table 8.2.2.1-1: Chronic toxicity of BAS 750 F to sheepshead minnow (*Cyprinodon variegatus*) in a fish early life-stage test (35 d)

Concentration [mg a.s./L] (nominal)	**Control**	**Vehicle control**	**0.010**	**0.020**	**0.040**	**0.080**	**0.16**
Concentration [mg a.s./L] (mean measured)	**< MQL**	**< MQL**	**0.00861**	**0.0172**	**0.0356**	**0.0725**	**0.147**
Hatching success [%]	91	93	96	84	88	90	88
Start of hatch [d]	6	6	6	6	6	6	6
Time to 95% hatch [d]	7	8	7	8	7	7	7
End of hatch [d]	7	9	7	8	7	7	7
Post-hatch survival [%] on day 35	97	93	96	97	94	97	97
Mean standard length on day 35 [mm] (standard deviation)	14.1 (1.2)	14.2 (1.4)	14.2 (0.72)	14.4 (1.3)	14.7 (0.85)	14.1 (1.3)	14.0 (1.3)
Mean blotted wet weight on day 35 [g] (standard deviation)	0.0836 (0.0193)	0.0887 (0.0198)	0.0969 (0.0818)	0.0859 (0.0293)	0.0911 (0.0158)	0.0826 (0.0202)	0.0882 (0.0215)
Endpoints [mg BAS 750 F/L] (nominal)							
$NOEC_{overall}$ (35 d)	**0.16**						

MQL = Minimal Quantifiable Limit

The NOEC is given as primary endpoint, since no dose-response relationship was derived from the study which could be used for EC_x calculations.

III. CONCLUSION

In an early life stage study with sheepshead minnow (*Cyprinodon variegatus*), the overall NOEC (35 d) for BAS 750 F was determined to be 0.16 mg a.s./L, based on nominal concentrations.

Report:	CA 8.2.2.1/2 [redacted] 2015 a BAS 750 F - Early life-stage toxicity test on the zebrafish (Danio rerio) in a flow through system 2014/1262160
Guidelines:	OECD 210, EPA 72-4 (a), EPA 850.1400, EPA 540/9-86-138
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The chronic toxicity of BAS 750 F to zebrafish (*Danio rerio*) was evaluated in a 36-day early life-stage test under flow-through conditions. Embryos were exposed to a dilution water control and to nominal test item concentrations of 0.010, 0.024, 0.060, 0.150 and 0.375 mg BAS 750 F/L (corresponding to mean measured concentrations of 0.011, 0.027, 0.063, 0.172 and 0.444 mg a.s./L). Hatchability, post-hatch survival rate, time to hatch and swim-up, and growth parameters of zebrafish embryos were assessed throughout the study.

The results are based on mean measured concentrations. Hatching started simultaneously in all test groups on day 3 and was complete by day 6. No test substance-related effect was observed on the time to start or end of hatching. Hatching success ranged from 96-100% for the replicates of the control group and there was no statistically significant decrease in hatching success in any of the treatment groups in comparison to the control group. Survival from hatch to the end of swim-up (day 7) was 100% for all replicates of the control group and there was no statistically significant decrease in survival in any of the treatment groups in comparison to the control group.
In all test groups, larval swim-up started on day 5 of exposure and was complete simultaneously with the control on day 7. No test substance-related effect was observed on the time to swim-up. From the end of swim-up to the end of exposure (day 7-36) survival was 96-100% for the replicates of the control group. The survival from the end of swim-up to the end of exposure (day 7-36) as well as the overall survival (day 0-36) was statistically significantly decreased in the treatment groups of 0.172 and 0.444 mg a.s./L and in comparison to the control group.
There were no signs of toxicity or abnormalities observed among the replicates of the control group and the treatment groups. In comparison to the control group the total body lengths of the surviving fish at the end of the exposure period were statistically significantly decreased in the test groups of 0.063, 0.172 and 0.444 mg a.s./L. In comparison to the control group, the mean wet weights of the surviving fish at the end of the exposure period were statistically significantly decreased in the test groups of 0.172 and 0.444 mg a.s./L.

The NOEC is given as primary endpoint, since from the dose-response it is obvious that effects on survival or growth with ≥ 10% (EC_{10}, EC_{20}) occur only at higher test concentrations than the NOEC. The effect level of the most sensitive parameter (length) is less than 5% at the NOEC. Hence, the NOEC is protective and used as the most reliable endpoint for the risk assessment. EC_x were not calculated.

In an early life stage study with zebrafish (*Danio rerio*) the overall NOEC (36 d) for BAS 750 F was determined to be 0.027 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Test substance no.: 11/0741-8), batch no. COD 001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Zebrafish (*Danio rerio*), eggs less than 6 hours old, source: in-house rearing.

Test design: Flow through system (36 d); 5 test item concentrations plus a dilution water control, 4 replicates per treatment with 25 fertilized eggs in each. Eggs and larvae were exposed in cylindrical glass vessels (egg cups); surviving larvae were released to their respective test vessels on day 20. The test solution flowed continuously from the mixing tank into an "udder" which splitted the test water into 4 equal parts for the 4 replicate test aquaria. On day 36 fish were sacrificed and the body length and weight of surviving individuals were determined. Daily assessment of hatch, swim-up, survival, signs of toxicity and abnormal behavior.

Endpoints: NOEC values based on hatch rate, post-hatch survival, toxic signs, growth and time spans to hatch and swim-up.

Test concentrations: Control (dilution water), 0.010, 0.024, 0.060, 0.150 and 0.375 mg BAS 750 F/L (nominal), corresponding to mean measured concentrations of <LoQ (Limit of quantification), 0.011, 0.027, 0.063, 0.172 and 0.444 mg a.s./L.

Test conditions: Test vessels: stainless steel aquaria (29 x 21 x 22 cm), water volume: 9 L; egg cups: cylindrical glass vessels, diameter 19 cm (water volume: 1.7 L). Dilution water: non-chlorinated, filtered tap water (diluted with deionized water); temperature 23.8 - 25.5 °C; pH 7.8 - 8.3; oxygen content 6.1 mg/L - 8.3 mg/L; water hardness: 1.11 - 1.23 mmol/L; conductivity: 277 - 282 µS/cm; acid capacity: 2.59 mmol/L. Light intensity: 73 - 148 lux; photoperiod: 16 hours light : 8 hours dark; flow rates: 2.25 L/hour/test vessel. Feeding: freshly hatched *Artemia nauplii* and commercial fish diet (Tetramin, supplied by Tetra-Werke; Seramicron, supplied by Sera) from day 7 on; slight aeration from day 24 on.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS detection.

Statistics: Descriptive statistics; one-sided Jonkheere-Terpstra for embryo, larvae and juvenile fish survival; one-sided William´s test for weight and length data, one-sided Wilcoxon-test for variability between replicates.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in all test item concentrations at weekly intervals until day 36. Mean measured concentrations of BAS 750 F ranged from 95.4% to 126.1% of nominal over the exposure period. Most individually measured concentration values were within a range of ± 20% of the nominal values. The following biological results are based on mean measured concentrations.

Biological results: Hatching started simultaneously in all test groups on day 3 and was complete by day 6. No test substance-related effect was observed on the time to start or end of hatching. Hatching success ranged from 96-100% for the replicates of the control group and there was no statistically significant decrease in hatching success in any of the treatment groups in comparison to the control group. Survival from hatch to the end of swim-up (day 7) was 100% for all replicates of the control group and there was no statistically significant decrease in survival in any of the treatment groups in comparison to the control group (one-sided Jonkheere-Terpstra, $p \leq 0.05$).
In all test groups, larval swim-up started on day 5 of exposure and was complete simultaneously with the control on day 7. No test substance-related effect was observed on the time to swim-up. From the end of swim-up to the end of exposure (day 7–36) survival was 96-100% for the replicates of the control group. The survival from the end of swim-up to the end of exposure (day 7–36) as well as the overall survival (day 0–36) was statistically significantly decreased in the treatment groups of 0.172 and 0.444 mg a.s./L in comparison to the control group (one-sided Jonkheere-Terpstra, $p \leq 0.05$).
There were no signs of toxicity or abnormalities observed among the replicates of the control group and the treatment groups. In comparison to the control group the total body lengths of the surviving fish at the end of the exposure period were statistically significantly decreased in the test groups of 0.063, 0.172 and 0.444 mg a.s./L (one-sided Jonkheere-Terpstra test, $p \leq 0.05$ or one-sided William´s test, $p \leq 0.01$). In comparison to the control group, the mean wet weights of the surviving fish at the end of the exposure period were statistically significantly decreased in the test groups of 0.172 and 0.444 mg a.s./L (one-sided Jonkheere-Terpstra test, $p \leq 0.05$ or one-sided William´s test, $p \leq 0.05$. The results are summarized in Table 8.2.2.1-2.

Table 8.2.2.1-2: Chronic toxicity of BAS 750 F to zebrafish (*Danio rerio*) in a fish early life stage test (36 d)

Concentration (nominal) [mg a.s./L]	**Control**	**0.010**	**0.024**	**0.060**	**0.150**	**0.375**
Concentration (mean measured) [mg a.s./L]	**<LoQ**	**0.011**	**0.027**	**0.063**	**0.172**	**0.444**
Embryo survival until hatch [%]	98	100	99	100	99	98
Survival of larvae from hatch until end of swim-up (day 7) [%]	100	100	100	99	100	100
Survival of young fish (day 7 - 36) [%]	99	96	98	96	**94 ***	**95 ***
Survival from day 0 to test termination (36 d) [%]	97	96	97	95	**93 ***	**93 ***
Start of hatch [day] End of hatch [day]	3 6	3 6	3 6	3 6	3 6	3 6
Start of swim-up End of swim-up	5 7	5 7	5 7	5 7	5 7	5 7
Symptoms	none	none	none	none	none	none
Mean weight (36 d) [mg]	48.1	51.4	47.3	44.2	**42.9 ***	**32.9 ****
% of control	--	106.7	98.3	91.9	89.1	68.4
Mean length (36 d) [cm]	1.76	1.74	1.71	**1.68 ***	**1.66 ****	**1.50 ****
% of control	--	99.2	97.6	95.9	94.3	85.6
	Endpoints [mg BAS 750 F/L] (mean measured)					
$NOEC_{overall}$ (36 d)	0.027					

Values printed in **bold** show statistically significant differences compared to the control.
* Statistically significant differences compared to the control (one-sided Jonkheere-Terpstra test, $p \leq 0.05$).
****** Statistically significant differences compared to the control (one-sided William´s test, $p \leq 0.01$).

The NOEC is given as primary endpoint, since from the dose-response it is obvious that effects on survival or growth with ≥ 10% (EC_{10}, EC_{20}) occur only at higher test concentrations than the NOEC. The effect level of the most sensitive parameter (length) is less than 5% at the NOEC. Hence, the NOEC is protective and used as the most reliable endpoint for the risk assessment. EC_x were not calculated.

III. CONCLUSION

In an early life stage study with zebrafish (*Danio rerio*) the overall NOEC (36 d) for BAS 750 F was determined to be 0.027 mg a.s./L based on mean measured concentrations.

Report:	CA 8.2.2.1/3 [redacted] 2015 b BAS 750 F - Fish sexual development test on the zebrafish (Danio rerio) 2015/1099093
Guidelines:	OECD 234
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 8.2.2.1/4 Obermann M., 2014a Concentration control analysis of BAS 750 F, Reg.No. 5834378 in mixing-water, GV/T Project No. 56F0741/11E177 2014/1161851
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 8.2.2.1/5 Obermann M., 2015a Report Amendment No. 1 - Concentration control analysis of BAS 750 F, Reg.No. 5834378 in mixing-water, GV/T Project No. 56F0741/11E177 2015/1117846
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 8.2.2.1/6 Obermann M., 2015b Report amendment no. 2 to final report: Concentration control analysis of BAS 750 F, Reg.No. 5834378 in mixing-water, GV/T Project No. 56F0741/11E177 2015/1181295
Guidelines:	<none>
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The chronic toxicity of BAS 750 F to zebrafish (*Danio rerio*) was evaluated in a 69-day fish sexual development test under flow-through conditions. Embryos were exposed to a dilution water control and to nominal test item concentrations of 0.010, 0.021 and 0.041 mg BAS 750 F/L (corresponding to mean measured concentrations of 0.010, 0.022 and 0.045 mg a.s./L). During exposure the effect of the test substance on development, survival and growth was evaluated. After sacrifice, fish from all test groups were histologically evaluated for gonad development and sex ratio. Vitellogenin was determined in head and tail of all fish at the end of exposure.

The results are based on mean measured concentrations. Hatching started simultaneously in all test groups on day 3 and was complete by day 5. No test substance-related effect was observed on the time to start or end of hatching. Hatching success ranged from 93 - 100% for the replicates of the control group and there was no statistically significant decrease in hatching success in any of the treatment groups in comparison to the control group. Survival from hatch to the end of swim-up (day 5) was 100% for all replicates of the control group and there was no statistically significant decrease in survival in any of the treatment groups in comparison to the control group. In all test groups, larval swim-up started on day 5 of exposure and was complete simultaneously with the control on day 6. No test substance-related effect was observed on the time to swim-up. From the end of swim-up to the end of exposure (day 6 - 68/69) survival was 93 - 100% for the replicates of the control group. The survival from the end of swim-up to the end of exposure (day 6 - 68/69) as well as the overall survival (day 0 - 68/69) was not statistically decreased in any treatment group in comparison to the control group. There was no statistically significant test substance-related effect on survival in at any test concentration.
There were no signs of toxicity or abnormalities observed among the replicates of the control group and the treatment groups with two exceptions in the treatment group of 0.021 mg a.s./L. In this treatment group two deformed fish were identified at the end of exposure. Due to the low incidence and lack of any observations in the next higher treatment group (0.041 mg a.s./L), these are considered incidental findings and are not test substance related.
In comparison to the control group the mean wet weights and length of the surviving male and female fish at the end of the exposure period were not statistically significantly decreased in the treatment groups.
There was a mean of 50% male fish in the control and means of 50 - 56% males in the treatment groups. There was no statistically significant difference in sex ratio between the control and treatment groups. There were also no significant differences between controls and treatments in maturity index or biomarker vitellogenin concentration in head/tail homogenates.

The NOEC is given as primary endpoint, since no dose-response relationship was derived from the study which could be used for EC_x calculations.

In a sexual development study with zebrafish (*Danio rerio*) the overall NOEC for BAS 750 F was determined to be ≥ 0.045 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Test substance no.: 11/0741-8), batch no. COD 001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Zebrafish (*Danio rerio*), eggs less than 6 hours old, source: in-house rearing.

Test design: Flow through system (69 d); 3 test item concentrations plus a dilution water control, 4 replicates per treatment with 30 fertilized eggs per replicate. Eggs and larvae were exposed in egg cups; juveniles were transferred to stainless steel aquaria at a suitable size for the remainder of exposure. The test solution flowed continuously from the mixing tank through a flow splitter which splitted the test water into 4 equal parts for the 4 replicate test vessels. On day 68/69 fish were sacrificed (workload was too high to sacrifice all fish on the same day, therefore A and B replicates were sacrificed on day 68, C and D replicates were sacrificed on day 69) and the body length and weight of surviving individuals were determined. Daily assessment of survival and signs of toxicity or abnormal behavior. Histological evaluation of gonad development and sex ratio after sacrifice. Determination of vitellogenin in head and tail of all fish at the end of exposure.

Endpoints: NOEC values based on hatching success, survival, toxic signs, growth, sex ratio, gonad histopathology and vitellogenin.

Test concentrations: Control (dilution water), 0.010, 0.021 and 0.041 mg BAS 750 F/L (nominal), corresponding to mean measured concentrations of 0.010, 0.022 and 0.045 mg a.s./L.

Test conditions: Test vessels: stainless steel aquaria (38.5 x 23.5 x 29.0 cm), test volume: 24 L; egg cups: cylindrical glass vessels, diameter 19 cm (water volume: 1.7 L). Dilution water: non-chlorinated drinking water (diluted with deionized water); temperature 25.4 – 26.9 °C; pH 7.9 - 8.1; oxygen content 5.6 mg/L - 8.7 mg/L; water hardness: 1.04 - 1.13 mmol/L; conductivity: 275 - 282 µS/cm; acid capacity: 2.48 – 2.52 mmol/L. Light intensity: 91 - 170 lux; photoperiod: 16 hours light : 8 hours dark; flow rate: 5 L/hour/test vessel. Feeding: live brine shrimp nauplii (*Artemia* sp.) and commercial fish diet (“Tetramin” and/or “Sera Micron”) from day 6 on; slight aeration from day 34 on.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS detection.

Statistics: Descriptive statistics; one-sided Jonkheere-Terpstra test or one-sided Wilcoxon-test for survival; one-sided William´s test or one-sided Dunnett's test for weight and length data, one-sided Williams test or one-sided Dunnett's test for maturity index; two-sided Jonkheere-Terpstra test or two-sided Wilcoxon test for sex ratio.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in all test item concentrations at test initiation, at weekly intervals until day 63 and at test termination (day 68). Mean measured concentrations of BAS 750 F ranged from 82.0% to 120.0% of nominal over the exposure period. The following biological results are based on mean measured concentrations.

Biological results: Hatching started simultaneously in all test groups on day 3 and was complete by day 5. No test substance-related effect was observed on the time to start or end of hatching. Hatching success ranged from 93 - 100% for the replicates of the control group and there was no statistically significant decrease in hatching success in any of the treatment groups in comparison to the control group. Survival from hatch to the end of swim-up (day 5) was 100% for all replicates of the control group and there was no statistically significant decrease in survival in any of the treatment groups in comparison to the control group. In all test groups, larval swim-up started on day 5 of exposure and was complete simultaneously with the control on day 6. No test substance-related effect was observed on the time to swim-up. From the end of swim-up to the end of exposure (day 6 - 68/69) survival was 93 - 100% for the replicates of the control group. The survival from the end of swim-up to the end of exposure (day 6 - 68/69) as well as the overall survival (day 0 - 68/69) was not statistically decreased in any treatment group in comparison to the control group. There was no statistically significant test substance-related effect on survival in any test concentration.
There were no signs of toxicity or abnormalities observed among the replicates of the control group and the treatment groups with two exceptions in the treatment group of 0.021 mg a.s./L. In this treatment group two deformed fish were identified at the end of exposure. Due to the low incidence and lack of any observations in the next higher treatment group (0.041 mg a.s./L), these are considered incidental findings and are not test substance related.
In comparison to the control group the mean wet weights and length of the surviving male and female fish at the end of the exposure period were not statistically significantly decreased in the treatment groups.
There was a mean of 50% male fish in the control and means of 50 - 56% males in the treatment groups. There was no statistically significant difference in sex ratio between the control and treatment groups. There were also no significant differences between controls and treatments in maturity index or biomarker vitellogenin concentration in head/tail homogenates. The results are summarized in Table 8.2.2.1-3.

Table 8.2.2.1-3: Chronic toxicity of BAS 750 F to zebrafish (*Danio rerio*) in a fish sexual development test (69 d)

Concentration (nominal) [mg a.s./L]		**Control**	**0.010**	**0.021**	**0.041**
Concentration (mean measured) [mg a.s./L]		**--**	**0.010**	**0.022**	**0.045**
Mean hatching success [%]		98	97	98	98
Mean survival of larvae from hatch until end of swim-up (day 6) [%]		100	100	100	100
Mean survival of juveniles (day 6 – 68/69) [%]		97	99	97	97
Mean survival from day 0 to test termination (day 68/69) [%]		94	96	95	96
Wet weight (day 68/69) [mg] #	males	561.4 ± 19.9	559.8 ± 25.6	564.4 ± 24.8	556.2 ± 22.9
	females	774.9 ±16.8	751.6 ±57.7	823.4 ±31.8	780.8 ±25.2
Total length (day 68/69) [cm] #	males	3.9 ± 0.02	3.8 ± 0.02	3.8 ± 0.07	3.9 ± 0.06
	females	4.1 ± 0.01	4.0 ± 0.05	4.1 ± 0.02	4.0 ± 0.01
Vitellogenin concentration in fish (day 68/69) [ng/mg] #	males	3.3 ± 1.71	3.8 ± 1.82	2.3 ± 0.64	2.4 ± 0.81
	females	450.6 ± 174.76	536.7 ± 217.53	547.5 ± 27.83	490.7 ± 141.97
Sex ratio	males	57	61	64	58
	females	56	53	50	57
	% males	50	53°	56	50
Maturity index #	males	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
	females	3.01 ± 0.083	3.02 ± 0.092	3.07 ± 0.136	2.94 ± 0.041
		Endpoints [mg BAS 750 F/L] (mean measured)			
$NOEC_{overall}$		≥ 0.045			

Mean value ± standard deviation
° One fish was excluded from statistical evaluation due to lack of gonadal tissue

The NOEC is given as primary endpoint, since no dose-response relationship was derived from the study which could be used for EC_x calculations.

III. CONCLUSION

In a sexual development study with zebrafish (*Danio rerio*) the overall NOEC for BAS 750 F was determined to be ≥ 0.045 mg a.s./L based on mean measured concentrations.

CA 8.2.2.2 Fish full life cycle test

According to regulation 283/2013 a fish full life cycle test with the active substance BAS 750 F is not required for registration in the EU. The potential for endocrine mediated effects was investigated in the ELS and fish sexual development studies with *D. rerio* and *C. variegatus* (2014/122160, 2015/1099093 and 2015/7000619), see also section CA 8.2.3. Therefore, no fish full life cycle study was conducted.

CA 8.2.2.3 Bioconcentration in fish

Report: CA 8.2.2.3/1
[redacted] 2015 a
14C-BAS 750 F (label: triazole-3(5)-C14) - Bioconcentration study in the rainbow trout (Oncorhynchus mykiss)
2015/1122811
Guidelines: EPA 850.1730, OECD 305 (October 2012)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

This study determined the bioconcentration potential of BAS 750 F (mixture of radiolabeled and unlabeled test item (ratio 2+1)) in rainbow trout. The fish were exposed to one concentration of test item at 0.010 mg/L in a flow-through system for an uptake period of 14 days. Additionally, a control was set up. The depuration period was 7 days. Test item concentrations in water and fish as well as wet weight of fish were determined throughout the study. Mortality and signs of toxicity were assessed daily.

No mortalities or signs of toxicity were observed in the control and treatment group over the 23 day test period. There was no statistically significant difference in fish growth rate between control and treatment group during the experiment, therefore data from both groups were combined to determine the overall growth rate (k_g) for “growth-corrected” calculations. The lipid content of control fish sampled over the test period remained constant considering the variability of individual values and the mean lipid content from the uptake period (2.1%) was used for lipid normalization calculations.

The concentration in fish reached 95% steady state within 2.6 days based on the kinetic calculations. Overall the measured steady state bioconcentration factor (BCF_{ss}) values were very similar to the calculated kinetic (BCF_K) values indicating that steady state was reached and that uptake and depuration follow first order kinetics. The accumulation in the edible fish portions was less than in the non-edible portions. However the most relevant BCF is the growth corrected kinetic BCF normalized to 5% lipid content (BCF_{KLg}) for the whole fish because it incorporates all measurements during uptake and depuration and since it removes the influence of the test fish lipid content. In conclusion the bioconcentration factor **BCF_{KLg}** was **385** for the whole fish based on total radioactive residues of BAS 750 F.

In a flow-through bioconcentration study, rainbow trout were exposed to BAS 750 F at 0.010 mg/L (nominal) in water for an uptake period of 14 days. 95% steady state was reached within 2.6 days. The most relevant BCF is the growth corrected kinetic BCF normalized to 5% lipid content (BCF_{KLg}) for the whole fish, which was determined to be 385 based on total radioactive residues of BAS 750 F.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Mixture of radiolabeled and unlabeled test item (ratio 2+1):
Non-radiolabelled test item: BAS 750 F (Reg. no.: 5 834 378), batch no. COD-001740, purity 98.8%.
Radiolabelled test item: ^{14}C-BAS 750 F (label: triazole-3(5)-C14); batch no. 1062-2001; specific activity: 5.46 MBq/mg; chemical purity: 98.9%, radiochemical purity: 98.8%.

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss*); mean body weight: 2.00 ±0.23 g; mean body length: 6.1 ± 0.2 cm; age: approximately 4 months; source: "Forellenzucht Trostadt GbR", Trostadt, Germany.

Test design: Flow-through system (14 days uptake, 7 days depuration); one test vessel per treatment with 80 fish per test vessel (loading 0.32 g fish/L at start of the uptake period); test item concentrations in water and fish as well as wet weight of fish were determined throughout the study; total radioactive residues in fish were measured separately in edible (e.g. fillet) and nonedible (e.g. remaining carcass) portions and the whole fish value was calculated from the weight normalized sum of the individually measured portions; lipid content was determined in fish from control group at the start and end of uptake and at the end of depuration; mortality and signs of toxicity were assessed daily.

Endpoints: Bioconcentration potential (bioconcentration factors BCFss, $BCF_{SSL,}$ BCF_{K}, $BCF_{Kg,}$ BCF_{KLg}); uptake rate; depuration rate; depuration half-life; time to 95% steady state.

Test concentrations: Control, 0.010 mg BAS 750 F/L (nominal).

Test conditions:	Silicon-sealed glass aquaria (80 x 35 x 55 cm); test volume: 100 L; aerated non-chlorinated drinking water diluted with deionized water; flow rate: approx. 21 L/h/test aquarium (= approx. 5-fold volume exchange/day/test vessel); temperature: 13 °C; pH 7.9 - 8.0; oxygen content: 9.0 mg/L - 9.8 mg/L; total organic carbon: 0.8 - 1.0 mg/L; photoperiod 16 h light : 8 h dark; no aeration; feeding: commercial fish diet (BioMar) at approx. 1% of the body weight per day in 2 applications per day.
Analytics:	Determination of test item concentrations in water and fish was conducted by measuring total radioactivity using Liquid Scintillation Counting.
Statistics:	Descriptive statistics; BCF values were calculated based on steady state concentration in fish (BCF_{ss}) and based on the uptake and depuration curves by using a first order (one-compartment) biokinetic model (BCF_k); BCF values were further normalized to 5% fish lipid content and corrected for growth during the experiment (according to OECD test guideline 305).

II. RESULTS AND DISCUSSION

Mortality and signs of toxicity
No mortalities or signs of toxicity were observed in the control and treatment group over the 23 day test period.

Fish growth
There was no statistically significant difference in fish growth rate between control and treatment group during the experiment, therefore data from both groups were combined to determine the overall growth rate (k_g) for "growth-corrected" calculations. The combined growth rate of both groups (k_g) was 0.0132/day.

Lipid content of fish
The mean lipid content of control fish was 2.3% at the start of exposure, 1.9% at the end of exposure and 3.2% at the end of the test period. The lipid content of the control fish remained constant considering the variability of the individual measurements. Since there was no statistical difference in growth, for calculation of the lipid corrected BCF the mean lipid content in control fish of the uptake period (2.1%) was used.

Analytical results in water and fish tissue
During the uptake period (to day 14) the concentration of the test item in water remained within ± 20% of the nominal concentration based on radioactivity measurement. Also the additional daily measurements indicated no deviation of > 20% from the nominal concentration. The mean concentrations of ^{14}C-BAS 750 F in water during the uptake phase (through day 14) were 9.34 ± 0.43 µg/L (93.4% of the nominal concentration). By day 2 of the depuration period, concentrations were less than 1% of nominal.

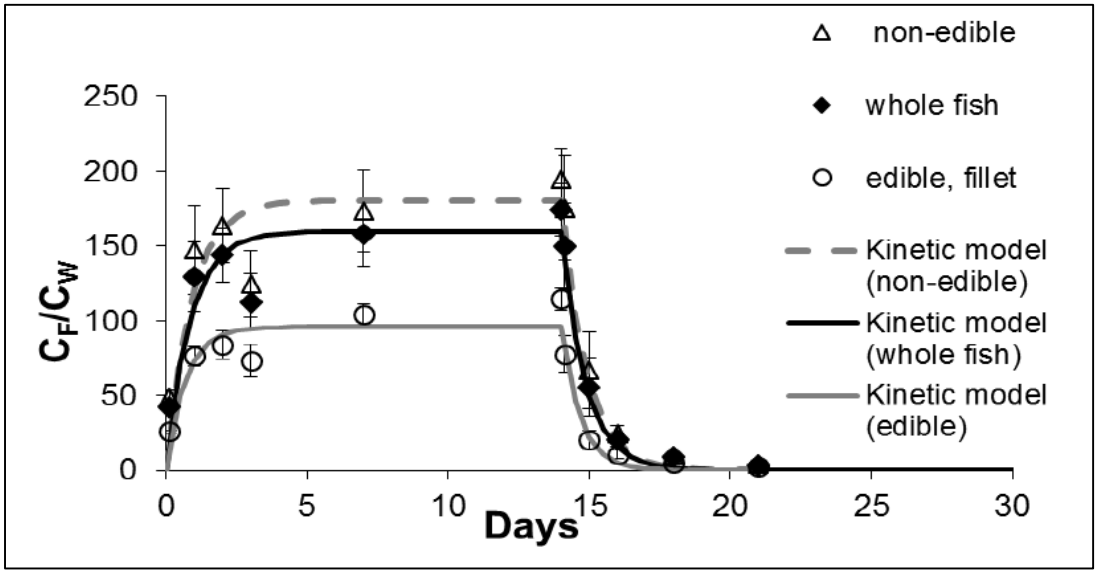
The accumulation of test item in the edible fish portions was less than in the non-edible portions. The concentration of ^{14}C-BAS 750 F in the whole fish on day 2 was within 20% of the highest measured concentration in fish during the uptake period, indicating that the uptake had become approximately asymptotic with respect to time (see Figure 8.2.2.3-1). Consequently the measured fish values from day 2 onward were considered for the calculation of the steady state bioconcentration factor (BCF_{ss}). The BCF_{ss} was 147, determined as mean value of $C_F(t)/C_W$ from days 2 to 14. When normalized to a 5% lipid content in fish, the steady state bioconcentration factor (BCF_{ssL}) was 350.

Bioconcentration kinetics

The measured data from whole fish as well as edible and non-edible portions fit well to a first order kinetic model allowing an estimation of the uptake and depuration rate constants based on simultaneous curve fitting. The data illustrate that steady state was quickly reached during the uptake period, after the sampling on day 2 (see Figure 8.2.2.3-1). Kinetic calculations indicate that concentration in fish reached 95% steady state within 2.6 days.

After the start of depuration, the concentrations in fish progressively declined. After 7 days in clean water the whole body residues in fish had declined to 3% of the mean steady state concentration (CF_{ss}).

Figure 8.2.2.3-1: Plot of the uptake and depuration curves as BCF ($C_F(t)/C_W$) in whole fish, fillet and non-edible portions. Not corrected for growth or lipid content.



The lipid normalized steady state BCF (BCF_{SSL}) values were 350, 224, 391 for the whole fish, edible and non-edible portions respectively. Based on the kinetic model, the calculated uptake rate constants (k_1) were 187, 140, and 204 day^{-1} for the whole fish, edible and non-edible portions respectively. The calculated growth corrected depuration rate constants (k_{2g}) were 1.16, 1.45, and 1.12 day^{-1} for the whole fish, edible and non-edible portions respectively. The results are summarized in Table 8.2.2.3-1.

Overall the measured BCF_{ss} values were very similar to the calculated BCF_K values indicating that steady state was reached and that uptake and depuration follow first order kinetics. The most relevant BCF is the growth corrected kinetic BCF normalized to 5% lipid content (BCF_{KLg}) because it incorporates all measurements during uptake and depuration and since it removes the influence of the test fish lipid content. In conclusion the bioconcentration factor BCF_{KLg} was 385 for the whole fish based on total radioactive residues of ^{14}C-BAS 750 F.

Table 8.2.2.3-1: Uptake and depuration rate constants and bioconcentration factors (BCF) for the whole fish, edible and non-edible portions based on measured and calculated data.

Parameter	Whole fish	Edible	Non-edible
k_g (growth rate constant; day^{-1}) (standard error)	0.0132 (0.0011)	-	-
k_1, (overall uptake rate constant, L/kg/day) (95% confidence interval)	187 (149-225)	140 (113-168)	204 (160-248)
k_2, (overall depuration rate constant, day^{-1}) (95% confidence interval)	1.17 (0.93-1.41)	1.46 (1.17-1.76)	1.13 (0.88-1.38)
k_{2g} (growth-corrected depuration rate constant, day^{-1})	1.16	1.45	1.12
$C_F$ss, (concentration in fish at steady-state, µg/kg) (mean (days 2-14) ± standard deviation)	1375 ± 245	877 ± 176	1533 ± 273
C_W (concentration in the water, µg/L) (mean (days 0 – 14) ±standard deviation)	9.34 ± 0.43	--	--
L_n (lipid normalization factor) (mean during uptake)	0.021	--	--
BCFss (steady-state BCF; L/kg) (mean (days 2-14) ± standard deviation)	147 ± 26	94 ± 19	164 ± 29
BCF_{SSL} (lipid normalized steady-state BCF; L/kg)	350	224	391
BCF_K (kinetic BCF; L/kg)	160	96	181
BCF_{Kg} (growth-corrected kinetic BCF; L/kg)	162	97	183
BCF_{KLg} (lipid-normalized kinetic BCF_{Kg}; L/kg) [a]	**385**	–	–
$t_{1/2}$, (depuration half-life; day)	0.59	0.47	0.61
$t_{1/2g}$ (growth-corrected half-life, day)	0.60	0.48	0.62
Time to 95% steady state (growth-corrected, day)	2.6	2.1	2.7

[a] The most relevant BCF in this study is the growth corrected kinetic BCF normalized to 5% lipid content, BCF_{KLG}.

III. CONCLUSION

In a flow-through bioconcentration study, rainbow trout were exposed to BAS 750 F at 0.010 mg/L (nominal) in water for an uptake period of 14 days. 95% steady state was reached within 2.6 days. The most relevant BCF is the growth corrected kinetic BCF normalized to 5% lipid content (BCF_{KLg}) for the whole fish which was determined to be 385 based on total radioactive residues of BAS 750 F.

CA 8.2.3 Endocrine disrupting properties

BAS 750 F is a DMI (de-methylase inhibiting) fungicide belonging to the triazole chemical class. This class of chemistry interacts with the sterol biosynthesis of the target organisms by inhibiting the fungal CYP51 (lanosterol 14-alpha-demethylase) which shows some structural analogy to CYP19 (aromatase). Aromatase has a decisive role in sexual differentiation by catalyzing the transformation of testosterone to 17β-estradiol. Its inhibition may thus influence the hormone levels of the above-mentioned steroids. Therefore, potential interaction of BAS 750 F with CYP19 aromatase was addressed with specific fish testing.

In fish, the phenotypic sex determination is not only controlled by genetics, but also by hormones and environmental factors (von Hofsten and Olsson 2005; Le Page et al. 2010). It has been shown for many teleosts that sex determination can be influenced by hormone treatment (e.g. Johnstone et al. 1978, Baroiller et al. 1999, Kitano et al. 2000). Being part of this complex process of sexual determination, aromatase activity can potentially change the phenotype of the fish (e.g. Ankley et al. 2005, Kinnberg et al. 2007). Therefore, sex ratio in fish is considered as a specific and sensitive endpoint to address concerns due to potential interaction of chemicals with the aromatase enzyme.
In addition to sex ratio, decreased vitellogenin (VTG, an estrogen-responsive yolk protein) in females has proven to be a sensitive biomarker in fish of potential effects of aromatase inhibitors (Andersen et al. 2004; Ankley et al. 2002, 2005; Kinnberg et al. 2007; Miller et al. 2007).

In the past, the fish full life cycle test (FFLC; OPPTS 850.1500) was requested for DMI fungicides in the EU regulatory process, including optional endpoints like sex ratio, histopathology and VTG. In July 2011, the fish sexual developmental test (FSDT, OECD test guideline 234) guideline was developed and published after ring testing and thorough validation (OECD 2011, 2012). The FSDT can be regarded as an extension of the fish early life stage test (OPPTS 850.1400 or OECD TG 210) and is prolonged until sexual differentiation of the fish. It includes VTG measurement and the endpoints sex ratio and gonad histopathology in addition to the standard apical endpoints like survival and growth of embryos, larvae, and juveniles. The FSDT design is optimized to detect effects of sexual endocrine disruptors during a very sensitive life stage with suitable fish numbers and replication. Holbech et al. (2012) summarizes the results of the validation program for the FSDT as follows: “Based on the literature and the present results it is suggested that the FSDT can be used for risk assessment of aromatase inhibiting chemicals”.

Hence, the FSDT can be regarded as the most suitable screening tool for DMI fungicides like BAS 750 F. It focuses on the early life stages of fish, the critical period for sexual determination (van Hofsten and Olsson 2005) and hence for assessing potential effects of aromatase inhibitors. Knacker et al. (2010) suggested the FSDT as a reliable test (and also comparable to a Fish Full Life Cycle Test) if aromatase inhibition is the relevant mode of action of the test substance. Data confirm that the most critical life stages are covered with respect to sensitivity of effects (see also Teigeler et al. 2007). Therefore, the FSDT was selected to address the potential for endocrine disruption of BAS 750 F.

The concentrations tested in the FSDT were carefully selected based on the available fish data. In order to avoid systemic and unspecific toxicity, the test concentrations were chosen based on the findings in the ELS test conducted with the same test species (zebra fish, *Danio rerio*, DocID 2014/122160). The highest concentration tested in the FSDT was the geometric mean of LOEC (63 µg a.s./L) and NOEC (27 µg a.s./L) as observed in the ELS study with BAS 750 F. This highest test concentration is based on consideration that testing should clarify potential (specific) effects on the endocrine system. Unspecific responses that might be caused by systemic toxicity should be avoided (see also Wheeler et al. 2013).

The fish sexual development test with zebra fish (DocID 2015/1099093) shows no effects in endocrine related endpoints (sex ratio, histological gonad examination, vitellogenin measurements) nor in standard apical endpoints (survival and growth parameters) up to and including the highest test concentration of 45 µg a.s./L (mean measured). Mean hatching success ranged from 97 to 98% and survival of larvae and fish until study termination ranged from 94 to 100% in treatments and control. Mean weight and length differed by less than 10% between control and treatments without dose-response or statistical significance. For the parameter sex ratio, a mean of 50% males was found in the control and 50 – 56% males in the treatments. Mean vitellogenin concentration in females was 451 ng/mg in the control and ranged from 491 - 548 ng/mg in the treatments.

In conclusion, there is no indication for endocrine potential of BAS 750 F in fish.

CA 8.2.4 Acute toxicity to aquatic invertebrates

CA 8.2.4.1 Acute toxicity to Daphnia magna

Report:	CA 8.2.4.1/1 Brzozowska K., 2014a BAS 750 F (Reg.No. 5834378) - Daphnia magna, acute immobilization test 2013/1250866
Guidelines:	OECD 202 (2004), EPA 850.1010
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 48-hour static acute toxicity laboratory study, the effect of BAS 750 F on water flea neonates was investigated. Due to the poor solubility of the test item, a filtrate of the loading of 10 mg BAS 750 F/L was used as the highest test item concentration, while the six lower test item concentrations consisted of 11.42-fold, 7.61-fold, 5.07-fold, 3.38-fold, 2.25-fold and 1.5-fold dilutions of the filtrate of the loading of 10 mg a.s./L, respectively. This approach resulted in geometric mean measured concentrations of 0.156, 0.254, 0.373, 0.591, 0.838, 1.225 and 1.854 mg BAS 750 F/L. Additionally, a dilution water control was set up. Daphnids were exposed in 4 replicates per concentration, containing 5 daphnids each. The daphnids were observed for immobility 24 and 48 hours after start of exposure.

The following biological results are based on geometric mean measured concentrations. After 48 h of exposure, no immobility of daphnids was observed in the control and at test item concentrations of up to and including 0.254 mg a.s./L, whereas 20%, 30%, 45% and 65% immobility were observed at the test item concentrations of 0.373, 0.591, 0.838 and 1.225 mg a.s./L, respectively. At the highest test item concentration, 75% of the daphnids were immobile after 48 hours of exposure. Statistically significant effects on mobility of daphnids were detected at the five highest test item concentrations.

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of BAS 750 F was determined to be 0.944 mg a.s./L based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no. 5834378), batch no. COD - 001740; purity: 98.8%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; < 24 h old at test initiation.

Test design: Static system (48 hours), 7 test item concentrations plus control, 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control, 11.42-fold diluted filtrate, 7.61-fold diluted filtrate, 5.07-fold diluted filtrate, 3.38-fold diluted filtrate, 2.25-fold diluted filtrate, 1.5-fold diluted filtrate and filtrate of the loading of 10 mg BAS 750 F/L, corresponding to geometric mean measured concentrations of < LoD (limit of detection), 0.156, 0.254, 0.373, 0.591, 0.838, 1.225 and 1.854 mg a.s./L.

Test conditions: 150 ml glass beakers, test volume 100 mL, dilution water "M7" (Elendt medium); pH 7.37 - 7.58; oxygen concentration: 88% - 98%; temperature: 20.3 - 21.7 °C; photoperiod: 16 h light : 8 h dark; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with DAD detection.

Statistics: Descriptive statistics, Probit analysis for determination of the EC_{50} values; Williams Multiple Sequential t-test Procedure ($\alpha = 0.05$) for determination of the NOEC value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item concentrations was conducted at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were: Control (< LoD), 0.155, 0.261, 0.368, 0.585, 0.908, 1.322 and 1.776 mg a.s./L. The test item concentrations determined in samples collected at exposure termination were in the range of 85.1% to 109.0% of initial concentrations. This confirms that the test item concentrations were stable under test conditions. The following biological results are based on geometric mean measured concentrations.

Biological results: After 48 h of exposure, no immobility of daphnids was observed in the control and at test item concentrations of up to and including 0.254 mg a.s./L, whereas 20%, 30%, 45% and 65% immobility were observed at the test item concentrations of 0.373, 0.591, 0.838 and 1.225 mg a.s./L, respectively. At the highest test item concentration, 75% of the daphnids were immobile after 48 hours of exposure. Statistically significant effects on mobility of daphnids were detected at the five highest test item concentrations (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$). For results see Table 8.2.4.1-1.

Table 8.2.4.1-1: Effects of BAS 750 F on *Daphnia magna* mobility

Concentration [mg a.s./L] (geometric mean measured)	**Control**	**0.156**	**0.254**	**0.373**	**0.591**	**0.838**	**1.225**	**1.854**
Immobility (24 h) [%]	0	0	0	5	5	25	20	30
Immobility (48 h) [%]	0	0	0	20 *	30 *	45 *	65 *	75 *
	Endpoints [mg BAS 750 F/L] (geometric mean measured)							
EC_{50} (48 h)	0.944 (95% confidence limits: 0.770 - 1.213)							
NOEC (48 h)	0.254							

* Statistically significant differences compared to control (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$); statistically significant differences compared to control were only determined after 48 h of exposure.

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of BAS 750 F was determined to be 0.944 mg a.s./L based on geometric mean measured concentrations.

Report:	CA 8.2.4.1/2 Backfisch K.,Haerthe N., 2015 a Acute toxicity of Reg.No. 6003432 (M750F007; metabolite of BAS 750 F) to Daphnia magna STRAUS in a 48 hour static test 2015/1003915
Guidelines:	OECD 202, EPA 850.1010 draft April 1996
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 48-hour static acute toxicity laboratory study, the effect of the metabolite M750F007 on water flea neonates was investigated. The following nominal test concentrations were tested: 0 (control), 0.625, 1.25, 2.5, 5.0 and 10.0 mg/L. Daphnids were exposed in 4 replicates per concentration, containing 5 daphnids each. The daphnids were observed for immobility 24 and 48 hours after start of exposure.

The following biological results are based on nominal concentrations. After 48 h of exposure, no immobility of daphnids was observed in the control and at all test item concentrations.

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} and the NOEC of the metabolite M750F007 were determined to be > 10.0 mg/L based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F007 (metabolite of BAS 750 F, Reg. no. 6003432), batch no. L87-32-1; purity: 97%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; > 2 < 24 h old at test initiation.

Test design: Static system (48 hours), 5 test item concentrations plus control, 4 replicates per treatment and the control with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control, 0.625, 1.25, 2.5, 5.0 and 10.0 mg/L (nominal).

Test conditions: Glass beakers, test volume 50 mL, dilution water "M4" (Elendt medium); pH 7.92 - 8.08; oxygen content: 8.23 - 8.53 mg/L; temperature: 21.4 - 22.2 °C; total hardness: 2.47 mmol/L, conductivity: 689 µS/cm (at test initiation) photoperiod: 16 h light : 8 h dark; light intensity: 490 - 550 lux; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item concentrations was determined at the beginning and at the end of the test. The mean measured values were in range of 78.9 to 100% at test initiation and 76.7 to 98.2% at test termination. The following biological results are based on nominal concentrations.

Biological results: No significant effects on the mobility of *Daphnia magna* could be observed at all concentrations after 24 and 48 h. For results see Table 8.2.4.1-2

Table 8.2.4.1-2: Effects of BAS 750 F on *Daphnia magna* mobility

Concentration (nominal)	**Control**	**0.625**	**1.25**	**2.5**	**5.0**	**10.0**
Immobility (24 h) [%]	0	0	0	0	0	0
Immobility (48 h) [%]	0	0	0	0	0	0
	Endpoints [mg M750F007/L] (nominal)					
EC_{50} (48 h)	> 10					
NOEC (48 h)	> 10					

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} and the NOEC of the metabolite M750F007 were determined to be > 10.0 mg/L based on nominal concentrations.

Report:	CA 8.2.4.1/3 Rzodeczko H., 2015 c Reg.No. 5863469 (metabolite of BAS 750 F, M750F006) - Daphnia magna, acute immobilization test 2015/1001492
Guidelines:	OECD 202 (2004), EPA 850.1010
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 48-hour static acute toxicity laboratory study, the effect of the metabolite M750F006 on water flea neonates was investigated. Water flea neonates were exposed to a filtrate of a loading of 10 mg M750F006/L and its 16-fold, 8-fold, 4-fold and 2-fold dilution (corresponding to geometric mean measured concentrations of 0.47, 0.84, 1.83, 3.68 and 7.67 mg/L). Additionally, a dilution water control and a solvent control were set up. Daphnids were exposed in 4 replicates per concentration, containing 5 daphnids each. The daphnids were observed for immobility 24 and 48 hours after start of exposure.

The following biological results are based on geometric mean measured concentrations. After 48 hours of exposure, no immobility of daphnids was observed in the control and at the lowest test item concentration of 0.47 mg M750F006/L, whereas 15%, 20%, 55% and 60% immobility occurred at the test item concentrations of 0.84, 1.83, 3.68 and 7.67 mg/L, respectively. Statistically significant effects on mobility of daphnids after 48 hours of exposure were observed at the two highest tested concentrations of 3.68 and 7.67 mg/L. No other sub-lethal effects were observed during the test.

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F006 was determined to be 4.42 mg/L based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F006 (metabolite of BAS 750 F, Reg. no. 5863469), batch no. L87-30; purity: 98.9%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; < 24 h old at test initiation.

Test design: Static system (48 hours), 5 test item concentrations plus control and solvent control (N,N-dimethylformamide (DMF)); 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control, solvent control (DMF), 16-fold dilution, 8-fold dilution, 4-fold dilution and 2-fold dilution of the filtrate of a loading of 10 mg M750F006/L and filtrate of a loading of 10 mg M750F006/L, corresponding to geometric mean measured concentrations of 0.47, 0.84, 1.83, 3.68 and 7.67 mg/L.

Test conditions: 150 mL glass beakers, test volume 100 mL, dilution water "M7" (Elendt medium); pH 7.41 - 7.53; oxygen concentration: 91% - 94%; temperature: 20.7 - 21.6 °C; total hardness: 188.0 - 196.4 mg $CaCO_3$/L; conductivity: 548 - 555 µS/cm; photoperiod: 16 h light : 8 h dark; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics; probit analysis for calculation of EC_{50}; Fisher's Exact Binominal Test with Bonferroni Correction for determination of the NOEC ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each test item concentration at the beginning of the test and at the end of the test. The concentrations of M750F006 determined in samples collected at exposure initiation were 7.74 mg/L in the filtrate of a loading of 10 mg/L, 3.67 mg/L in its 2-fold dilution, 1.81 mg/L in its 4-fold dilution, 0.91 mg/L in its 8-fold dilution and 0.44 mg/L in its 16-fold dilution. The geometric mean concentrations of the test item were 7.67, 3.68, 1.83, 0.84, 0.47 mg/L, respectively. The concentrations of test item determined in samples collected at exposure termination were in the range of 85.68% to 111.71% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: After 48 hours of exposure, no immobility of daphnids was observed in the control and at the lowest test item concentration of 0.47 mg M750F006/L, whereas 15%, 20%, 55% and 60% immobility occurred at the test item concentrations of 0.84, 1.83, 3.68 and 7.67 mg/L, respectively. Statistically significant effects on mobility of daphnids after 48 hours of exposure were observed at the two highest tested concentrations of 3.68 and 7.67 mg/L (Fisher's Exact Binominal Test with Bonferroni Correction, $\alpha = 0.05$). No other sub-lethal effects were observed during the test. For results see Table 8.2.4.1-3.

Table 8.2.4.1-3: Effects of M750F006 (metabolite of BAS 750 F) on *Daphnia magna* mobility

Concentration [mg/L] (geometric mean measured)	**Control**	**Solvent Control**	**0.47**	**0.84**	**1.83**	**3.68**	**7.67**
Immobility (24 h) [%]	0	0	0	0	0	35	55
Immobility (48 h) [%]	0	0	0	15	20	55 *	60 *
	Endpoints [mg M750F006/L] (geometric mean measured)						
EC_{50} (48 h)	4.42 (95% confidence limits: 3.03 – 8.05)						
NOEC (48 h)	1.83						

* Statistically significantly different compared to the control after 48 h of exposure (Fisher's Exact Binominal Test with Bonferroni Correction, $\alpha = 0.05$).

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F006 was determined to be 4.42 mg/L based on geometric mean measured concentrations.

Report:	CA 8.2.4.1/4 Rzodeczko H., 2015 d Reg.No. 6003433 (metabolite of BAS 750 F, M750F005) - Daphnia magna, acute immobilization test 2015/1001490
Guidelines:	OECD 202 (2004), EPA 850.1010
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 48-hour static acute toxicity laboratory study, the effect of the metabolite M750F005 on water flea neonates was investigated. Water flea neonates were exposed to a filtrate of a loading of 10 mg M750F005/L and its 16-fold, 8-fold, 4-fold and 2-fold dilution (corresponding to geometric mean measured concentrations of 0.53, 1.07, 2.13, 4.23 and 8.58 mg/L). Additionally, a dilution water control and a solvent control were set up. Daphnids were exposed in 4 replicates per concentration, containing 5 daphnids each. The daphnids were observed for immobility 24 and 48 hours after start of exposure.

The following biological results are based on geometric mean measured concentrations. After 48 hours of exposure, no immobility of daphnids was observed in the control and all test item concentrations.

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F005 was determined to be > 8.58 mg/L based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F005 (metabolite of BAS 750 F, Reg. No. 6003433), batch no. L87-34; purity: 99.4%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; < 24 h old at test initiation.

Test design: Static system (48 hours), 5 test item concentrations plus control and solvent control (N,N-dimethylformamide (DMF)); 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control, solvent control (DMF), 16-fold dilution, 8-fold dilution, 4-fold dilution and 2-fold dilution of the filtrate of a loading of 10 mg M750F005/L and filtrate of a loading of 10 mg M750F005/L, corresponding to geometric mean measured concentrations of 0.53, 1.07, 2.13, 4.23 and 8.58 mg/L.

Test conditions: 150 mL glass beakers, test volume 100 mL, dilution water "M7" (Elendt medium); pH 7.39 - 7.50; oxygen concentration: 88% - 94%; temperature: 20.7 - 21.6 °C; total hardness: 198.4 - 202.8 mg $CaCO_3$/L; conductivity: 543 - 551 µS/cm; photoperiod: 16 h light : 8 h dark; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each test item concentration at the beginning of the test and at the end of the test. The concentrations of M750F005 determined in samples collected at exposure initiation were 8.56 mg/L in the filtrate of a loading of 10 mg/L, 4.28 mg/L in its 2-fold dilution, 2.16 mg/L in its 4-fold dilution, 1.07 mg/L in its 8-fold dilution and 0.54 mg/L in its 16-fold dilution. The geometric mean concentrations of the test item were 8.58, 4.23, 2.13, 1.07 and 0.53 mg/L, respectively. The concentrations of test item determined in samples collected at exposure termination were in the range of 97.59% to 100.55% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: After 48 hours of exposure, no immobility of daphnids was observed in the control and all test item concentrations. For results see Table 8.2.4.1-4.

Table 8.2.4.1-4: Effects of M750F005 (metabolite of BAS 750 F) on *Daphnia magna* mobility

Concentration [mg/L] (geometric mean measured)	**Control**	**Solvent Control**	**0.53**	**1.07**	**2.13**	**4.23**	**8.58**
Immobility (24 h) [%]	0	0	0	0	0	0	0
Immobility (48 h) [%]	0	0	0	0	0	0	0
	Endpoints [mg M750F005/L] (geometric mean measured)						
EC_{50} (48 h)	> 8.58						
NOEC (48 h)	≥ 8.58						

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F005 was determined to be > 8.58 mg/L based on geometric mean measured concentrations.

Report:	CA 8.2.4.1/5 Rzodeczko H., 2015 e Reg.No. 6010286 (metabolite of BAS 750 F, M750F008) - Daphnia magna, acute immobilization test 2015/1001493
Guidelines:	OECD 202 (2004), EPA 850.1010
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 48-hour static acute toxicity laboratory study, the effect of the metabolite M750F008 on water flea neonates was investigated. Water flea neonates were exposed to a filtrate of a loading of 10 mg M750F008/L and its 16-fold, 8-fold, 4-fold and 2-fold dilution (corresponding to geometric mean measured concentrations of 0.47, 0.97, 1.90, 3.90 and 8.07 mg/L). Additionally, a dilution water control and a solvent control were set up. Daphnids were exposed in 4 replicates per concentration, containing 5 daphnids each. The daphnids were observed for immobility 24 and 48 hours after start of exposure.

The following biological results are based on geometric mean measured concentrations. After 48 hours of exposure, no immobility of daphnids was observed in the control and all test item concentrations.

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F008 was determined to be > 8.07 mg/L based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F008 (metabolite of BAS 750 F, Reg. No. 6010286), batch no. L85-94; purity: 96.5%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; < 24 h old at test initiation.

Test design: Static system (48 hours), 5 test item concentrations plus control and solvent control (N,N-dimethylformamide (DMF)); 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control, solvent control (DMF), 16-fold dilution, 8-fold dilution, 4-fold dilution and 2-fold dilution of the filtrate of a loading of 10 mg M750F008/L and filtrate of a loading of 10 mg M750F008/L, corresponding to geometric mean measured concentrations of 0.47, 0.97, 1.90, 3.90 and 8.07 mg/L.

Test conditions: 150 mL glass beakers, test volume 100 mL, dilution water "M7" (Elendt medium); pH 7.37 - 7.53; oxygen concentration: 89% - 96%; temperature: 19.3 - 20.2 °C; total hardness: 196.0 - 199.8 mg $CaCO_3$/L; conductivity: 539 - 549 µS/cm; photoperiod: 16 h light : 8 h dark; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each test item concentration at the beginning of the test and at the end of the test. The concentrations of M750F008 determined in samples collected at exposure initiation were 8.13 mg/L in the filtrate of a loading of 10 mg/L, 3.94 mg/L in its 2-fold dilution, 1.90 mg/L in its 4-fold dilution, 0.97 mg/L in its 8-fold dilution and 0.45 mg/L in its 16-fold dilution. The geometric mean concentrations of the test item were 8.07, 3.90, 1.90, 0.97, 0.47 mg/L, respectively. The concentrations of test item determined in samples collected at exposure termination were in the range of 97.79% to 108.89% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: After 48 hours of exposure, no immobility of daphnids was observed in the control and all test item concentrations. For results see Table 8.2.4.1-5.

Table 8.2.4.1-5: Effects of M750F008 (metabolite of BAS 750 F) on *Daphnia magna* mobility

Concentration [mg/L] (geometric mean measured)	**Control**	**Solvent Control**	**0.47**	**0.97**	**1.90**	**3.90**	**8.07**
Immobility (24 h) [%]	0	0	0	0	0	0	0
Immobility (48 h) [%]	0	0	0	0	0	0	0
	Endpoints [mg M750F008/L] (geometric mean measured)						
EC_{50} (48 h)	> 8.07						
NOEC (48 h)	≥ 8.07						

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} of the metabolite M750F008 was determined to be > 8.07 mg/L based on geometric mean measured concentrations.

CA 8.2.4.2 Acute toxicity to an additional aquatic invertebrate species

Report:	CA 8.2.4.2/1 VanHooser A., 2014 a BAS 750 F: Acute toxicity test with the saltwater mysid, Americamysis bahia, determined under flow-through test conditions 2014/7002845
Guidelines:	EPA 850.1035
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a flow-through acute toxicity laboratory study, saltwater mysids (*Americamysis bahia*) were exposed to a dilution water control, a solvent control and to nominal concentrations of 0.32, 0.63, 1.3, 2.5 and 5.0 mg BAS 750 F/L (corresponding to mean measured concentrations of 0.227, 0.415, 0.896, 1.76 and 3.29 mg a.s./L) in two replicates per treatment containing 10 mysids each. Saltwater mysids were observed for survival and symptoms of toxicity 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations of the test item. After 48 hours of exposure no mortality and no other toxic effects were observed in the control, the solvent control and at test item concentrations of up to and including 0.896 mg a.s./L, whereas 70% mortality and sub-lethal effects (*i.e.* lethargy) were observed at the second highest test item concentration of 1.76 mg a.s./L. At the highest test item concentration of 3.29 mg a.s./L, 100% mortality occurred.

In a flow-through acute toxicity study with saltwater mysids (*Americamysis bahia*) the LC_{50} (48 h) for BAS 750 F was determined to be 1.53 mg a.s./L.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Saltwater mysid (*Americamysis bahia*), juveniles, age: less than 24 hours old; source: in-house cultures.

Test design: Flow-through system (96 hours); 5 test item concentrations plus a control and a solvent control, 2 replicates per treatment; 10 mysids per replicate; 2 retention baskets per aquarium; daily assessment of mortality and symptoms of toxicity.

Endpoints: LC_{50} (48 h), mortality and sub-lethal effects.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L) and 0.32, 0.63, 1.3, 2.5 and 5.0 mg BAS 750 F/L (nominal), corresponding to mean measured concentrations of 0.227, 0.415, 0.896, 1.76 and 3.29 mg a.s./L.

Test conditions: Glass aquaria (14 cm x 23 cm x 16.5 cm), test volume approx. 4 L; retention baskets: glass petri dish base (approx. 1.5 cm x 10 cm) with a stainless steel screen collar (stainless steel mesh, approximate mesh opening 381 µm); dilution water: artificial saltwater, aerated, filtered and sterilized; flow rate: approx. 12 volume additions per 24 hours; salinity: 19.9 - 20.1‰; temperature: 24.0 °C - 24.6 °C; pH 7.9 - 8.1; oxygen content: 5.2 - 7.4 mg/L; photoperiod 14 h light : 10 h dark; light intensity: 793 lux; feeding: live brine shrimp nauplii (*Artemia* sp.) at least once daily.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with MS/MS-detection.

Statistics: Descriptive statistics; probit, Trimmed Spearman Karber or Spearman-Karber method for calculation of the LC_{50}.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The analytically determined concentrations of BAS 750 F ranged from 67% to 77% of nominal concentrations at test initiation and from 62% to 68% of nominal at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 48 hours of exposure no mortality and no other toxic effects were observed in the control, the solvent control and at test item concentrations of up to and including 0.896 mg a.s./L, whereas 70% mortality and sub-lethal effects (*i.e.* lethargy) were observed at the second highest test item concentration of 1.76 mg a.s./L. At the highest test item concentration of 3.29 mg a.s./L, 100% mortality occurred. The results are summarized in Table 8.2.4.2-1.

Table 8.2.4.2-1: Acute toxicity (48h) of BAS 750 F to saltwater mysids (*Americamysis bahia*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.32**	**0.63**	**1.3**	**2.5**	**5.0**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.227**	**0.415**	**0.896**	**1.76**	**3.29**
Mortality [%] (48 h)	0	0	0	0	0	70	100
Symptoms after 48 h [#]	none	none	none	none	none	L	n.d.
	Endpoints [mg BAS 750 F/L] (mean measured)						
LC_{50} (48 h)	1.53 (95% confidence limits: 1.34 - 1.74)						

[#] Symptoms after 48 h: L = lethargy
n.d. = not determined; all animals dead

III. CONCLUSION

In a flow-through acute toxicity study with saltwater mysids (*Americamysis bahia*) the LC_{50} (48 h) for BAS 750 F was determined to be 1.53 mg a.s./L.

Report:	CA 8.2.4.2/2 VanHooser A., 2015 a BAS 750 F: Effect on new shell growth of the eastern oyster (Crassostrea virginica) 2015/7000021
Guidelines:	EPA 850.1025, EPA 72-3(e)
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 96-hour acute toxicity laboratory study the effect of BAS 750 F on shell deposition of eastern oysters (*Crassostrea virginica*) was investigated under flow-through conditions. The eastern oysters were exposed to a dilution water control, a solvent control and to nominal concentrations of 0.14, 0.25, 0.45, 0.80, 1.4 and 2.6 mg BAS 750 F/L (corresponding to mean measured concentrations of 0.111, 0.174, 0.335, 0.623, 1.12 and 1.80 mg a.s./L) in groups of 10 oysters per replicate with two replicates per treatment. Eastern oysters were observed for survival and symptoms of toxicity daily during the exposure period. Measurements of shell deposition for each oyster were made after 96 hours.

The biological results are based on mean measured concentrations of the test item. There was a noticeable reduction in fecal material observed at the test item concentrations of 1.12 mg a.s./L and 1.80 mg a.s./L as compared to the control throughout the test. After 96 hours of exposure, no mortality of oysters occurred in the control and the solvent control and at test item concentrations of up to and including 1.12 mg BAS 750 F/L, whereas 20% mortality was observed at the highest test item concentration of 1.80 mg a.s./L. Shell growth was statistically significantly inhibited at the three highest test item concentrations compared to the control.

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*), the LC_{50} (96 h) for BAS 750 F was > 1.80 mg a.s./L and the EC_{50} (96 h) was 0.947 mg a.s./L based on mean measured concentrations. The NOEC (96 h) was determined to be 0.335 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), lot no. COD-001740, purity: 98.8% (tolerance ± 1%).

B. STUDY DESIGN

Test species: Eastern oyster (*Crassostrea virginica*), mean valve height: 35.4 mm ± 1.9 mm; source: “Circle C Oyster Ranch”, ridge, Maryland, USA.

Test design: Flow-through system (96 hours); 6 test item concentrations plus a control and a solvent control, 2 replicates for each test item concentration and the controls with 10 oysters per replicate (20 animals per treatment); daily assessment of mortality and symptoms of toxicity; measurements of shell deposition 96 hours after start of exposure.

Endpoints: LC_{50}, EC_{50} and NOEC for mortality, shell growth inhibition and symptoms of toxicity.

Test concentrations: Control (dilution water), solvent control (0.050 mL dimethylformamide/L), 0.14, 0.25, 0.45, 0.80, 1.4 and 2.6 mg BAS 750 F/L (nominal), corresponding to mean measured concentrations of 0.111, 0.174, 0.335, 0.623, 1.12 and 1.80 mg a.s./L.

Test conditions: Glass aquaria (21.5 cm x 37.0 cm x 18.0 cm), test volume approx. 8.4 L; artificial seawater, filtered, -aerated and sterilized; flow rate: approx. 12.9 volume additions per 24 hours in each test vessel; salinity: 19.4 - 19.5‰; temperature: 19.1 °C - 20.6 °C; pH 7.9 - 8.5; oxygen content: 5.0 mg/L - 7.6 mg/L; photoperiod 16 h light : 8 h dark; light intensity: 420 - 541 lux; feeding: marine microalgal concentrate.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with MS/MS-detection.

Statistics: Descriptive statistics; four-parameter logistic (sigmoid-shaped) model for calculation of EC_{50}, Dunnett's Test for determination of the NOEC value for shell deposition data ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at test initiation and at test termination. Mean measured concentrations for BAS 750 F ranged from 65% to 78% of nominal concentrations at test initiation and from 71% to 87% of nominal at test termination. The following biological results are based on mean measured concentrations.

Biological results: There was a noticeable reduction in fecal material observed at the test item concentrations of 1.12 mg a.s./L and 1.80 mg a.s./L as compared to the control throughout the test. After 96 hours of exposure, no mortality of oysters occurred in the control and the solvent control and at test item concentrations of up to and including 1.12 mg BAS 750 F/L, whereas 20% mortality was observed at the highest test item concentration of 1.80 mg a.s./L. Shell growth was statistically significantly inhibited at the three highest test item concentrations compared to the control (Dunnett's Test, $p < 0.05$). The results are summarized in Table 8.2.4.2-2.

Table 8.2.4.2-2: Acute toxicity (96 h) of BAS 750 F to eastern oysters (*Crassostrea virginica*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.14**	**0.25**	**0.45**	**0.80**	**1.4**	**2.6**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.111**	**0.174**	**0.335**	**0.623**	**1.12**	**1.80**
Mortality after 96 h [%]	0	0	0	0	0	0	0	20
Inhibition of shell growth after 96 h [% of control]	--	-7	1	-6	8	27 *	60 *	92 *
	Endpoints [mg BAS 750 F/L] (mean measured)							
LC_{50} (96 h)	> 1.80							
EC_{50} (96 h)	0.947 (95% confidence limits: 0.856 - 1.04)							
NOEC (96 h)	0.335							

* Statistically significant difference compared to the control (Dunnett's Test, $p < 0.05$).

III. CONCLUSION

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*), the LC_{50} (96 h) for BAS 750 F was > 1.80 mg a.s./L and the EC_{50} (96 h) was 0.947 mg a.s./L based on mean measured concentrations. The NOEC (96 h) was determined to be 0.335 mg a.s./L (mean measured).

CA 8.2.5 Long-term and chronic toxicity to aquatic invertebrates

CA 8.2.5.1 Reproductive and development toxicity to Daphnia magna

Report:	CA 8.2.5.1/1 Janson G., 2014a Chronic toxicity of the BAS 750 F (Reg.No.5834387) to Daphnia magna Straus in a 21 day semi-static test 2014/1098028
Guidelines:	OECD 211, EPA 850.1300
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 21-day semi-static toxicity test, effects of BAS 750 F to water fleas (*Daphnia magna*) were examined. Neonates less than 24 hours old were exposed to nominal concentrations of 0.005, 0.010, 0.020, 0.040 and 0.080 mg BAS 750 F/L (corresponding to time-weighted mean measured concentrations of 0.0045, 0.0091, 0.0184, 0.0378 and 0.0773 mg a.s./L, respectively.). Additionally, a dilution water control and a solvent control were set up. All treatment groups and the controls consisted of 10 replicates containing one daphnid. Assessment of parent mortality and reproduction was conducted daily except for days 3 and 4. Measurements of growth (body weight and length) were made at test termination.

The biological results are based on nominal concentrations and additionally on time-weighted mean measured concentrations. Since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.
After 21 days of exposure, no parent mortality occurred in the control groups and at the test item concentrations of up to and including the highest concentration tested. Statistically significant differences in the number of offspring per parent were observed at the three highest test item concentrations. The intrinsic rate of increase and day of first brood were significantly affected at the two highest test item concentrations. Body length of the parent animals was only significantly affected at the highest test item concentration. Due to the low variation in reproductive parameters, it is feasible to use an EC_x approach in this study and therefore the EC_{10} is the more relevant endpoint of this study.

In a 21-day semi-static toxicity study with *Daphnia magna* the NOEC of BAS 750 F was determined to be 0.010 mg a.s./L. The EC_{10} value was 0.0175 mg a.s./L, based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% (tolerance ± 1%).

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates from in-house culture; originally obtained from Institute National de Recherche Chimique Appliquee, France, > 2 h and < 24 hours old at test initiation.

Test design: Semi-static system (21 days), 5 test concentrations plus control and solvent control, 10 replicates per treatment; one animal per test vessel; daily assessment (except day 3 and 4) of parent mortality and reproduction; determination of body length and dry weight at test termination.

Endpoints: EC_{10}; NOEC, parent mortality, reproduction, growth (parent length and dry weight) and population growth rate.

Test concentrations: Control (dilution water), solvent control (dimethylformamide), 0.005, 0.010, 0.020, 0.040 and 0.080 mg BAS 750 F/L (nominal); corresponding to time-weighted mean measured concentrations of 0.0045, 0.0091, 0.0184, 0.0378 and 0.0773 mg a.s./L, respectively.

Test conditions: Glass vessels; test volume: 50 mL; dilution water "M4" (Elendt medium); temperature: 20.7 °C - 22.2 °C; pH 7.69 - 8.26; oxygen content: 8.08 mg/L - 9.03 mg/L; total hardness of old test solutions: 2.4 - 2.5 mmol/L; alkalinity of old test solutions: 0.8 - 0.9 mmol/L; conductivity of dilution water: 621 - 642 µS/cm; photoperiod 16 hours light : 8 hours dark; light intensity: 467 - 970 lux; feeding: daily (except day 3 and 4) with algae (*Desmodesmus subspicatus*); no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS-detection.

Statistics: Descriptive statistics; calculation of EC_{10} using probit-analysis; ANOVA followed by Dunnett's test for determination of the NOEC values ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the active substance concentrations was conducted in all treatments at day 0, 2, 9, 12, 14, 16, 19 and 21. Recoveries of BAS 750 F in fresh solutions (measured on days 0, 9, 14 and 19) were in the range of 91% to 101% of nominal concentrations. Measured concentrations of the test item in old solutions (measured on days 2, 12, 16 and 21) ranged from 81% to 97%. The following biological results are based on nominal concentrations and additionally on time-weighted mean measured concentrations. However, since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.

Biological results: Statistical analyses in this study were performed by comparing the pooled control data to the treatment data for the respective parameters. After 21 days of exposure, no parent mortality occurred in the control groups and at the test item concentrations of up to and including the highest concentration tested. Statistically significant differences in the number of offspring per parent were observed at the three highest test item concentrations (ANOVA followed by Dunnett's test; $p < 0.05$). The intrinsic rate of increase and day of first brood were significantly affected at the two highest test item concentrations (ANOVA followed by Dunnett's test; $p < 0.05$). Body length of the parent animals was only significantly affected at the highest test item concentration (ANOVA followed by Dunnett's test; $p < 0.05$). Looking on the percentage of reduction of the cumulative offspring, it is seen that at the NOEC level of 0.010 mg a.s./L (nominal) a reduction of only 0.7% occurs and at the LOEC level of 0.020 mg a.s./L (nominal) only a reduction of 11%. Due to the low variation in reproductive parameters, it is feasible to use an EC_x approach in this study. The EC_{10} is the more relevant endpoint of this study. The results are summarized in Table 8.2.5.1-1.

Table 8.2.5.1-1: Effects of BAS 750 F on *Daphnia magna* parent mortality, reproduction and growth after 21 days of exposure

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**Pooled control**	**0.005**	**0.010**	**0.020**	**0.040**	**0.080**
Concentration [mg a.s./L] (time-weighted mean measured)	--	--	--	**0.0045**	**0.0091**	**0.0184**	**0.0378**	**0.0773**
Parent mortality [%]	0	0	--	0	0	0	0	0
Offspring / parent	182	186	184	193	183	163 *	138 *	121 *
CV of offspring / parent [%]	9.5	8.2	--	5.9	8.0	8.4	14.6	16.2
Inhibition in mean cumulative offspring [% of pooled control]	--	--	--	-5.0	0.7	11.3	25.0	34.6
Day of first brood	7.5	7.7	--	7.7	7.7	7.7	8.3 *	9.4 *
Body weight [mg]	0.881	0.891	--	0.874	0.886	0.874	0.861	0.841
Body length [mm]	4.9	4.9	--	5.0	4.9	4.9	4.8	4.7 *
Intrinsic rate of increase (population growth rate)	0.419	0.427	--	0.427	0.416	0.408	0.371 *	0.350 *
	Endpoints [mg BAS 750 F/L]							
EC_{10} (21 d) [a)]	**Nominal: 0.0175 (95% confidence limits: 0.0130 - 0.0237)** Mean measured: 0.0161 (95% confidence limits: 0.0118 - 0.0220)							
$NOEC_{overall}$ (21 d)	**Nominal: 0.010** Mean measured: 0.0091							

CV = coefficient of variation
* Statistically significant effects compared to the pooled control (ANOVA followed by Dunnett's test; $p < 0.05$).
[a)] EC_{10} based on cumulative offspring per female data.

III. CONCLUSION

In a 21-day semi-static toxicity study with *Daphnia magna* the NOEC of BAS 750 F was determined to be 0.010 mg a.s./L. The EC_{10} value was 0.0175 mg a.s./L, based on nominal concentrations.

CA 8.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species

Report:	CA 8.2.5.2/1 Janson G.-M., 2015 b Chronic toxicity of BAS 750 F (Reg.No. 5834378) to Daphnia longispina in a 21 day semi-static test 2015/1003912
Guidelines:	OECD 211, EPA 850.1300
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)
Report:	CA 8.2.5.2/2 Janson G.-M., 2015 c Report Amendment No.1 - Chronic toxicity of BAS 750 F (Reg.No. 5834378) to Daphnia longispina in a 21 day semi-static test 2015/1251197
Guidelines:	OECD 211, EPA 850.1300
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 21-day semi-static toxicity test, effects of BAS 750 F to water fleas (*Daphnia longispina*) were examined. Neonates less than 24 hours old were exposed to nominal concentrations of 0.015, 0.0225, 0.0338, 0.0507 and 0.0761 mg BAS 750 F/L (corresponding to time-weighted mean concentrations of 0.0148, 0.0223, 0.0342, 0.0511 and 0.0775 mg a.s./L, respectively). Additionally, a dilution water control and a solvent control were set up. All treatment groups and the controls consisted of 10 replicates containing one daphnid. Assessment of parent mortality and reproduction was conducted daily except for days 3 and 4. Measurements of growth (body weight and length) were made at test termination.

The biological results are based on nominal concentrations and additionally on time-weighted mean concentrations. Since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.
After 21 days of exposure, no parent mortality occurred in the control groups and at all tested test item concentrations. Statistically significant differences in the number of offspring per parent were observed at the two highest test item concentrations and in the intrinsic rate of increase at the highest test item concentration (ANOVA followed by Dunnett's test; $p < 0.05$). Day of first brood, body length, body weight, mobility and age at first reproduction were not affected up to and including the highest test concentration tested. Due to the low variation in reproductive parameters, it is feasible to use an EC_x approach in this study and therefore the EC_{10} is the more relevant endpoint of this study.

In a 21-day semi-static toxicity study with *Daphnia longispina* the NOEC of BAS 750 F was determined to be 0.0338 mg a.s./L. The EC_{10} value was 0.0558 mg a.s./L, based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% (± 1.0% tolerance).

B. STUDY DESIGN

Test species: Water flea (*Daphnia longispina*), neonates from in-house culture (non-GLP); originally obtained from Molecular Ecology University in Landau, Germany; > 2 h and < 24 hours old at test initiation.

Test design: Semi-static system (21 days), 5 test concentrations plus control and solvent control (0.01 mL DMF/L), 10 replicates per treatment; one animal per test vessel; daily assessment (except day 3 and 4) of parent mortality and reproduction; determination of body length and dry weight at test termination.

Endpoints: EC_{10}; NOEC, parent mortality, reproduction, growth (parent length and dry weight) and population growth rate.

Test concentrations: Control (dilution water), solvent control (dimethylformamide), 0.015, 0.0225, 0.0338, 0.0507 and 0.0761 mg BAS 750 F/L (nominal); corresponding to time-weighted mean concentrations of 0.0148, 0.0223, 0.0342, 0.0511 and 0.0775 mg a.s./L, respectively.

Test conditions: Glass vessels; test volume: 50 mL; dilution water "M4" (Elendt medium); temperature: 20.9 °C - 21.9 °C; pH 7.90 - 8.15; oxygen content: 8.23 - 9.30 mg/L; total hardness of old test solutions: 2.39 - 2.53 mmol/L; alkalinity of old test solutions: 0.84 - 0.90 mmol/L; conductivity of dilution water: 653 - 664 µS/cm; photoperiod 16 hours light : 8 hours dark; light intensity: 320 - 570 lux; feeding: algae (*Desmodesmus subspicatus*); no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS-detection.

Statistics: Descriptive statistics; calculation of EC_{10} using probit-analysis; ANOVA followed by Dunnett's test for determination of the NOEC values ($p < 0.05$) based on reproduction, intrinsic rate of increase, body length and dry weight. Bonferroni U-Test for day of first brood. Fisher´s exact test for mortality.

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the active substance concentrations was conducted in all treatments at day 0, 2, 9, 12, 14, 16, 19 and 21. Recoveries of BAS 750 F in fresh solutions were in the range of 97% to 108% of nominal concentrations. Measured concentrations of the test item in old solutions ranged from 95% to 106%. The following biological results are based on nominal concentrations and additionally on time-weighted mean measured concentrations. Since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.

Biological results: After 21 days of exposure, no parent mortality occurred in the control groups and at all tested test item concentrations. Statistically significant differences in the number of offspring per parent were observed at the two highest test item concentrations and in the intrinsic rate of increase at the highest test item concentration (ANOVA followed by Dunnett's test; $p < 0.05$). Day of first brood, body length, body weight, mobility and age at first reproduction were not affected up to and including the highest test concentration tested. The results are summarized in Table 8.2.5.2-1.

Table 8.2.5.2-1: Effects of BAS 750 F on *Daphnia longispina* parent mortality, reproduction and growth after 21 days of exposure

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.0150**	**0.0225**	**0.0338**	**0.0507**	**0.0761**
Concentration [mg a.s./L] (time-weighted mean)	**--**	**--**	**0.0148**	**0.0223**	**0.0342**	**0.0511**	**0.0775**
Parent mortality [%]	0	0	0	0	0	0	0
Offspring / parent	205	206	201	211	207	192 *	141 *
CV of offspring / parent [%]	2.8	3.7	8.9	2.7	2.2	4.3	10.5
Inhibition in mean cumulative offspring [% of control]	--	--	2.1	-2.9	-1.1	6.4	31.1
Day of first brood	6.7	6.7	6.7	6.7	6.7	6.8	7.0
Body weight [mg]	0.322	0.335	0.321	0.343	0.326	0.324	0.309
Body length [mm]	2.6	2.6	2.6	2.6	2.6	2.5	2.5
Intrinsic rate of increase (population growth rate)	0.439	0.438	0.438	0.443	0.437	0.430	0.401 *
	Endpoints [mg BAS 750 F/L]						
EC_{10} (21 d) [a)]	**Nominal: 0.0558 (95% confidence limits: 0.0517 - 0.0603)** Mean measured: 0.0564 (95% confidence limits: 0.0521 - 0.0610)						
$NOEC_{overall}$ (21 d)	**Nominal: 0.0338** Mean measured: 0.0342						

CV = coefficient of variation
* Statistically significant effects compared to the control (ANOVA followed by Dunnett's test; $p < 0.05$).
[a)] EC_{10} based on cumulative offspring per female data.

III. CONCLUSION

In a 21-day semi-static toxicity study with *Daphnia longispina* the NOEC of BAS 750 F was determined to be 0.0338 mg a.s./L. The EC_{10} value was 0.0558 mg a.s./L, based on nominal concentrations.

Report:	CA 8.2.5.2/3 Janson G.-M., 2015a Chronic toxicity of BAS 750 F (Reg.No. 5834378) to Daphnia pulex in a 21 day semi-static test 2015/1003913
Guidelines:	EPA 850.1300, OECD 211 (2008), OECD 211 (2012)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 21-day semi-static toxicity test, effects of BAS 750 F to water fleas (*Daphnia pulex*) were examined. Neonates less than 24 hours old were exposed to nominal concentrations of 0.0125, 0.0188, 0.0282, 0.0423 and 0.0635 mg BAS 750 F/L (corresponding to time-weighted mean measured concentrations of 0.0121, 0.0184, 0.0276, 0.0407 and 0.0631 mg a.s./L, respectively.). Additionally, a dilution water control and a solvent control were set up. All treatment groups and the controls consisted of 10 replicates containing one daphnid. Assessment of parent mortality and reproduction was conducted daily except for days 3 and 4. Measurements of growth (body weight and length) were made at test termination.

The biological results are based on nominal concentrations and additionally on time-weighted mean measured concentrations. Since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.
After 21 days of exposure, no parent mortality occurred in the control groups and at the test item concentrations of up to and including the highest concentration tested. Statistically significant differences in the number of offspring per parent and in the intrinsic rate of increase were observed at the two highest test item concentrations. Day of first brood, body length, body weight, mobility and age at first reproduction were not affected up to and including the highest test concentration tested.

In a 21-day semi-static toxicity study with *Daphnia pulex* the NOEC of BAS 750 F was determined to be 0.0282 mg a.s./L. The EC_{10} value was 0.0573 mg a.s./L, based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% (± 1.0% tolerance).

B. STUDY DESIGN

Test species: Water flea (*Daphnia pulex*), neonates from in-house culture (non-GLP); originally obtained from an ornamental fish shop (Bachflohkrebse, Stuttgart, Germany), > 2 h and < 24 hours old at test initiation.

Test design: Semi-static system (21 days), 5 test concentrations plus control and solvent control (0.01 mL DMF/L), 10 replicates per treatment; one animal per test vessel; daily assessment (except day 3 and 4) of parent mortality and reproduction; determination of body length and dry weight at test termination.

Endpoints: EC_{10}; NOEC, parent mortality, reproduction, growth (parent length and dry weight) and population growth rate.

Test concentrations: Control (dilution water), solvent control (dimethylformamide), 0.0125, 0.0188, 0.0282, 0.0423 and 0.0635 mg BAS 750 F/L (nominal); corresponding to time-weighted mean measured concentrations of 0.0121, 0.0184, 0.0276, 0.0407 and 0.0631 mg a.s./L.

Test conditions: Glass vessels; test volume: 50 mL; dilution water "M4" (Elendt medium); temperature: 20.3 °C - 21.7 °C; pH 7.90 - 8.21; oxygen content: 8.50 mg/L - 9.42 mg/L; total hardness of old test solutions: 2.53 - 2.63 mmol/L; alkalinity of old test solutions: 0.84 - 0.86 mmol/L; conductivity of dilution water: 602 - 694 µS/cm; photoperiod 16 hours light : 8 hours dark; light intensity: 270 - 550 lux; feeding: algae (*Desmodesmus subspicatus*); no aeration.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS-detection.

Statistics: Descriptive statistics; calculation of EC_{10} using probit-analysis; ANOVA followed by Dunnett's test for determination of the NOEC values ($p < 0.05$) based on reproduction, intrinsic rate of increase, body length and dry weight. Bonferroni U-Test for day of first brood. Fisher´s exact test for mortality.

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the active substance concentrations was conducted in all treatments at day 0, 2, 9, 12, 14, 16, 19 and 21. Recoveries of BAS 750 F in fresh solutions were in the range of 92.2% to 104% of nominal concentrations. Measured concentrations of the test item in old solutions ranged from 85.2% to 106%. The following biological results are based on nominal concentrations and additionally on time-weighted mean measured concentrations. Since all analytical measurements are within 80 - 120% of nominal, the nominal endpoints shall be taken for a risk assessment.

Biological results: After 21 days of exposure, no parent mortality occurred in the control groups and at the test item concentrations of up to and including the highest concentration tested. Statistically significant differences in the number of offspring per parent and in the intrinsic rate of increase were observed at the two highest test item concentrations (ANOVA followed by Dunnett's test; $p < 0.05$). Day of first brood, body length, body weight, mobility and age at first reproduction were not affected up to and including the highest test concentration tested. The results are summarized in Table 8.2.5.2-2.

Table 8.2.5.2-2: Effects of BAS 750 F on *Daphnia pulex* parent mortality, reproduction and growth after 21 days of exposure

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.0125**	**0.0188**	**0.0282**	**0.0423**	**0.0635**
Concentration [mg a.s./L] (time-weighted mean measured)	--	--	**0.0121**	**0.0184**	**0.0276**	**0.0407**	**0.0631**
Parent mortality [%]	0	0	0	0	0	0	0
Offspring / parent	253	255	259	259	263	234 *	224 *
CV of offspring / parent [%]	3.5	2.7	3.1	3.8	1.9	4.3	11.5
Inhibition in mean cumulative offspring [% of control]	--	--	-2.2	-2.3	-3.8	7.7	11.4
Day of first brood	7.0	7.0	7.0	7.0	7.0	7.0	7.3
Body weight [mg]	0.217	0.212	0.200	0.199	0.191	0.200	0.184
Body length [mm]	2.6	2.6	2.6	2.6	2.5	2.5	2.5
Intrinsic rate of increase (population growth rate)	0.470	0.469	0.475	0.474	0.475	0.455 *	0.439 *
	Endpoints [mg BAS 750 F/L]						
EC_{10} (21 d) [a)]	**Nominal: 0.0573 (95% confidence limits: 0.050 - 0.0657)** Mean measured: 0.0567 (95% confidence limits: 0.0491 - 0.0654)						
$NOEC_{overall}$ (21 d)	**Nominal: 0.0282** Mean measured: 0.0276						

CV = coefficient of variation
* Statistically significant effects compared to the control (ANOVA followed by Dunnett's test; $p < 0.05$).
[a)] EC_{10} based on cumulative offspring per female data.

III. CONCLUSION

In a 21-day semi-static toxicity study with *Daphnia pulex* the NOEC of BAS 750 F was determined to be 0.0282 mg a.s./L. The EC_{10} value was 0.0573 mg a.s./L, based on nominal concentrations.

Report:	CA 8.2.5.2/4 Dinehart S., 2016 a BAS 750 F: Life-cycle toxicity test of the saltwater mysid, Americamysis bahia, conducted under flow-through conditions 2016/7001293
Guidelines:	EPA 850.1350, EPA 72-4
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The chronic toxicity of BAS 750 F to saltwater mysids (*Americamysis bahia*) was evaluated in a 28-day life cycle test under flow-through conditions. Mysids were exposed to a dilution water control, a solvent control (10 µL dimethylformamide/L) and to nominal test item concentrations of 1.0, 2.0, 4.0, 8.0 and 16 µg BAS 750 F/L (corresponding to mean measured concentrations of 0.931, 1.50, 3.42, 6.57 and 13.2 µg a.s./L). Survival, reproductive success and symptoms of toxicity were assessed throughout the study. Body length and dry weight of the males and females was determined at test termination.

The biological results are based on mean measured concentrations. No statistically significant and biologically relevant differences were determined between the control and the solvent control data. Thus, data of the test substance treatments were compared to the dilution water control. There was no statistically significant reduction in survival, reproduction, time of first brood release and growth for any test substance treatment.

In a flow-through chronic toxicity study with saltwater mysids (*Americamysis bahia*), the overall NOEC (28 d) for BAS 750 F was determined to be ≥ 13.2 µg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg.No.: 5834378), batch no.: COD-001740; purity: 98.8%.

B. STUDY DESIGN

Test species: Saltwater mysid (*Americamysis bahia*), juveniles, age: less than 24 hours old; source: in-house culture.

Test design: Flow-through system (28 d); 5 test item concentrations plus a control and a solvent control (10 µL dimethylformamide/L), 3 replicates for each test item concentration, the control and the solvent control; 30 mysids per glass aquaria (15 mysids per retention chamber); mysids were maintained in retention chambers until sexual maturity; at time of sexual maturity (day 13) male-female pairs were transferred into brood cups (7 brood cups per glass aquaria); remaining mysids (after isolation of male-female pairs) were pooled and placed in separate brood cups within glass aquaria; dead parental mysids and juveniles released during the test were removed daily; daily assessment of survival and symptoms of toxicity, assessment of reproduction (number of offspring produced by each female) from day 14 on; determination of length of F0 mysids on day 14 and 28 and F1 mysids after 12 d of exposure; determination of F0 dry weight at test termination.

Endpoints: NOEC based on survival, reproductive success (offspring per female and days to first brood release), length and dry weight.

Test concentrations: Control (dilution water), solvent control (10 µL dimethylformamide/L); 1.0, 2.0, 4.0, 8.0 and 16 µg BAS 750 F/L (nominal), corresponding to mean measured concentrations of <MQL (control), <MQL (solvent control), 0.931, 1.50, 3.42, 6.57 and 13.2 µg a.s./L.

Test conditions: Test chambers: glass aquaria (19.3 L, 19 cm x 39 - 78cm x 21 cm: 2 cylindrical retention baskets per test chamber, one for reproduction observation and the other for growth observation; retention basket consisted of a glass Petri dish base (approximately 1.5 × 15 cm) with a stainless steel screen collar (mesh with an approximate mesh opening of 381 µm); brood cups consisting of a glass Petri dish base (approximately 1.5 × 10 cm) with the same type of stainless steel screen collar; stainless steel screens were attached to the Petri dish with translucent silicone sealant; dilution water: commercial sea salt mix (Crystal Sea Marinemix; Marine Enterprises International, Inc. Baltimore, Maryland) added to laboratory freshwater, flow rate: approximately 8 volume exchanges/aquarium/24 h; salinity: 19.2 - 21.0‰; temperature: 24.6 - 26.2 °C; pH 7.8 - 8.2; oxygen content: 4.0 mg/L - 7.8 mg/L;

photoperiod 14 h light : 10 h dark with two 30-minute transition periods; light intensity: 479 - 652 lux; mysids were fed with live brine shrimps (*Artemia* sp.) three times daily, with the exception of the first and last days of the study when mysids were fed twice, in combination with a commercial enrichment mixture and rotifers once per day.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with MS/MS detection.

Statistics: Descriptive statistics; Statistical comparison of the control and solvent control data with test item treatments using SAS software; Dunnett's test and Williams' test for determination of NOEC values ($\alpha = 0.05$) when assumptions on normality and homogeneity of variance were met, otherwise non-parametric analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at test initiation and after 7, 14, 21, and 28 days. Measured concentrations for BAS 750 F were between 80% and 90% of nominal at test initiation. Measured concentrations after 7, 14 and 21 days ranged from 75% to 92%, from 69% to 102% and from 76% to 90% of nominal, respectively. At test termination, measured concentrations were between 75% and 102% of nominal. The following biological results are based on mean measured concentrations.

Biological results: No statistically significant (and biologically relevant) differences were determined between the control and the solvent control data. Thus, data of the test substance treatments were compared to the dilution water control. There was no statistically significant reduction in survival, reproduction, growth and time of first brood release for any test substance treatment. The results are summarized in Table 8.2.5.2-3.

Table 8.2.5.2-3: Chronic toxicity (28 d) of BAS 750 F to saltwater mysids (*Americamysis bahia*)

Concentration [µg a.s./L] (nominal)	**Control**	**Solvent control**	**1.0**	**2.0**	**4.0**	**8.0**	**16**
Concentration [µg a.s./L] (mean measured)	**--**	**--**	**0.931**	**1.50**	**3.42**	**6.57**	**13.2**
Survival on day 28 of the first generation (F0) [%]	93	86	87	93	84	85	93
Survival on day 12 of the second generation (F1) [%]	96	87	93	98	93	98	98
F0: Reproductive success [offspring per female] (± SD)	20.1 ± 4.32	19.1 ± 3.44	27.4 ± 4.00	25.7 ± 1.92	29.7 ± 4.57	28.7 ± 2.16	30.4 ± 1.57
F0: Days to first brood release (± SD)	16.3 ± 0.378	16.3 ± 0.143	15.4 ± 0.378	15.2 ± 0.360	15.5 ± 0.192	15.3 ± 0.515	15.4 ± 0.143
F0: Mean body length on day 28, males (± SD) [mm]	6.03 ± 0.190	6.12 ± 0.102	6.10 ± 0.0431	6.28 ± 0.0595	6.12 ± 0.107	6.24 ± 0.100	6.35 ± 0.0266
F0: Mean body length on day 28, females (± SD) [mm]	6.37 ± 0.103	6.40 ± 0.0729	6.67 ± 0.0512	6.65 ± 0.0436	6.57 ± 0.0389	6.68 ± 0.0648	6.68 ± 0.0715
F0: Mean dry weight on day 28, males (± SD) [mg]	0.960 ± 0.0254	0.944 ± 0.0664	0.954 ± 0.0110	1.09 ± 0.0223	1.02 ± 0.0826	1.07 ± 0.0783	1.06 ± 0.0710
F0: Mean dry weight on day 28, females (± SD) [mg]	1.36 ± 0.0557	1.37 ± 0.0877	1.64 ± 0.0479	1.53 ± 0.0950	1.53 ± 0.0595	1.60 ± 0.0755	1.55 ± 0.118
F1: Mean body length after 12 days of exposure, males (± SD) [mm]	4.82 ± 0.196	4.98 ± 0.142	4.93 ± 0.149	5.06 ± 0.0381	5.03 ± 0.181	4.92 ± 0.169	4.96 ± 0.119
F1: Mean body length after 12 days of exposure, females (± SD) [mm]	4.87 ± 0.241	5.12 ± 0.192	5.09 ± 0.113	5.34 ± 0.0714	5.16 ± 0.218	5.13 ± 0.222	5.12 ± 0.0818
Endpoints [µg BAS 750 F/L] (mean measured)							
$NOEC_{overall}$ (28 d)	≥ 13.2						

SD = standard deviation

III. CONCLUSION

In a flow-through chronic toxicity study with saltwater mysids (*Americamysis bahia*), the overall NOEC (28 d) for BAS 750 F was determined to be ≥ 13.2 µg a.s./L based on mean measured concentrations.

CA 8.2.5.3 Development and emergence in Chironomus riparius

Report: CA 8.2.5.3/1
Clark R., 2015 a
BAS 750 F - 10-day toxicity test exposing midge (Chironomus dilutus) to a test substance applied to sediment under static-renewal conditions
2015/7000621
Guidelines: EPA 850.1735
GLP: yes
(certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 10-day semi-static spiked sediment study with renewals of the overlying water, non-biting midge larvae (*Chironomus dilutus*) were exposed to BAS 750 F. Nominal test concentrations were 6.3, 13, 25, 50 and 100.0 mg BAS 750 F/kg dry sediment (corresponding to mean measured concentrations of 7.2, 13, 22, 53 and 97 mg BAS 750 F/kg dry sediment). Additionally, a dilution water control and a solvent control were set up. All test item concentrations and the controls had 8 replicates for the biological observations and 3 replicates were tested for the chemical analysis. 10 larvae were added to each test vessel.

The biological results are based on mean measured concentrations. Following 10 days of exposure, midge survival in both the control and solvent control averaged 96%. During the same period, midge growth in the control and solvent control averaged 2.16 and 2.05 mg ash-free dry weight per larva, respectively. At test termination, a significant difference was determined in survival among midge larvae exposed to the 53 and 97 mg/kg treatment level when compared to the control. Statistical analysis of the relevant treatment levels (7.2, 13 and 22 mg/kg treatment levels) determined a significant difference in growth among midge larvae exposed to the 13 and 22 mg/kg sediment dry weight treatment level compared to the control.

In a 10-day semi-static sediment test with *Chironomus dilutus* the LC_{50} and EC_{50} values of BAS 750 F for survival and growth were determined to be both ≥ 97 mg/kg dry sediment based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (CAS No. 1417782-03-6), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Non-biting midge (*Chironomus dilutus*), third instar larvae, 9-10 days old at test initiation; source: Smithers Viscient culture.

Test design: Semi-static system (10 days); renewal of overlying water, 5 test concentrations plus a water control and a solvent control (acetone), 11 replicates per test item concentration and for the controls (8 replicates for biological response of test organisms and 3 replicates for chemical analysis), 10 larvae per test vessel; assessment of survival, abnormal behavior and growth.

Endpoints: NOEC and LC_{50} / EC_{50} (regarding survival and growth).

Test concentrations: Water control, solvent control, 6.3, 13, 25, 50 and 100.0 mg BAS 750 F/kg dry sediment (nominal), corresponding to mean measured sediment concentrations of 7.2, 13, 22, 53 and 97 mg BAS 750 F/kg dry sediment.

Test conditions: 300 mL glass beakers with 100 mL (approx. 4 cm layer) spiked wet artificial sediment (according to OECD 218), 175 mL laboratory well water; the overlying water was initially renewed by adding two volume additions per test vessel per day using delivery system and water-distribution system (days -1 to 3: 350 mL per day and test vessel, days 4 to 6: 700 mL per day and test vessel, days 7 to 10: 1050 mL per day and test vessel); pH 6.4 - 7.2; oxygen concentration: 3.5 - 7.3 mg/L; total hardness: 68 - 80 mg $CaCO_3$/L; conductivity: 470 - 530 µS/cm; ammonia: $\leq$ 0.10 - 0.44 mg/L; water temperature: 22 °C - 23 °C; light intensity: 280 lux - 800 lux; photoperiod: 16 h light : 8 h dark; no aeration; daily feeding of ground flaked fish food suspended in laboratory well water (4.0 mg/mL), 1.5 mL of food suspension per test vessel.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with MS/MS detection.

Statistics: Descriptive statistics, Wilcoxon's Rank Sum Two-Sample Test and Unequal Variance Two-Sample t-Test (α = 0.05) for comparison of the survival and growth data in the control groups; Dunnett's Multiple Comparison for comparison of the growth data and Steel's Many-One Rank Sum Test for comparison of survival in the treatment groups to the negative control (α = 0.05).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations in the sediment, the overlaying water and the pore water were conducted in each concentration at the beginning and at the end of the test. Recoveries in the sediment were in a range between 104% - 135% of the nominal concentrations at test initiation. At test termination the detected concentrations ranged from 76% - 94% of the nominal values. Overlaying water concentrations ranged from < 0.010 to 0.28 mg BAS 750 F/L on day 0 and from < 0.0083 to 0.056 mg BAS 750 F/L on day 10. The pore water concentrations measured on day 0 were between 0.050 and 1.6 mg BAS 750 F/L and between 0.027 and 1.1 mg BAS 750 F/L on day 10. The following biological results are based on the mean measured sediment concentrations.

Biological results: Following 10 days of exposure, midge survival in both the control and solvent control averaged 96%. During the same period, midge growth in the control and solvent control averaged 2.16 and 2.05 mg ash-free dry weight per larva, respectively. At test termination, a significant difference was determined in survival among midge larvae exposed to the 53 and 97 mg/kg treatment level when compared to the control (Steel's Many-One Rank Sum Test, $\alpha = 0.05$). Statistical analysis (Dunnett's Multiple Comparison Test, $\alpha = 0.05$) of the relevant treatment levels (7.2, 13 and 22 mg/kg treatment levels) determined a significant difference in growth among midge larvae exposed to the 13 and 22 mg/kg sediment dry weight treatment level compared to the control. The results are summarized in Table 8.2.5.3-1.

Table 8.2.5.3-1: Effects of BAS 750 F on survival and growth of *Chironomus dilutus* (10 d)

Concentration [mg BAS 750 F/kg dry sediment] (nominal)	**Water control**	**Solvent control**	**6.3**	**13.0**	**25.0**	**50.0**	**100.0**
Concentration [mg BAS 750 F/kg dry sediment] (mean measured)	--	--	**7.2**	**13.0**	**22.0**	**53.0**	**97.0**
Mean survival (10 d) [%] (SD)	96 (7)	96 (5)	96 (5)	94 (7)	95 (8)	79 (10) *	84 (7) *
Mean ash-free dry weight per larva (10 d) [mg] (SD)	2.16 (0.64)	2.05 (0.18)	1.90 (0.48)	1.56 (0.50) #	1.34 (0.37) #	2.05 (0.34) &	1.73 (0.38) &
	Endpoints [mg BAS 750 F/kg dry sediment] (mean measured)						
LC_{50} / EC_{50}	> 97						
NOEC	7.2						

SD: standard deviation

* Significant difference compared to the control, based on Steel's Many-One Rank Sum Test ($\alpha = 0.05$).

\# Significant difference compared to the control, based on Dunnett's Multiple Comparison Test ($\alpha = 0.05$).

& Based on the survival effect observed, this treatment level was excluded from statistical analysis of growth (ash free dry weight per larva) for the determination of the NOEC value.

III. CONCLUSION

In a 10-day semi-static sediment test with *Chironomus dilutus* the LC_{50} and EC_{50} values of BAS 750 F for survival and growth were determined to be both ≥ 97 mg/kg dry sediment based on mean measured concentrations.

Report:	CA 8.2.5.3/2 Backfisch K.,Weltje L., 2015 a Chronic toxicity of Reg.No. 5924326 (M750F003; metabolite of BAS 750 F) to the non-biting midge Chironomus riparius - a spiked sediment study 2015/1003916
Guidelines:	OECD 218: Sediment-water chironomid toxicity test using spiked sediment (April 2004)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 28-day static spiked sediment study, non-biting midge larvae (*Chironomus riparius*) were exposed to M750F003 (metabolite of BAS 750 F) at nominal test item concentrations of 0.200, 0.400, 0.800, 1.600 and 3.200 mg M750F003/kg dry sediment (corresponding to initial measured concentrations of 0.125, 0.272, 0.504, 0.983 and 1.944 mg/kg dry sediment). Additionally, a solvent (acetone) control and a water control were set up. All test item concentrations and the water control had 4 replicates, whereas 6 replicates were tested for the solvent control. 20 larvae were added to each test vessel.

The biological results are based on initial measured concentrations of the test item. On DAI 13 (= day after insertion of the larvae), the first emerged midges were observed. No statistically significant differences compared to the control treatments were found for emergence rate and development rate up to and including the highest test item concentration.

In a 28-day static sediment test with *Chironomus riparius* the NOEC of M750F003 (metabolite of BAS 750 F) was determined to be ≥ 1.944 mg a.s./kg dry sediment based on emergence and development rate (initial measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F003 (Reg. No. 5924326; metabolite of BAS 750 F), batch no. L84-250, purity: 99.6%.

B. STUDY DESIGN

Test species: Non-biting midge (*Chironomus riparius*), first instar larvae from in-house culture (non-GLP), originally obtained from "the Zoological Institute of the J.W. Goethe University" Frankfurt, Germany.

Test design: Static system (28 days); 5 test concentrations plus a solvent (acetone) control and a water control, 4 replicates per test item concentration and for the water control, 6 replicates for the solvent control; 20 larvae were added to each vessel; assessment of emergence rate and development rate.

Endpoints: NOEC and EC_{50} (regarding emergence rate and development rate).

Test concentrations: Solvent control, water control, 0.200, 0.400, 0.800, 1.600 and 3.200 mg M750F003/kg dry sediment (nominal), corresponding to initial measured concentrations of 0.125, 0.272, 0.504, 0.983 and 1.944 mg/kg dry sediment.

Test conditions: 600 mL glass vessels with 100 g spiked wet artificial sediment (according to OECD 218), 400 mL M4 water (Elendt medium) corresponding to a water layer of about 8.0 cm; pH 7.70 - 8.36; oxygen content: 7.73 - 9.44 mg/L (saturation > 60%); total hardness: 2.5 mmol/L; conductivity: 665 µS/cm; ammonia: 0.5 mg/L at test initiation and 4.5 mg/L at test termination; water temperature: 20.3 °C - 20.7 °C; light intensity: 550 - 950 lux; photoperiod: 16 h light : 8 h dark; gentle aeration; food: TetraMin (0.5 mg food/larva/day).

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS-detection.

Statistics: Descriptive statistics, Williams' Multiple sequential t-test for determination of the NOEC based on emergence and development rate (α = 0.05); Probit and Weibull analysis for determination of EC_x values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations in the overlying water, the pore water and the sediment was conducted in the controls and in each concentration at the beginning and the end of the test. Recoveries in the sediment were in the range between 60.8% and 68.0% of the nominal concentrations at test initiation and between 20.6% and 28.8% of nominal at test termination. Overlying water concentrations ranged from 0.0119 to 0.212 mg/L at test start and from 0.0209 to 0.363 mg/L at test end. The pore water concentrations ranged from 0.0061 to 1.050 mg/L at test start and from 0.0215 to 0.384 mg/L at test termination.

The analytical results show that M750F003 has quite a low affinity for sediment and quickly redistributed into the pore water and the overlying water. To quantify the redistribution, mass balance calculations were made on basis of the measured amounts in sediment, overlaying water and pore water. On test initiation approximately 35% of the test item was found in pore water and overlaying water. The mass balance on DAT 2 shows, that the total recovery in the whole system was between 95.9% and 104.3%. This confirms the correct application of the test item on test initiation. Therefore, the following biological results are based on the initial measured sediment concentrations.

Biological results: On DAI 13 (= day after insertion of the larvae), the first emerged midges were observed. No statistically significant differences compared to the control treatments were found for emergence rate and development rate up to and including the highest test item concentration (Williams' Multiple t-test, $\alpha = 0.05$). Hence, all endpoints (EC_x and NOEC) are equal or above the highest test concentration. The results are summarized in Table 8.2.5.3-2.

Table 8.2.5.3-2: Effects of M750F003 (metabolite of BAS 750 F) on emergence and development of *Chironomus riparius*

Concentration [mg M750F003/kg dry sediment] (nominal)	**Control**	**Solvent control**	**0.200**	**0.400**	**0.800**	**1.600**	**3.200**
Concentration [mg M750F003/kg dry sediment] (initial measured)	**--**	**--**	**0.125**	**0.272**	**0.504**	**0.983**	**1.944**
Emergence rate (ER) (28 d) [#]	0.9125 ± 0.0479	0.8750 ± 0.0524	0.9125 ± 0.0854	0.8500 ± 0.1354	0.9000 ± 0.0707	0.9250 ± 0.1190	0.7875 ± 0.0629
Development rate (DR) (28 d) [#]	0.0701 ± 0.0012	0.0689 ± 0.0016	0.0694 ± 0.0003	0.0683 ± 0.0004	0.0686 ± 0.0015	0.0685 ± 0.0015	0.0699 ± 0.0005
Endpoints [mg M750F003/kg dry sediment] (initial measured)							
EC_{50} emergence & development rate (28 d)	> 1.944						
EC_{10} emergence & development rate (28 d)	> 1.944						
NOEC emergence & development rate (28 d)	≥ 1.944						

[#] Values represent mean and standard deviation from all replicates, each with 20 larvae.

III. CONCLUSION

In a 28-day static sediment test with *Chironomus riparius* the NOEC of M750F003 (metabolite of BAS 750 F) was determined to be ≥ 1.944 mg a.s./kg dry sediment based on emergence and development rate (initial measured).

Report:	CA 8.2.5.3/3 Backfisch K.,Weltje L., 2015a Chronic toxicity of Reg.No. 5834378 to the non-biting midge Chironomus riparius - A spiked sediment study 2014/1243181
Guidelines:	OECD 218 (2004)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 28-day static spiked sediment study, non-biting midge larvae (*Chironomus riparius*) were exposed to BAS 750 F at nominal concentrations of 0.075, 0.150, 0.300, 0.600 and 1.200 mg a.s./kg dry sediment (corresponding to initial measured concentrations of 0.0755, 0.155, 0.303, 0.557 and 1.158 mg a.s./kg dry sediment). Additionally, a solvent (acetone) control and a water control were set up. All test item concentrations and the water control had 4 replicates, whereas 6 replicates were tested for the solvent control. 20 larvae were added to each test vessel.

The biological results are based on initial measured concentrations of the test item. On DAI 15 (= day after insertion of the larvae), the first emerged midges were observed. No statistically significant differences compared to the control treatments were found for emergence rate and development rate up to and including the highest test item concentration.

In a 28-day static sediment test with *Chironomus riparius*, the NOEC value of BAS 750 F was determined to be ≥ 1.158 mg a.s./kg dry sediment (initial measured) based on emergence rate and development rate.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg No. 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Non-biting midge (*Chironomus riparius*), first instar larvae; source: in-house culture (non-GLP), originally obtained from "Zoological Institute of the J.W. Goethe University", Frankfurt am Main, Germany.

Test design: Static system (28 days); 5 test concentrations plus a solvent (acetone) control and a water control, 4 replicates per test item concentration and for the water control, 6 replicates for the solvent control; 20 larvae were added to each test vessel; assessment of emergence rate and development rate.

Endpoints: NOEC, EC_{50} (regarding emergence rate and development rate).

Test concentrations: Solvent (acetone) control, water control, 0.075, 0.150, 0.300, 0.600 and 1.200 mg a.s./kg dry sediment (nominal), corresponding to initial measured concentrations of 0.0755, 0.155, 0.303, 0.557 and 1.158 mg a.s./kg dry sediment.

Test conditions: 600 mL glass vessels with 100 g spiked wet artificial sediment (according to OECD 218), 400 mL M4 water (Elendt medium) corresponding to a water layer of about 8.0 cm; pH 7.63 - 8.26; oxygen content: 7.36 - 8.79 mg/L (saturation > 60%); total hardness: 2.45 mmol/L; conductivity: 613 µS/cm; ammonia: 2.0 mg/L at test initiation and 4.5 mg/L at test termination; water temperature: 19.7 °C - 20.3 °C; light intensity: 520 - 980 lux; photoperiod: 16 h light : 8 h dark; gentle aeration; food: TetraMin (0.5 mg food/larva/day).

Analytics: Analytical verification of test item concentrations in overlaying water and sediment was conducted using a HPLC-method with MS detection.

Statistics: Descriptive statistics, Williams' Multiple sequential t-test for determination of the NOEC based on emergence and development rate ($\alpha = 0.05$); Probit and Weibull analysis for determination of EC_x values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations in the overlying water, the pore water and the sediment was conducted in each concentration at the beginning and the end of the test. Recoveries in the sediment were in a range between 93% and 103% of the nominal concentrations at test initiation and 83% and 89% of nominal at test termination. BAS 750 F concentrations found in the overlaying water ranged from < LOD (limit of detection = 0.001 mg/L) to 0.007 mg a.s./L at test initiation and from < LOD to 0.0029 mg a.s./L at test end. Measured pore water concentrations were between 0.0094 and 0.0126 mg a.s./L at the beginning of the test and between < LOD and 0.00233 mg a.s./L at test termination. The following biological results are based on initial measured sediment concentrations.

Biological results: On DAI 15 (= day after insertion of the larvae), the first emerged midges were observed. No statistically significant differences compared to the control treatments were found for emergence rate and development rate up to and including the highest test item concentration (Williams' Multiple sequential t-test, $\alpha = 0.05$). Hence, all endpoints (EC_x and NOEC) are equal or above the highest test concentration. The results are summarized in Table 8.2.5.3-3.

Table 8.2.5.3-3: Effects of BAS 750 F on emergence and development of *Chironomus riparius*

Concentration [mg a.s./kg dry sediment] (nominal)	**Control**	**Solvent control**	**0.075**	**0.150**	**0.300**	**0.600**	**1.200**
Concentration [mg a.s./kg dry sediment] (initial measured)	**--**	**--**	**0.0755**	**0.155**	**0.303**	**0.557**	**1.158**
Emergence rate (ER) [#]	0.9625 ± 0.0479	0.9250 ± 0.0689	0.9125 ± 0.0479	0.9125 ± 0.0629	0.9375 ± 0.0629	0.8500 ± 0.0408	0.8570 ± 0.1190
Development rate (DR) [#]	0.0585 ± 0.0040	0.0606 ± 0.0022	0.0607 ± 0.0039	0.0608 ± 0.0023	0.0596 ± 0.0010	0.0594 ± 0.0014	0.0588 ± 0.0026
Endpoints [mg BAS 750 F/kg dry sediment] (initial measured)							
EC_{50} emergence rate, development rate (28 d)	> 1.158						
EC_{10} emergence rate, development rate (28 d)	> 1.158						
NOEC emergence rate, development rate (28 d)	≥ 1.158						

[#] Values represent mean and standard deviation from all replicates, each with 20 larvae.

III. CONCLUSION

In a 28-day static sediment test with *Chironomus riparius*, the NOEC value of BAS 750 F was determined to be ≥ 1.158 mg a.s./kg dry sediment (initial measured) based on emergence rate and development rate.

CA 8.2.5.4 Sediment dwelling organisms

Report:	CA 8.2.5.4/1 Clark R., 2015 b BAS 750 F - 10-Day toxicity test exposing freshwater Amphipods (Hyalella azteca) to a test substance applied to sediment under static renewal conditions 2015/7000622
Guidelines:	EPA 850.1735, OECD 218: Sediment-water chironomid toxicity test using spiked sediment (April 2004)
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 10-day semi-static acute spiked sediment study, amphipods (*Hyalella azteca*) were exposed to BAS 750 F at nominal concentrations of 6.3, 13, 25, 50 and 100 mg BAS 750 F/kg dry sediment (corresponding to mean measured concentrations of 6.6, 12, 22, 50 and 100 mg BAS 750 F/kg dry sediment). Additionally, a solvent control and a dilution water control were set up. All test item concentrations and the controls had 8 replicates for the biological observations and 3 replicates were used for the chemical analysis. 10 larvae were added to each test vessel. Assessment of survival and abnormal behaviour growth of the amphipods was conducted daily. Growth was determined at test end.

The biological results are based on mean measured sediment concentrations. After 10-days of exposure, statistical analysis determined no significant difference in survival and growth among amphipods exposed to any of the treatment levels tested compared to the water control.

In a 10-day semi-static acute sediment test with *Hyalella azteca* the LC_{50} / EC_{50} of BAS 750 F was determined to be > 100 mg BAS 750 F/kg dry sediment based on mean measured concentrations. The NOEC was ≥ 100 mg a.s./kg dry sediment (mean measured).

I. MATERIAL AND METHODS

Test item:	BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%
Test species:	Amphipods (*Hyalella azteca*), 8 days old, source: laboratory cultures maintained at Smithers Viscient.
Test design:	Semi-static system (10 days); 5 test concentrations plus a solvent (acetone) control and a water control; 11 replicates per test item concentration and for the controls (8 replicates for biological response of test organisms and 3 replicates for chemical analysis); 10 amphipods per test vessel; daily assessment of survival and abnormal behaviour; determination of dry weight at test end.
Endpoints:	NOEC and LC_{50} / EC_{50} (regarding survival and growth).
Test concentrations:	Control (dilution water), solvent control (acetone), 6.3, 13, 25, 50 and 100 mg BAS 750 F/kg dry sediment (nominal), corresponding to mean measured concentrations of 6.6, 12, 22, 50 and 100 mg BAS 750 F/kg dry sediment.
Test conditions:	300 mL glass vessels with 100 mL spiked artificial sediment (OECD 218) and 175 mL overlaying laboratory well water; pH 6.7 - 7.5; oxygen content: 2.6 mg/L - 8.4 mg/L; total hardness: 68 – 80 mg $CaCO_3$/L; total alkalinity: 20 – 24 mg $CaCO_3$/L; conductivity: 380 – 480 µS/cm; ammonia: $\leq$ 0.10 - 0.44 mg/L (as nitrogen); water temperature: 22 °C - 23 °C; photoperiod: 16 hours light : 8 hours darkness; light intensity: 210 – 520 lux; feeding: 1.5 mL of yeast, cereal leaves and flaked fish food suspension daily.
Analytics:	Analytical verification of test item concentrations was conducted using an LC-method with MS/MS detection.
Statistics:	Descriptive statistics, Wilcoxon's Rank Sum Test and Equal Variance Two-Sample t-Test (both $\alpha = 0.05$) for comparison of control and solvent control data; Steel's Many-One Rank Sum Test for determination of NOEC values ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in each concentration at test initiation and at test termination. Measured concentrations of BAS 750 F in sediment ranged from 99% - 120% of nominal test item concentrations at test initiation and from 76% to 97% of nominal at test termination. Overlaying water concentrations of BAS 750 F were between 0.015 and 0.20 mg a.s./L on day 0 and between 0.012 and 0.17 mg a.s./L on day 10. Measured pore water concentrations of BAS 750 F ranged from 0.12 to 2.0 mg a.s./L on day 0 and from 0.076 to 1.4 mg a.s./L on day 10. The following biological results are based on mean measured concentrations.

Biological results: After 10-days of exposure, statistical analysis determined no significant difference in survival and growth among amphipods exposed to any of the treatment levels tested compared to the water control (Steel's Many-One Rank Sum Test, $\alpha = 0.05$). The results are summarized in Table 8.2.5.4-1.

Table 8.2.5.4-1: Effect of BAS 750 F on survival and growth of *Hyalella azteca*

Concentration [mg a.s./kg dry sediment] (nominal)	**Control**	**Solvent control**	**6.3**	**13**	**25**	**50**	**100**
Concentration [mg a.s./kg dry sediment] (mean measured)	**--**	**--**	**6.6**	**12**	**22**	**50**	**100**
Mean survival (SD) (10 d) [%]	96 (5)	98 (5)	100 (0)	98 (7)	100 (0)	100 (0)	95 (11)
Mean individual dry weight (SD) (10 d) [%]	0.20 (0.024)	0.20 (0.027)	0.20 (0.020)	0.18 (0.013)	0.19 (0.011)	0.18 (0.010)	0.18 (0.045)
Endpoints [mg a.s./kg dry sediment] (mean measured)							
LC_{50} / EC_{50} (10 d)	> 100						
NOEC (10 d)	≥ 100						

SD = Standard Deviation

III. CONCLUSION

In a 10-day semi-static acute sediment test with *Hyalella azteca* the LC_{50} / EC_{50} of BAS 750 F was determined to be > 100 mg BAS 750 F/kg dry sediment based on mean measured concentrations. The NOEC was ≥ 100 mg a.s./kg dry sediment (mean measured).

Report:	CA 8.2.5.4/2 Clark R., 2015 c BAS 750 F - 10-Day toxicity test exposing estuarine amphipods (Leptocheirus plumulosus) to a test substance applied to sediment under static conditions 2015/7000623
Guidelines:	EPA 850.1740
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 10-day static acute spiked sediment study, marine amphipods (*Leptocheirus plumulosus*) were exposed to BAS 750 F at nominal concentrations of 6.3, 13, 25, 50 and 100 mg BAS 750 F/kg dry sediment (corresponding to mean measured concentrations of 6.7, 12, 25, 48 and 95 mg BAS 750 F/kg dry sediment). Additionally, a solvent control (acetone) and a water control were tested. All test item concentrations and the controls had 5 replicates for biological observations and 4 replicates were used for chemical analysis. 20 larvae were added to each test vessel. Assessment of survival and abnormal behaviour was conducted daily.

The biological results are based on the mean measured sediment concentrations. After 10 days of exposure amphipod survival in both the control and solvent control averaged 100%. No statistically significant differences were detected for amphipod survival up to and including the highest tested concentration compared to the water control.

In a 10-day static acute sediment test with *Leptocheirus plumulosus* the NOEC of BAS 750 F was determined to be ≥ 95 mg a.s./kg dry sediment based on mean measured concentrations. The LC_{50} was > 95 mg BAS 750 F/kg dry sediment (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Marine Amphipod (*Leptocheirus plumulosus*), juveniles, 2 - 4 mm in length at test initiation; source: Chesapeake Cultures, Inc., Hayes, VA, USA.

Test design: Static system (10 days); 5 test concentrations plus control and solvent control, 9 replicates per test item concentration and for the controls (5 replicates for biological response of test organisms and 4 replicates for chemical analysis and monitoring of water quality), 20 amphipods per replicate; daily assessment of survival and abnormal behavior.

Endpoints: NOEC and LC_{50}.

Test concentrations: Control (dilution water), solvent control (acetone), 6.3, 13, 25, 50 and 100 mg BAS 750 F/kg dry sediment (nominal), corresponding to mean measured concentrations of 6.7, 12, 25, 48 and 95 mg BAS 750 F/kg dry sediment.

Test conditions: 1000 mL glass beakers; 175 mL sediment (natural sediment collected from Sequim Bay, Port Gamble, Washington; 3.2% organic carbon, particle size distribution: 37% sand, 37% silt and 26% clay, pH 7.8) and 725 mL overlying water (filtered natural seawater), total overlying water/sediment volume: approx. 900 mL; continuous illumination; light intensity: 600 - 880 lux; gentle aeration; no feeding.
Overlying water: salinity: 20 - 21‰; pH 7.2 - 8.4; oxygen content: 5.7 mg/L - 7.9 mg/L; water temperature: 24°C - 25°C; ammonia: 1.1 - 1.9 mg/L as nitrogen.
Pore water: salinity: 24 - 28‰; pH 7.0 - 7.5; ammonia: 0.65 - 12 mg/L as nitrogen.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with MS/MS detection.

Statistics: Descriptive statistics, Wilcoxon's Rank Sum Two-Sample Test ($\alpha = 0.05$) for comparison of control and solvent control data; Steel's Many-One Rank Sum Test for determination of NOEC value ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in each concentration at test initiation and at test termination. Measured concentrations of BAS 750 F in sediment ranged from 93% - 110% of nominal test item concentrations at test initiation and from 93% to 100% of nominal at test termination. Overlaying water concentrations of BAS 750 F were between < 0.012 mg a.s./L and 0.13 mg a.s./L at day 0 and between 0.038 mg a.s./L and 0.46 mg a.s./L at day 10. Pore water concentrations of BAS 750 F rangend from 0.058 mg a.s./L to 1.0 mg a.s./L at day 0 and from 0.071 mg a.s./L to 0.65 mg a.s./L at day 10. The biological results are based on the mean measured sediment concentrations.

Biological results: After 10 days of exposure amphipod survival in both the control and solvent control averaged 100%. No statistically significant differences were detected for amphipod survival up to and including the highest tested concentration compared to the water control (Steel's Many-One Rank Sum Test, $\alpha = 0.05$). The results are summarized in Table 8.2.5.4-2.

Table 8.2.5.4-2: Effect of BAS 750 F on survival of *Leptocheirus plumulosus*

Concentration [mg a.s./kg dry sediment] (nominal)	**Control**	**Solvent control**	**6.3**	**13**	**25**	**50**	**100**
Concentration [mg a.s./kg dry sediment] (mean measured)	**--**	**--**	**6.7**	**12**	**25**	**48**	**95**
Mean survival (SD) [%]	100 (0)	100 (0)	98 (1)	99 (1)	99 (1)	100 (0)	98 (1)
Endpoints [mg BAS 750 F/kg dry sediment] (mean measured)							
LC_{50}	> 95 (95% confidence limits: n.d. [#])						
NOEC	≥ 95						

SD = Standard Deviation
[#] 95% confidence limits could not be calculated as LC_{50} value was empirically determined.

III. CONCLUSION

In a 10-day static acute sediment test with *Leptocheirus plumulosus* the NOEC of BAS 750 F was determined to be ≥ 95 mg a.s./kg dry sediment based on mean measured concentrations. The LC_{50} was > 95 mg BAS 750 F/kg dry sediment (mean measured).

CA 8.2.6 Effects on algal growth

CA 8.2.6.1 Effects on growth of green algae

Report:	CA 8.2.6.1/1 Brzozowska K., 2014b BAS 750 F (Reg. No. 5834378) - Pseudokirchneriella subcapitata SAG 61.81 - Growth inhibition test 2013/1250865
Guidelines:	OECD 201 (2006), EPA 850.4500
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz,

EXECUTIVE SUMMARY

In a 96 hour static acute toxicity laboratory study, the effect of BAS 750 F on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. Due to the poor solubility of the test item, a filtrate of the loading of 10 mg BAS 750 F/L was used as the highest test item concentration, while the four lower test item concentrations consisted of 16-fold, 8-fold, 4-fold and 2-fold dilutions of the filtrate of the loading of 10 mg a.s./L, respectively. This approach resulted in geometric mean measured concentrations of 0.103, 0.209, 0.416, 0.914 and 1.899 mg BAS 750 F/L. Additionally, a dilution water control was set up. Assessment of growth was conducted 24, 48, 72 and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations. No morphological effects on algae were observed in the control and at test item concentrations of up to and including 0.416 mg a.s./L. At 0.914 mg a.s./L about 15% and 20% of the cells were opalescent after 72 and 96 hours of exposure, respectively, while at 1.899 mg a.s./L about 20% of the cells were opalescent and 20% of the cells were comma-shaped after 72 and 96 hours of exposure. After 72 h of exposure, statistically significant effects compared to the control were detected at the four highest and at the five highest test item concentrations for growth rate and yield, respectively. After 96 h of exposure, statistically significant effects compared to the control occurred at the three highest test item concentrations for both growth rate and yield.

In a 96-hour algae test with *Pseudokirchneriella subcapitata,* the respective E_rC_{50} was determined to be 1.352 mg a.s./L (geometric mean measured) after 72 hours of exposure.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata*, (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz), SAG 61.81; stock obtained from the "The Culture Collection of Algae", Göttingen University, Germany.

Test design: Static system; test duration 96 hours; 5 test concentrations, each with 4 replicates per treatment plus a control with 8 replicates; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 hours and 96 hours.

Test concentrations: Control, 16-fold diluted filtrate, 8-fold diluted filtrate, 4-fold diluted filtrate, 2-fold diluted filtrate and filtrate of the loading of 10 mg BAS 750 F/L, corresponding to geometric mean measured concentrations of < LoD (limit of detection), 0.103, 0.209, 0.416, 0.914, 1.899 mg a.s./L.

Test conditions: Erlenmeyer glass flasks; test volume 100 mL; test medium: AAP medium; pH 7.03 - 8.98; temperature: 24.1 - 24.4 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at 4363 - 4480 lux; constant shaking.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics; probit analysis for determination of EC_x values; Williams Multiple Sequential t-test Procedure and Multiple Sequentially–rejective U-test after Bonferroni-Holm for determination of the NOEC value ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were: control (< LoD), 0.115, 0.235, 0.466, 0.946 and 1.914 mg a.s./L. The test item concentrations determined in samples collected at exposure termination were in the range of 78.7% to 98.4% of initial concentrations, therefore the following biological results are based on geometric mean measured concentrations.

Biological results: No morphological effects on algae were observed in the control and at test item concentrations of up to and including 0.416 mg a.s./L. At 0.914 mg a.s./L about 15% and 20% of the cells were opalescent after 72 and 96 hours of exposure, respectively, while at 1.899 mg a.s./L about 20% of the cells were opalescent and 20% of the cells were comma-shaped after 72 and 96 hours of exposure. After 72 h of exposure, statistically significant effects compared to the control were detected at the four highest and at the five highest test item concentrations for growth rate and yield, respectively (Multiple Sequentially-rejective U-test after Bonferroni-Holm for growth rate, Williams Multiple Sequential t-test Procedure for yield, $\alpha = 0.05$ in both cases). After 96 h of exposure, statistically significant effects compared to the control occurred at the three highest test item concentrations for both growth rate and yield (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$). The effects on algal growth are summarized in Table 8.2.6.1-1.

Table 8.2.6.1-1: Effect of BAS 750 F on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration [mg a.s./L] (geometric mean measured)	**Control**	**0.103**	**0.209**	**0.416**	**0.914**	**1.899**
Inhibition in 72 h (growth rate) [%]	0.0	2.0	4.8 *, b)	6.7 *, b)	10.6 *, b)	86.0 *, b)
Inhibition in 72 h (yield) [%]	0.0	9.1 *, a)	20.3 *, a)	26.6 *, a)	39.0 *, a)	99.0 *, a)
Inhibition in 96 h (growth rate) [%]	0.0	0.0	0.6	2.2 *, a)	3.5 *, a)	89.9 *, a)
Inhibition in 96 h (yield) [%] #	0.0	-0.2	2.8	10.9 *, a)	16.6 *, a)	99.6 *, a)

	Endpoints [mg BAS 750 F/L] (geometric mean measured)
E_rC_{50} (72 h)	**1.352 (95% confidence limits: 1.272-1.434)**
E_rC_{10} (72 h)	0.904 (95% confidence limits: 0.812-0.983)
E_yC_{50} (72 h)	0.777 (95% confidence limits: 0.606-1.012)
E_yC_{10} (72 h)	0.215 (95% confidence limits: 0.102-0.315)
E_rC_{50} (96 h)	1.404 (95% confidence limits: 1.368-1.437)
E_rC_{10} (96 h)	1.036 (95% confidence limits: 0.990-1.078)
E_yC_{50} (96 h)	1.163 (95% confidence limits: 1.073-1.268)
E_yC_{10} (96 h)	0.744 (95% confidence limits: 0.646-0.824)

Negative values indicate stimulated growth compared to the control.
* Statistically significant differences compared to the control ($\alpha = 0.05$).
a) Williams Multiple Sequential t-test Procedure.
b) Multiple Sequentially-rejective U-test after Bonferroni-Holm.

III. CONCLUSION

In a 96-hour algae test with *Pseudokirchneriella subcapitata,* the respective E_rC_{50} was determined to be 1.352 mg a.s./L (geometric mean measured) after 72 hours of exposure.

Report:	CA 8.2.6.1/2 Backfisch K., 2015 a Effect of Reg.No. 6003432 (M750F007, metabolite of BAS 750 F) on the growth of the green alga Pseudokirchneriella subcapitata 2015/1003914
Guidelines:	OECD 201, EPA 850.5400
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

In a 72 hour static acute toxicity laboratory study, the effect of M750F007 (metabolite of BAS 750 F) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. The following nominal test concentrations were tested: 0 (control), 0.625, 1.25, 2.5, 5.0 and 10.0 mg M750F007/L. Assessment of growth was conducted 24, 48, 72 h after test initiation.

The biological results are based on nominal concentrations. No morphological effects on algae were observed in the control and at all test item concentrations. After 72 h of exposure, no statistically significant effects compared to the control were detected at all test item concentrations for growth rate and yield.

In a 72-hour algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F007 was determined to be > 10 mg/L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F007 (metabolite of BAS 750 F, Reg. no.: 6003432), batch no. L87-32-1, purity: 97.0%.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata*, (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz), SAG 61.81; stock obtained from the "The Culture Collection of Algae", Göttingen University, Germany.

Test design: Static system; test duration 72 hours; 5 test concentrations, each with 5 replicates per treatment plus a control with 10 replicates; daily assessment of growth.

Endpoints: EC_{50} with respect to growth rate and yield after exposure over 72 hours.

Test concentrations: Control, 0.625, 1.25, 2.5, 5 and 10 mg M750F007/L.

Test conditions: 100 mL Erlenmeyer dimple flasks; test volume 60 mL; test medium: OECD medium; pH 7.92 - 8.04; temperature: 22 ± 1 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at about 8000 lux; constant shaking (130 rpm).

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with MS detection.

Statistics: Descriptive statistics; probit analysis for determination of EC_x values ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were in the range of 93% and 99%. The test item concentrations determined in samples collected at exposure termination were in the range of 90% to 94% of nominal concentrations, therefore the following biological results are based on nominal concentrations.

Biological results: No morphological effects on algae were observed in the control and at all test item concentrations. After 72 h of exposure, no statistically significant effects compared to the control were detected at all test item concentrations for growth rate and yield. The results are summarized in Table 8.2.6.1-2.

Table 8.2.6.1-2: Effect of M750F007 (metabolite of BAS 750 F) on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration [mg/L] (nominal)	**Control**	**0.625**	**1.25**	**2.5**	**5**	**10**
Inhibition in 72 h (growth rate) [%] #	--	- 0.5	1.0	1.6	2.4	- 1.7
Inhibition in 72 h (yield) [%] #	--	- 1.9	2.9	7.2	6.5	- 7.2
Endpoints [mg M750F007/L] (nominal)						
E_rC_{50} (72 h)	> 10					
E_yC_{50} (72 h)	> 10					

Negative values indicate stimulated growth compared to the control.

III. CONCLUSION

In a 72-hour algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F007 was determined to be > 10 mg/L (nominal).

Report:	CA 8.2.6.1/3 Brzozowska-Wojoczek K., 2015 a Reg.No. 6010286 (metabolite of BAS 750 F, M750F008) - Pseudokirchneriella subcapitata SAG 61.81 - Growth inhibition test 2015/1001491
Guidelines:	OECD 201 (2006), EPA 850.4500
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 96 hour static acute toxicity laboratory study, the effect of M750F008 (metabolite of BAS 750 F) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. Due to the poor solubility of the test item, a filtrate of the loading of 10 mg M750F008/L was used as the highest test item concentration, while the five lower test item concentrations consisted of 7.6-fold, 5.06-fold, 3.38-fold, 2.25-fold and 1.5-fold dilutions of the filtrate of the loading of 10 mg a.s./L. This approach resulted in geometric mean measured concentrations of 7.08, 0.83, 1.24, 1.93, 3.08 and 4.67 mg M750F008/L, respectively. Additionally, a dilution water control and a solvent control (N,N-dimethylformamide (DMF)) were set up. Assessment of growth was conducted 24, 48, 72 and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations. At exposure termination in the test item concentration of 1.93mg/L and 3.08 mg/L opalescent rod shaped cells were observed. In the test item concentrations of 4.67 and 7.08 mg/L cells were bigger, opalescent and rod shaped. In the remaining test item concentrations no differences in shape, size and color of algae cells were reported as compared to the algae cells in the control and the solvent control. After 72 and 96 h of exposure, statistically significant effects compared to the control were detected at the five highest test item concentrations for growth rate and yield.

In a 96-hour static algae test with *Pseudokirchneriella subcapitata,* the respective E_rC_{50} was determined to be 4.08 mg/L (geometric mean measured) after 72 hours of exposure.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F008 (metabolite of BAS 750 F, Reg. no.: 6010286), batch no. L85-94, purity: 96.5 ± 1%.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata*, (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz), SAG 61.81; stock obtained from the "The Culture Collection of Algae", Göttingen University, Germany.

Test design: Static system; test duration 96 hours; 6 test concentrations, each with 4 replicates per treatment plus a control and a solvent control with 8 replicates, each; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 hours and 96 hours.

Test concentrations: Control, solvent control (0.1 mL DMF/L), 7.6-fold diluted filtrate, 5.06-fold diluted filtrate, 3.38-fold diluted filtrate, 2.25-fold diluted filtrate, 1.5-fold diluted filtrate and filtrate of the loading of 10 mg M750F008/L, corresponding to geometric mean measured concentrations of 0.83, 1.24, 1.93, 3.08, 4.67 and 7.08 mg/L.

Test conditions: 250 mL Erlenmeyer glass flasks; test volume 100 mL; test medium: AAP medium; pH 7.02 - 7.36 at test initiation and pH 7.61 - 9.06 at test termination; temperature: 22.9 - 23.5 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at 3975 - 4200 lux; constant shaking.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics; Student-t-test for comparison of control and solvent control; probit analysis for determination of EC_x values, determination of NOEC: Jonckheere-Terpstra Test for growth rate, Welch-t-test with Bonferroni-Holm adjustment for yield ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were 7.07 mg/L in the filtrate of a loading of 10 mg/L, 4.74 mg/L in its 1.5-fold dilution, 3.16 mg/L in its 2.25-fold dilution, 2.09 mg/L in its 5.06-fold dilution, 1.38 mg/L in its 5.06-fold dilution and 0.91 mg/L in its 7.6-fold dilution. The geometric mean concentrations of the test item were 7.08, 4.67, 3.08, 1.93, 1.24 and 0.83 mg/L, respectively. The test item concentrations determined in samples collected at exposure termination were in the range of 81.2% to 100.3% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: At exposure termination in the test item concentration of 1.93mg/L and 3.08 mg/L opalescent rod shaped cells were observed. In the test item concentrations of 4.67 and 7.08 mg/L cells were bigger, opalescent and rod shaped. In the remaining test item concentrations no differences in shape, size and color of algae cells were reported as compared to the algae cells in the control and the solvent control. After 72 and 96 h of exposure, statistically significant effects compared to the control were detected at the five highest test item concentrations for growth rate and yield (Jonckheere-Terpstra Test for growth rate, Welch-t-test with Bonferroni-Holm adjustment for yield, $\alpha = 0.05$ for both tests). The effects on algal growth are summarized in Table 8.2.6.1-3.

Table 8.2.6.1-3: Effect of M750F008 on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration [mg M750F008/L] (geometric mean measured)	**Control**	**Solvent control**	**0.83**	**1.24**	**1.93**	**3.08**	**4.67**	**7.08**
Inhibition in 72 h (growth rate) [%] [a)]	--	0.0	0.8	3.5 *	12.1 *	45.7 *	54.7 *	70.7 *
Inhibition in 72 h (yield) [%] [b)]	--	0.0	4.0	15.6 *	43.5 *	89.0 *	93.0 *	97.2 *
Inhibition in 96 h (growth rate) [%] [a)]	--	0.0	0.1	4.2 *	7.2 *	26.8 *	45.7 *	75.3 *
Inhibition in 96 h (yield) [%] [b)]	--	0.0	0.3	20.5 *	32.6 *	77.3 *	92.2 *	98.7 *
Endpoints [mg M750F008/L] (geometric mean measured)								
E_rC_{50} (72 h)	**4.08 (95% confidence limits: 3.83 - 4.34)**							
E_rC_{10} (72 h)	1.40 (95% confidence limits: 1.33 - 1.47)							
E_yC_{50} (72 h)	2.01 (95% confidence limits: 1.72 - 2.33)							
E_rC_{50} (96 h)	4.78 (95% confidence limits: 4.23 - 5.38)							
E_rC_{10} (96 h)	2.12 (95% confidence limits: 1.92 - 2.34)							
E_yC_{50} (96 h)	2.21 (95% confidence limits: 1.86 - 2.63)							

* Statistically significant differences compared to the control ($\alpha = 0.05$).
a) Jonckheere-Terpstra Test
b) Welch-t-test with Bonferroni-Holm adjustment

III. CONCLUSION

In a 96-hour static algae test with *Pseudokirchneriella subcapitata,* the respective E_rC_{50} was determined to be 4.08 mg/L (geometric mean measured) after 72 hours of exposure.

Report:	CA 8.2.6.1/4 Rzodeczko H., 2016 a Reg.No. 5863469 (metabolite of BAS 750 F, M750F006) - Pseudokirchneriella subcapitata SAG 61.81 - Growth inhibition test 2015/1184815
Guidelines:	OECD 201 (2006)
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 72 hour static acute toxicity laboratory study, the effect of M750F006 (metabolite of BAS 750 F) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. Due to the poor solubility of the test item, a filtrate of the loading of 10 mg M750F006/L was used as the highest test item concentration, while the five lower test item concentrations consisted of 2.5-fold, 6.25-fold, 15.6-fold, 39.0-fold and 97.7-fold dilutions of the filtrate of the loading of 10 mg a.s./L. This approach resulted in geometric mean measured concentrations of 7.754, 3.159, 1.268, 0.485, 0.200 and 0.075 mg M750F006/L, respectively. Additionally, a dilution water control and a solvent control (N,N-dimethylformamide (DMF)) were set up. Assessment of growth was conducted 24, 48 and 72 h after test initiation.

The biological results are based on geometric mean measured concentrations. At exposure termination in the filtrate of a loading of 10 mg/L and its 2.5-fold, 6.25-fold, 15.6-fold dilutions all observed algae cells were deformed. In its 39.0-fold dilution 80% of the algae cells were deformed. In the lowest test item concentration and in the control groups no morphological effects were observed. No statistically significant effects were observed between the control groups (Student-t-test, $\alpha = 0.05$). At the end of the test, statistically significant effects compared to the solvent control occurred at all test item concentration for both growth rate and yield.

In a 72-hour static algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F006 was determined to be 1.424 mg/L, based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F006 (metabolite of BAS 750 F, Reg. no.: 5863469), batch no. L87-30, purity: 98.9 ± 1%.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata*, (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz), SAG 61.81; stock obtained from the "The Culture Collection of Algae", Göttingen University, Germany.

Test design: Static system; test duration 72 hours; 6 test concentrations, each with 3 replicates per treatment plus a control and a solvent control with 6 replicates, each; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 hours.

Test concentrations: Control, solvent control (0.1 mL DMF/L), 2.5-fold diluted filtrate, 6.25-fold diluted filtrate, 15.6-fold diluted filtrate, 39.0-fold diluted filtrate, 97.7-fold diluted filtrate and filtrate of the loading of 10 mg M750F006/L, corresponding to geometric mean measured concentrations of 3.159, 1.268, 0.485, 0.200, 0.075 and 7.754 mg a.s./L.

Test conditions: 250 mL Erlenmeyer glass flasks; test volume 100 mL; test medium: AAP medium; pH 7.20 - 7.24 at test initiation and pH 7.17 - 8.82 at test termination; temperature: 22.4 - 23.0 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at 6330 - 7080 lux; constant shaking.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics; Student-t-test for comparison of control and solvent control; probit analysis for determination of EC_x values, Williams Multiple Sequential t-test Procedure ($\alpha = 0.05$) for determination of the NOEC value ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were 7.676 mg/L in the filtrate of a loading of 10 mg/L, 3.144 mg/L in its 2.5-fold dilution, 1.264 mg/L in its 6.25-fold dilution, 0.477 mg/L in its 15.6-fold dilution, 0.201 mg/L in its 39.0-fold dilution and 0.086 mg/L in its 97.7-fold dilution. The geometric mean concentrations of the test item were 7.754, 3.159, 1.268, 0.485, 0.200 and 0.075 mg/L, respectively. The test item concentrations determined in samples collected at exposure termination were in the range of 76.74% to 103.35% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: At exposure termination in the filtrate of a loading of 10 mg/L and its 2.5-fold, 6.25-fold, 15.6-fold dilutions all observed algae cells were deformed. In its 39.0-fold dilution 80% of the algae cells were deformed. In the lowest test item concentration and in the control groups no morphological effects were observed. At the end of the test, statistically significant effects compared to the solvent control occurred at all test item concentration for both growth rate and yield (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$). The effects on algal growth are summarized in Table 8.2.6.1-4.

Table 8.2.6.1-4: Effect of M750F006 on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration [mg M750F006/L] (geometric mean measured)	**Control**	**Solvent control**	**0.075**	**0.200**	**0.485**	**1.268**	**3.159**	**7.754**
Inhibition in 72 h (growth rate) [%]	0.91	--	2.10 *	17.91 *	48.98 *	57.05 *	58.58 *	63.97 *
Inhibition in 72 h (yield) [%]	4.91	--	10.51 *	61.35 *	92.86 *	95.50 *	95.90 *	97.03 *
Endpoints [mg M750F006/L] (geometric mean measured)								
E_rC_{50} (72 h)	1.424 (95% confidence limits: 0.937 – 2.293)							
E_rC_{10} (72 h)	0.041 (95% confidence limits: 0.009 – 0.095)							
E_yC_{50} (72 h)	0.168 (95% confidence limits: 0.156 – 0.180)							

* Statistically significant differences compared to solvent control (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$).

III. CONCLUSION

In a 72-hour static algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F006 was determined to be 1.424 mg/L, based on geometric mean measured concentrations.

Report:	CA 8.2.6.1/5 Rzodeczko H., 2016 b Reg.No. 6003433 (metabolite of BAS 750 F, M750F005) - Pseudokirchneriella subcapitata SAG 61.81 - Growth inhibition test 2015/1184816
Guidelines:	OECD 201 (2006)
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 72 hour static acute toxicity laboratory study, the effect of M750F005 (metabolite of BAS 750 F) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. Due to the poor solubility of the test item, a filtrate of the loading of 10 mg M750F005/L was used as the highest test item concentration, while the four lower test item concentrations consisted of 2-fold, 4-fold, 8-fold and 16-fold dilutions of the filtrate of the loading of 10 mg a.s./L. This approach resulted in geometric mean measured concentrations of 8.572, 4.273, 2.121, 1.027 and 0.497 mg M750F005/L, respectively. Additionally, a dilution water control and a solvent control (N,N-dimethylformamide (DMF)) were set up. Assessment of growth was conducted 24, 48 and 72 h after test initiation.

The biological results are based on geometric mean measured concentrations. No morphological effects on algae were observed in the control and at all test item concentrations. At the end of the test, statistically significant effects compared to the control occurred at the highest test item concentration for both growth rate and yield.

In a 72-hour static algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F005 was determined to be > 8.572 mg/L, based on geometric mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: M750F005 (metabolite of BAS 750 F, Reg. no.: 6003433), batch no. L87-34, purity: 99.4 ± 1%.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata*, (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz), SAG 61.81; stock obtained from the "The Culture Collection of Algae", Göttingen University, Germany.

Test design: Static system; test duration 72 hours; 5 test concentrations, each with 3 replicates per treatment plus a control and a solvent control with 6 replicates each; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 hours.

Test concentrations: Control, solvent control (0.1 mL DMF/L), 2-fold diluted filtrate, 4-fold diluted filtrate, 8-fold diluted filtrate, 16-fold diluted filtrate and filtrate of the loading of 10 mg M750F005/L, corresponding to geometric mean measured concentrations of 4.273, 2.121, 1.027, 0. 497 and 8.572 mg a.s./L.

Test conditions: 250 mL Erlenmeyer glass flasks; test volume 100 mL; test medium: AAP medium; pH 7.06 - 7.23 at test initiation and pH 7.67 - 8.74 at test termination; temperature: 22.4 - 23.0 C; initial cell densities 1 x 10^4 cells/mL; continuous light at 6460 - 7160 lux; constant shaking.

Analytics: Analytical verification of test item concentrations was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics; Student-t-test for comparison of control and solvent control; probit analysis for determination of EC_x values, Williams Multiple Sequential t-test Procedure ($\alpha = 0.05$) for determination of the NOEC value ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were 8.538 mg/L in the filtrate of a loading of 10 mg/L, 4.322 mg/L in its 2-fold dilution, 2.190 mg/L in its 4-fold dilution, 1.080 mg/L in its 8-fold dilution and 0.540 mg/L in its 16-fold dilution. The geometric mean concentrations of the test item were 8.572, 4.273, 2.121, 1.027 and 0.497 mg/L, respectively. The test item concentrations determined in samples collected at exposure termination were in the range of 84.81% to 100.80% of initial concentrations. The following biological results are based on geometric mean measured concentrations.

Biological results: No morphological effects on algae were observed in the control and at all test item concentrations. No statistically significant effects were observed between the control groups (Student-t-test, $\alpha = 0.05$). At the end of the test, statistically significant effects compared to the solvent control occurred at the highest test item concentration for both growth rate and yield (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$). The effects on algal growth are summarized in Table 8.2.6.1-5.

Table 8.2.6.1-5: Effect of M750F005 on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration [mg M750F005/L] (geometric mean measured)	**Control**	**Solvent control**	**0.497**	**1.027**	**2.121**	**4.273**	**8.572**
Inhibition in 72 h (growth rate) [%] [1)]	0.91	--	0.72	- 0.85	2.40	0.58	3.76 *
Inhibition in 72 h (yield) [%] [1)]	4.91	--	3.77	- 4.96	11.71	3.14	18.10 *
	Endpoints [mg M750F005/L] (geometric mean measured)						
E_rC_{50} (72 h)	> 8.572						
E_rC_{10} (72 h)	> 8.572						
E_yC_{50} (72 h)	> 8.572						

* Statistically significant differences compared to solvent control (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$).
1) Inhibition compared to solvent control; negative values indicate stimulated growth.

III. CONCLUSION

In a 72-hour static algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} for M750F005 was determined to be > 8.572 mg/L, based on geometric mean measured concentrations.

CA 8.2.6.2 Effects on growth of an additional algal species

Report: CA 8.2.6.2/1
Bergfield A., 2015 a
BAS 750 F: Growth inhibition test with the marine diatom, Skeletonema costatum
2015/7000620
Guidelines: EPA 850.4500
GLP: yes
(certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

The effect of BAS 750 F on the growth of the marine diatom *Skeletonema costatum* was investigated in a 96-hour static laboratory study. The following nominal concentrations were applied: 0 (control), 0 (vehicle control), 0.063, 0.13, 0.25, 0.50 and 1.0 mg BAS 750 F/L, corresponding to initial measured concentrations of < MQL (minimum quantification level), < MQL, 0.0529, 0.111, 0.217, 0.434 and 0.876 mg a.s./L. Assessment of growth was conducted 24, 48, 72 and 96 h after test initiation.

The biological results are based on initial measured concentrations of the test item. After 72 h of exposure, statistically significant effects compared to the control were detected at the three highest test item concentrations for growth rate and yield. After 96 h of exposure, statistically significant effects compared to the control occurred at the two highest test item concentrations for growth rate and yield.

In a 96 hour static toxicity test with *Skeletonema costatum* the E_rC_{50} of BAS 750 F was determined to be 0.723 mg a.s./L (72 h) based on initial measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Marine diatom, *Skeletonema costatum*, stock originally obtained from the "UTEX - Culture Collection of Algae", University of Texas, Austin, USA.

Test design: Static system; test duration 96 hours; 5 test item concentrations plus a dilution water control and a vehicle control (dimethyl formamide (DMF)) with 4 replicates per concentration and each control group; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 and 96 hours.

Test concentrations: Control (dilution water), control (vehicle control (0.050 mL DMF/L), 0.063, 0.13, 0.25, 0.50 and 1.0 mg BAS 750 F/L, corresponding to initial measured concentrations of < MQL, < MQL, 0.0529, 0.111, 0.217, 0.434 and 0.876 mg BAS 750 F/L.

Test conditions: 250 mL Erlenmeyer flasks; test volume 50 mL; filtered saltwater algal medium: commercial salt mix (Marinemix, Wiegandt GmbH) added to autoclaved reagent water; pH 7.7 - 8.2; temperature: 20.5 - 20.9 °C; initial cell densities 1×10^4 cells/mL; photoperiod: 14 hours light :10 hours dark; light intensity: about 4497 - 4860 lux; flasks were swirled by hand once per day.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with MS/MS detection.

Statistics: Descriptive statistics; t-test for comparison of control and vehicle control results. Further statistical analyses were conducted using the blank control. Dunnett's test for determination of the NOEC values ($\alpha = 0.05$); nonlinear modeling procedure for calculation of EC_x values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were in the range of 84% and 88%. The test item concentrations determined in samples collected at exposure termination were in the range of 67% to 83% of initial concentrations. The biological results are based on initial measured concentrations.

Biological results: After 72 h of exposure, statistically significant effects compared to the control were detected at the three highest test item concentrations for growth rate and yield (Dunnett's test, p = 0.05). After 96 h of exposure, statistically significant effects compared to the control occurred at the two highest test item concentrations for growth rate and yield (Dunnett's test, p = 0.05). The effects on algal growth are summarized in Table 8.2.6.2-1.

Table 8.2.6.2-1: Effect of BAS 750 F on the growth of the marine diatom *Skeletonema costatum*

Concentration [mg a.s./L] (nominal)	**Control**	**Vehicle control**	**0.063**	**0.13**	**0.25**	**0.50**	**1.0**
Concentration [mg a.s./L] (initial measured)	**< MQL**	**< MQL**	**0.0529**	**0.111**	**0.217**	**0.434**	**0.876**
Inhibition in 72 h (growth rate) [%]	--	0	1	0	4 *	15 *	66 *
Inhibition in 72 h (yield) [%] 1)	--	0	3	-1	11 *	36 *	90 *
Inhibition in 96 h (growth rate) [%] 1)	--	0	-1	-1	2	19 *	66 *
Inhibition in 96 h (yield) [%] 1)	--	-1	-5	-3	6	54 *	94 *

	Endpoints [mg BAS 750 F/L] (initial measured)
E_rC_{50} (72 h)	0.723 (95% confidence limits: 0.696 - 0.750)
E_rC_{10} (72 h)	0.381 (95% confidence limits: 0.330 - 0.431)
E_yC_{50} (72 h)	0.502 (95% confidence limits: 0.488 - 0.516)
E_yC_{10} (72 h)	0.284 (95% confidence limits: 0.266- 0.301)
E_rC_{50} (96 h)	0.704 (95% confidence limits: 0.691 - 0.717)
E_rC_{10} (96 h)	0.336 (95% confidence limits: 0.315 - 0.357)
E_yC_{50} (96 h)	0.417 (95% confidence limits: 0.411 - 0.424)
E_yC_{10} (96 h)	0.234 (95% confidence limits: 0.221 - 0.246)

* Significant reduction in growth rate/ yield as compared to the control (Dunnett's test, $\alpha = 0.05$).
1) Inhibition compared to control; negative values indicate stimulated growth.

III. CONCLUSION

In a 96 hour static toxicity test with *Skeletonema costatum* the E_rC_{50} of BAS 750 F was determined to be 0.723 mg a.s./L (72 h) based on initial measured concentrations.

Report:	CA 8.2.6.2/2 Bergfield A., 2015 b BAS 750 F: Growth inhibition test with the freshwater diatom, Navicula pelliculosa 2015/7000618
Guidelines:	EPA 850.4500
GLP:	yes (certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 96 hour static acute toxicity laboratory study, the effect of BAS 750 F on the growth of the diatom *Navicula pelliculosa* was investigated. The following nominal concentrations were applied: 0 (control), 0 (vehicle control, dimethylformamide (DMF)), 0.22, 0.44, 0.88, 1.8, 3.5 and 7.0 mg BAS 750 F/L, corresponding to initial measured concentrations of < MQL (minimum quantification level), < MQL, 0.176, 0.358, 0.724, 1.47, 2.86 and 3.20 mg a.s./L. Assessment of growth was conducted 24, 48, 72 and 96 h after test initiation.

The biological results are based on initial measured concentrations. After 72 h and 96 h of exposure, statistically significant effects compared to the control were detected at test item concentrations ≥ 0.724 mg a.s./L for growth rate and yield, respectively.

In a 96-hour algae test with *Navicula pelliculosa,* the E_rC_{50} (72 h) for BAS 750 F was determined to be 1.57 mg a.s./L (initial measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Fresh water diatom, *Navicula pelliculosa*; stock obtained from the Department of Botany, Culture Collection of Algae, University of Texas at Austin (UTEX), USA.

Test design: Static system; test duration 96 hours; 6 test concentrations, each with 4 replicates per treatment plus a control and vehicle control with 6 replicates; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 hours and 96 hours.

Test concentrations:	Control, vehicle control (0.050 mL DMF/L), 0.22, 0.44, 0.88, 1.8, 3.5 and 7.0 mg BAS 750 F/L, corresponding to initial measured concentrations of < MQL, < MQL, 0.176, 0.358, 0.724, 1.47, 2.86 and 3.20 mg a.s./L.
Test conditions:	250 mL Erlenmeyer flasks; test volume 100 mL; test medium: freshwater algal nutrient medium plus silicate; pH 7.5 - 8.4; temperature: 22.4 - 24.1 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at 4329 - 4549 lux; constant shaking (100 rpm).
Analytics:	Analytical verification of test item concentrations was conducted using an LC-method with MS/MS detection.
Statistics:	Descriptive statistics; t-test for comparison of control and vehicle control results. Further statistical analyses were conducted using the blank control and nominal test substance treatments ≤ 3.5 mg a.s./L, because undissolved test material was observed in the highest test concentration. One-tailed Dunnett's test for determination of the NOEC values (α = 0.05); nonlinear modeling procedure for calculation of EC_x values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The test item concentrations determined in samples collected at exposure initiation were in the range of 80% and 82% and collected at test termination in the range of 49% and 64% (excluding highest test concentration). The measured test item concentration of the nominal test treatment 7.0 mg a.s./L was 46% and 19% of nominal concentration at test initiation and test termination, respectively. Due to poor initial recovery and undissolved test material in the solution, the nominal 7.0 mg a.s./L treatment level was not used for statistical analysis. The following biological results are based on initial measured concentrations.

Biological results: After 72 h and 96 h of exposure, statistically significant effects compared to the control were detected at test item concentrations ≥ 0.724 mg a.s./L for growth rate and yield, respectively (Dunnett's test, α = 0.05 in both cases). The effects on algal growth are summarized in Table 8.2.6.2-2.

Table 8.2.6.2-2: Effect of BAS 750 F on the growth of the diatom *Navicula pelliculosa*

Concentration [mg a.s./L] (nominal)	**Control**	**Vehicle control**	**0.22**	**0.44**	**0.88**	**1.8**	**3.5**	**7.0**
Concentration [mg a.s./L] (initial measured)	**< MQL**	**< MQL**	**0.176**	**0.358**	**0.724**	**1.47**	**2.86**	**3.20**
Inhibition in 72 h (growth rate) [%] [#]	--	0	0	-1	15 *	52 *	72 *	76 [$]
Inhibition in 72 h (yield) [%] [#]	--	-1	-2	-3	44 *	88 *	96 *	97 [$]
Inhibition in 96 h (growth rate) [%]	--	0	0	0	15 *	31 *	78 *	81 [$]
Inhibition in 96 h (yield) [%]	--	0	1	1	53 *	80 *	99 *	99 [$]

Endpoints [mg BAS 750 F/L] (initial measured)	
E_rC_{50} (72 h)	**1.57 (95% confidence limits: 1.45 - 1.68)**
E_rC_{10} (72 h)	0.445 (95% confidence limits: 0.342 - 0.548)
E_yC_{50} (72 h)	0.789 (95% confidence limits: 0.756 - 0.822)
E_yC_{10} (72 h)	0.390 (95% confidence limits: 0.350 - 0.429)
E_rC_{50} (96 h)	1.89 (95% confidence limits: 1.83 - 1.96)
E_rC_{10} (96 h)	0.910 (95% confidence limits: 0.833 - 0.987)
E_yC_{50} (96 h)	0.753 (95% confidence limits: 0.691 - 0.814)
E_yC_{10} (96 h)	0.301 (95% confidence limits: 0.239 - 0.363)

MQL = 0.0250 mg a.s./L

[#] Negative values indicate stimulated growth compared to the control.

* Statistically significant differences compared to the control (Dunnett's test, p = 0.05).

[$] The nominal 7.0 mg a.s./L treatment level was not used for statistical analysis due to poor initial recovery (46%) and undissolved test material in the solution.

III. CONCLUSION

In a 96-hour algae test with *Navicula pelliculosa,* the E_rC_{50} (72 h) for BAS 750 F was determined to be 1.57 mg a.s./L (initial measured).

Report: CA 8.2.6.2/3
Bergfield A., 2015 c
BAS 750 F: Growth inhibition test with the Cyanobacterium, Anabaena flos-aquae
2015/7000617
Guidelines: EPA 850.4500
GLP: yes
(certified by United States Environmental Protection Agency)

EXECUTIVE SUMMARY

In a 96-hour static acute toxicity laboratory study, the effect of BAS 750 F on the growth of the freshwater blue-green alga *Anabaena flos-aquae* was investigated. The following nominal concentrations were applied: Control, vehicle control (dimethylformamide (DMF)), 0.31, 0.63, 1.3, 2.5 and 5.0 mg BAS 750 F/L (corresponding to initial measured concentrations of < MQL (minimum quantification level), < MQL, 0.247, 0.507, 1.09, 2.04 and 3.20 mg a.s./L). Assessment of growth was conducted 24 h, 48 h, 72 h and 96 h after test initiation.

The biological results are based on initial measured concentrations. After 96 hours of exposure, yield was statistically significantly reduced at the highest test item concentration compared to the control.

In a 96-hour algae test with *Anabaena flos-aquae,* the E_rC_{50} (72 h) value for BAS 750 F was determined to be > 3.20 mg a.s./L based on initial measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: Freshwater blue-green alga, *Anabaena flos-aquae*; in-house cultures; stock obtained from University of Texas at Austin (UTEX), USA.

Test design: Static system; test duration 96 hours; 5 test item concentrations plus a control and a vehicle control (dimethyl formamide (DMF)), each with 4 replicates per treatment and control group; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 72 and 96 hours.

Test concentrations:	Control, vehicle control (0.050 mL DMF/L), 0.31, 0.63, 1.3, 2.5 and 5.0 mg a.s./L (nominal), corresponding to initial measured concentrations of < MQL, < MQL, 0.247, 0.507, 1.09, 2.04 and 3.20 mg a.s./L.
Test conditions:	250 mL Erlenmeyer flasks; test volume 100 mL; freshwater algal nutrient medium; pH 7.6 - 9.0; temperature: 22.2 °C - 25.4 °C; initial cell densities 1 x 10^4 cells/mL; continuous light; light intensity 2247 - 2307 lux; flasks were swirled daily by hand.
Analytics:	Analytical verification of test item concentrations was conducted using a LC-method with MS/MS detection.
Statistics:	Descriptive statistics; t-test for comparison of control groups. statistical analyses were conducted using the blank control. Dunnett´s test for determination of the NOEC values ($p < 0.05$); EC_{50} values were calculated using logistic (sigmoid-shaped) model fit.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The measured values of BAS 750 F ranged from 64% to 84% of nominal at test initiation and from 59% to 71% of nominal at test termination. The following biological results are based on initial measured concentrations.

Biological results: After 96 hours of exposure, yield was statistically significantly reduced at the highest test item concentration compared to the control (Dunnett's test, $p < 0.05$). The effects on algal growth are summarized in Table 8.2.6.2-3.

Table 8.2.6.2-3: Effect of BAS 750 F on the growth of the freshwater blue-green alga *Anabaena flos-aquae*

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.31**	**0.63**	**1.3**	**2.5**	**5.0**
Concentration [mg a.s./L] (initial measured)	**< MQL**	**< MQL**	**0.247**	**0.507**	**1.09**	**2.04**	**3.20**
Inhibition in 72 h (growth rate) [%] #	--	--	-1	0	1	1	0
Inhibition in 72 h (yield) [%] #	--	--	-2	0	4	4	0
Inhibition in 96 h (growth rate) [%] #	--	--	-2	-2	-2	1	1
Inhibition in 96 h (yield) [%] #	--	--	-7	-8	-7	2	5 *
	Endpoints [mg a.s./L] (initial measured)						
E_rC_{50} / E_yC_{50} (96 h & 72 h)	> 3.20						
E_rC_{10} / E_yC_{10} (96 h & 72 h)							

Negative values indicate stimulated growth compared to the control.
* Statistically significant differences compared to the control (Dunnett's test, $p < 0.05$).

III. CONCLUSION

In a 96-hour algae test with *Anabaena flos-aquae*, the E_rC_{50} (72 h) value for BAS 750 F was determined to be > 3.20 mg a.s./L based on initial measured concentrations.

CA 8.2.7 Effects on aquatic macrophytes

Report:	CA 8.2.7/1 Swirkot A., 2014a BAS 750 F (Reg. No. 5834378) - Lemna gibba CPCC 310 growth ihibition test 2014/1001322
Guidelines:	OECD 221 (2006), EPA 850.4400
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

EXECUTIVE SUMMARY

In a 7-day static toxicity laboratory study, the effect of BAS 750 F on the growth of the duckweed *Lemna gibba* was investigated. Due to poor solubility of the test item, a filtrate of the loading of 10 mg a.s./L was used as the highest test concentration while the four lower test item concentrations consisted of 16-fold, 8-fold, 4-fold and 2-fold dilutions of the filtrate of the loading of 10 mg a.s./L, respectively. This approach resulted in time-weighted mean concentrations of 0.119, 0.233, 0.485, 0.921 and 1.894 mg a.s./L. Additionally, a dilution water control was set up. Assessment of growth and other effects were conducted 3, 5 and 7 days after test initiation. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and final yield for the parameters frond number and dry weight.

The biological results are based on the time-weighted mean of the analytically determined concentrations of the test item. The duckweed population in the control vessels showed exponential growth, increasing from 12 fronds per vessel to an average of 131.9 fronds per vessel, corresponding to an 11 x multiplication. The dry weight increased to an average of 22.4 mg per vessel in the control at test termination. No morphological effects on algae were observed up to and including the highest test item concentration. No statistically significant differences compared to the control were observed at any test item concentration for all measured parameters.

In a 7-day aquatic plant test with *Lemna gibba,* the E_rC_{50} for BAS 750 F was determined to be > 1.894 mg a.s./L based on both frond number and dry weight (time-weighted mean).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. no.: 5834378), batch no. COD-001740, purity: 98.8% ± 1.0%.

B. STUDY DESIGN

Test species: Duckweed (*Lemna gibba* G3), specification CPCC 310, inocula from 7 days old cultures; cultures maintained in-house; stock obtained from "Canadian Phycological Culture Centre (CPCC)", Department of Biology, University of Waterloo, Canada.

Test design: Static system (7 days); 6 treatment groups (5 test item concentrations plus control) with 4 replicates for the test item treatments and 8 replicates for the control; 3 plants with 4 fronds, total number of fronds at test initiation: 12 per replicate; assessment of growth and other effects on days 3, 5 and 7.
Due to poor solubility of the test item, a filtrate of the loading of 10 mg a.s./L was used as the highest test concentration.

Endpoints: EC_{10} and EC_{50} with respect to growth rate and yield after exposure over 7 days.

Test concentrations: Control, 16-fold diluted filtrate, 8-fold diluted filtrate, 4-fold diluted filtrate, 2-fold diluted filtrate and filtrate of the loading of 10 mg BAS 750 F/L, corresponding to time-weighted mean measured concentrations of < LoD (limit of detection), 0.119, 0.233, 0.485, 0.921 and 1.894 mg a.s./L.

Test conditions: Glass beakers, test volume 400 mL, 20 x-AAP nutrient medium, pH 7.58 - 7.79 at test initiation and pH 9.40 - 9.46 at test termination; temperature: 24.1 - 24.6 °C, continuous light, light intensity: 6510 - 6700 lux.

Analytics: Analytical verification of the test item was conducted using an LC-method with DAD detection.

Statistics: Descriptive statistics, probit analysis for determination of the EC_x values, Williams Multiple Sequential t-test Procedure ($\alpha = 0.05$) for determination of the NOEC values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each test concentration at the beginning and at the end of the test. The concentrations of the test item determined in samples collected at exposure initiation were: Control (< LoD), 0.132, 0.259, 0.541, 0.998 and 2.017 mg a.s./L. The test item concentrations determined in samples collected at exposure termination were in the range of 80.04% to 88.10% of initial concentrations. This confirms that the test item concentrations were stable under test conditions. The following biological results are based on the time-weighted mean of the analytically determined concentrations of the test item.

Biological results: The duckweed population in the control vessels showed exponential growth, increasing from 12 fronds per vessel to an average of 131.9 fronds per vessel, corresponding to an 11 x multiplication. The dry weight increased to an average of 22.4 mg per vessel in the control at test termination. No morphological effects on algae were observed up to and including the highest test item concentration. No statistically significant differences compared to the control were observed at any test item concentration for all measured parameters (Williams Multiple Sequential t-test Procedure, $\alpha = 0.05$). Effects on growth rate and yield are summarized in Table 8.2.7-1.

Table 8.2.7-1: Effect of BAS 750 F on the growth of duckweed *Lemna gibba*

Concentration [mg a.s./L] (time-weighted mean)	**Control**	**0.119**	**0.233**	**0.485**	**0.921**	**1.894**
Inhibition after 7 d [%] [#] (growth rate based on frond no.)	0.0	0.1	-1.7	-4.5	-1.0	0.3
Inhibition after 7 d [%] [#] (growth rate based on dry weight)	0.0	0.0	-2.8	-8.5	-1.3	-0.9
Inhibition after 7 d [%] [#] (yield based on frond no.)	0.0	0.3	-4.5	-12.4	-2.6	0.7
Inhibition after 7 d [%] [#] (yield based on dry weight)	0.0	0.0	-7.5	-23.0	-3.5	-2.0
Endpoints [mg BAS 750 F/L] (time-weighted mean)						
E_rC_{50} (7 d) based on frond no.	**> 1.894**					
E_rC_{10} (7 d) based on frond no	> 1.894					
E_yC_{50} (7 d) based on frond no	> 1.894					
E_yC_{10} (7 d) based on frond no	> 1.894					
E_rC_{50} (7 d) based on dry weight	**> 1.894**					
E_rC_{10} (7 d) based on dry weight	> 1.894					
E_yC_{50} (7 d) based on dry weight	> 1.894					
E_yC_{10} (7 d) based on dry weight	> 1.894					
NOEC overall	≥ 1.894					

[#] Negative values indicate stimulated growth compared to the control.

III. CONCLUSION

In a 7-day aquatic plant test with *Lemna gibba,* the E_rC_{50} for BAS 750 F was determined to be > 1.894 mg a.s./L based on both frond number and dry weight (time-weighted mean).

CA 8.2.8 Further testing on aquatic organisms

According to Regulation 283/2013, no further studies are required.

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CA 8.3 Effects on arthropods

CA 8.3.1 Effects on bees

For application for the approval of the active substance BAS 750 F, studies on honeybees have been performed with the active substance, which are considered in the honeybee risk assessment. Summaries of these studies are provided below and an overview on studies and endpoints is given in Table 8.3.1-1.

Table 8.3.1-1: List of studies and endpoints with honeybees and the active substance BAS 750 F

Substance	Test species	Endpoint	Value	Reference (BASF DocID)
BAS 750 F	honeybee	48 h acute oral LD_{50}	> 100 µg a.s./bee	2015/1128674
		48 h acute contact LD_{50}	> 100.0 µg a.s./bee	
		10 d chronic LD_{50}	> 110.5 µg a.s./bee/day	2013/1235086
		10 d chronic LC_{50}	> 2.562 g a.s./kg food	
		10 d chronic NOED	≥ 110.5 µg a.s./bee/day	
		10 d chronic NOEC	≥ 2.562 g a.s./kg food	
	honeybee larvae	8 d LD_{50}	43.9 µg a.s./larva	2013/1235087
		8 d LC_{50}	1.295 g a.s./kg food	
		8 d NOED	29.7 µg a.s./larva	
		8 d NOEC	0.875 g a.s./kg food	
	honeybee larvae	21 d ED_{50}	> 50.1 µg a.s./larva	2014/1327676
		21 d LC_{50}	> 325 mg a.s./kg food	
		21 d NOED	≥ 50.1 µg a.s./larva	
		21 d NOEC	≥ 325 mg a.s./kg food	

Substance	Test species	Endpoint	Value	Reference (BASF DocID)
	bumblebee	96 h acute oral LD_{50}	> 195.4 µg a.s./bee	2014/1275250
		96 h acute contact LD_{50}	> 200.0 µg a.s./bee	

CA 8.3.1.1 Acute toxicity to bees

CA 8.3.1.1.1 Acute oral toxicity

Report: CA 8.3.1.1.1/1
Franke M., 2015a
Acute toxicity of BAS 750 F to the honeybee Apis mellifera L. under laboratory conditions
2015/1128674
Guidelines: OECD 213 (1998), OECD 214 (1998)
GLP: yes
(certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an oral toxicity test, honeybees (young adult worker bees of *Apis mellifera* L.) were exposed to BAS 750 F (Reg. No. 5 834 378). The toxicity of the test item was determined at nominal dose rates of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee (based on analyzed purity); resulting in actual uptake of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee. Additionally, honeybees were treated with Dimethoate EC 400 as a reference item at dose rates ranging from 0.069 to 0.250 µg dimethoate/bee and with 50% (w/v) sucrose solution and 50% (w/v) sucrose solution containing 1% (v/v) acetone and 1% (v/v) Tween®80 as controls.

After 48 hours, no mortality occurred in the control groups fed with either pure sucrose solution or sucrose solution containing 1% acetone and 1% tween. In the test item treatment, no mortality occurred after oral consumption of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee, after 48 hours. No test item induced behavioral effects were observed.

In an acute oral toxicity study with BAS 750 F on honeybees, the LD_{50} value (48 h) was determined to be > 100 µg a.s./bee.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: *Apis mellifera* L. Buckfast (honeybee), young adult worker bees (about 3 - 5 weeks old) derived from a healthy and queen-right colony, source: BioChem agrar GmbH, Gerichshain, Germany; collected from the top of the bee hive in the morning prior to use.

Test design: In a 48 hour test, young adult worker bees of *Apis mellifera* L. were exposed orally to BAS 750 F via food (50% (w/v) aqueous sucrose solution). In total, 3 treatment groups were set up (5 dose rates of the test item, 2 untreated control groups and 4 dose rates of the reference item) with 3 replicates per treatment and 10 bees per replicate. Assessment of bee mortality and behavioral effects were done after 4, 24 and 48 hours.

Endpoints: Mortality (LD_{50}), behavioral impairments.

Reference item: Dimethoate EC 400 (dimethoate, 400 g/L nominal).

Test doses: BAS 750 F: 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee, resulting in an actual uptake of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee.
Control groups: 50% (w/v) sucrose solution, 50% (w/v) sucrose solution containing 1.0% (v/v) acetone and 1.0% (v/v) Tween®80.
Reference item: 0.069, 0.106, 0.163 and 0.250 µg dimethoate/bee.

Test conditions: Temperature: 24.0°C – 26.5°C; relative humidity: 45% - 67%; photoperiod: 24 h darkness; food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics; Multiple sequentially-rejective Fisher Test after Bonferroni-Holm for mortality data (one-sided greater, $\alpha = 0.05$); Probit maximum likelihood regression for calculation of the LD_{50} value of the reference item.

II. RESULTS AND DISCUSSION

After 48 hours, no mortality occurred in the control groups fed with either pure sucrose solution or sucrose solution containing 1 % acetone and 1 % tween.
In the test item treatment, no mortality occurred after oral consumption of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee, after 48 hours. No test item induced behavioral effects were observed. The results are summarized in Table 8.3.1.1.1-1.

Table 8.3.1.1.1-1: Toxicity of BAS 750 F to *Apis mellifera* L. (honeybee) in an oral toxicity test

Treatment	Dosage [consumed]	Mortality [%]		
		4 h	24 h	48 h
Control	Sucrose solution	0.0	0.0	0.0
	Acetone-Tween sucrose solution	0.0	0.0	0.0
BAS 750 F [µg a.s./bee]	6.2	0.0	0.0	0.0
	12.5	0.0	0.0	0.0
	25.0	0.0	0.0	0.0
	50.0	0.0	0.0	0.0
	100.0	0.0	0.0	0.0
Endpoint [µg consumed a.s./bee]				
LD_{50} (48 h)	> 100.0			

The LD_{50} value (24 h) for the reference item was determined to be 0.171 µg dimethoate/bee (95% confidence limits: 0.154 - 0.189 µg dimethoate/bee), based on consumption.

III. CONCLUSION

In an acute oral toxicity study with BAS 750 F on honeybees, the LD_{50} value (48 h) was determined to be > 100 µg a.s./bee.

Report:	CA 8.3.1.1.1/2 Amsel K., 2015a Acute toxicity of BAS 750 F to the bumblebee Bombus terrestris L. under laboratory conditions 2014/1275250
Guidelines:	OECD 213 (1998), OECD 214 (1998), Hanewald et al. (2013), Van der Steen (1996), Van der Steen (2001)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an acute oral toxicity test, young adult worker bumblebees (*Bombus terrestris*) were exposed to BAS 750 F. The toxicity of the test item was determined at analyzed doses of 12.5, 25.0, 50.0, 100.0 and 200.0 µg BAS 750 F/bumblebee, resulting in an actual uptake of 12.2, 24.7, 48.3, 97.7 and 195.4 µg BAS 750 F/bumblebee. Additionally, bumblebees were treated with Dimethoate EC 400 as a reference item at dose rates ranging from 0.25 to 1.50 µg dimethoate/bumblebee (nominal), or with a 50 % (w/v) sucrose solution or sucrose solution including 1 % acetone as controls.

After 96 hours of oral exposure, no mortality occurred in the control group fed with 50% (w/v) sucrose solution or with the sucrose solution including 1% acetone. In the test item treatment, no mortality occurred after oral consumption of 12.2, 24.7, 48.3, 97.7 and 195.4 µg a.s./bumblebee. No behavioral effects of surviving bumblebees occurred in all tested dose rates in the oral toxicity test when compared to the control.

In an acute oral toxicity study with BAS 750 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 195.4 µg consumed BAS 750 F/bumblebee (equivalent to > 200.0 µg BAS 750 F/bumblebee (nominal)).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378); batch no.: COD-001740; analyzed purity: 98.8%.

B. STUDY DESIGN

Test species: *Bombus terrestris* L. (bumblebee), young adult worker bumblebees derived from healthy and queen-right hives; source: Biobest Belgium N.V., Westerlo, Belgium; collected on the morning prior to use.

Test design: In a 96-hour test, adults of *Bombus terrestris* were exposed to 5 doses of BAS 750 F in treated food (50% (w/v) sucrose solution including 1% acetone). In total, 4 treatment groups were set up: 5 dose rates of the test item, 2 control groups and 4 dose rates of the reference item with 30 replicates per dose and 1 bumblebee per replicate, respectively. Assessments of bumblebee mortality and behavioral effects were done after 4, 24, 48, 72 and 96 hours.

Endpoints: Mortality, behavioral impairments.

Reference item: BAS 152 11 I (dimethoate, nominal 400.0 g/L).

Test doses: Sucrose control (50% (w/v) sucrose solution), sucrose control (50% (w/v) sucrose solution including 1% acetone); reference item at dose rates of 0.25, 0.45, 0.82 and 1.50 µg dimethoate/bumblebee; test item at dose rates of 12.5, 25.0, 50.0, 100.0 and 200.0 µg BAS 750 F/bumblebee (resulting in an actual uptake of 12.2, 24.7, 48.3, 97.7 and 195.4 µg BAS 750 F/bumblebee).

Test conditions: Temperature: 24.8 °C – 25.2 °C, relative humidity: 59.2% – 61.0%, photoperiod: 24 h darkness; food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics; Fisher's Exact Binominal Test with Bonferroni Correction for mortality data (one-sided greater, $\alpha = 0.05$). Logit analysis using linear weight regression for calculation of the LD_{50} values for the reference item.

II. RESULTS AND DISCUSSION

After 96 hours of oral exposure, no mortality occurred in the control group fed with 50% (w/v) sucrose solution and with sucrose solution including 1% acetone. In the test item treatment, no mortality occurred after oral consumption of 12.2, 24.7, 48.3, 97.7 and 195.4 µg a.s./bumblebee. No behavioral effects of surviving bumblebees occurred in all tested dose rates in the oral toxicity test when compared to the control. The results are summarized in Table 8.3.1.1.1-2.

Table 8.3.1.1.1-2: Toxicity of BAS 750 F to *Bombus terrestris* (bumblebee) in an oral toxicity test

Treatment	Dosage	Mortality [%]			
		24 h	48 h	72 h	96 h
Control	Sucrose	0.0	0.0	0.0	0.0
	Sucrose + 1% acetone	0.0	0.0	0.0	0.0
BAS 750 F [µg a.s./bumblebee]	12.2	0.0	0.0	0.0	0.0
	24.7	0.0	0.0	0.0	0.0
	48.3	0.0	0.0	0.0	0.0
	97.7	0.0	0.0	0.0	0.0
	195.4	0.0	0.0	0.0	0.0
Endpoint [µg a.s./bumblebee]					
LD_{50} (96 h)	> 195.4				

III. CONCLUSION

In an acute oral toxicity study with BAS 750 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 195.4 µg consumed BAS 750 F/bumblebee (equivalent to > 200.0 µg BAS 750 F/bumblebee (nominal)).

CA 8.3.1.1.2 Acute contact toxicity

Report:	CA 8.3.1.1.2/1 Franke M., 2015b Acute toxicity of BAS 750 F to the honeybee Apis mellifera L. under laboratory conditions 2015/1128674
Guidelines:	OECD 213 (1998), OECD 214 (1998)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an acute contact toxicity test, young adult honeybees (worker bees of *Apis mellifera* L.) were exposed to BAS 750 F (Reg. No. 5 834 378). The toxicity of the test item was determined at nominal dose rates of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee (based on analyzed purity). Additionally, honeybees were treated with Dimethoate EC 400 as a reference item at dose rates ranging from 0.106 to 0.250 µg dimethoate/bee, and furthermore with deionized water, tween solution (deionized water + wetting agent) and pure acetone as controls.
After 48 hours of contact exposure, no mortality occurred in the control groups treated with either deionized water, tween solution or acetone. In the test item treatment, no mortality occurred after thoracic application of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee, after 48 hours. No test item induced behavioral effects were observed.

In an acute contact toxicity study with BAS 750 F on honeybees, the LD_{50} value (48 h) was determined to be > 100.0 µg a.s./bee.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8%.

B. STUDY DESIGN

Test species: *Apis mellifera* L. Buckfast (honeybee), young adult worker bees (about 3 – 5 weeks old) derived from a healthy and queen-right colony, source: BioChem agrar GmbH, Gerichshain, Germany; collected from the top of the bee hive in the morning of use.

Test design: In a 48 hour test, young adult worker bees of *Apis mellifera* L. were exposed to 5 dose rates of BAS 750 F in an appropriate carrier (pure acetone) placed on the dorsal bee thorax. In total, 3 treatment groups were set up (5 dose rates of the test item, 3 untreated control groups and 4 dose rates of the reference item) with 3 replicates per treatment and 10 bees per replicate. Assessment of bee mortality and behavioral effects were done after 4, 24 and 48 hours.

Endpoints: Mortality (LD_{50}), behavioral impairments.

Reference item: Dimethoate EC 400 (dimethoate, 400 g/L nominal).

Test doses: BAS 750 F: 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee.
Control groups: water control (deionized water), Tween control (deionized water + 1.0% v/v wetting agent (Tween®80)) and acetone control.
Reference item: 0.106, 0.141, 0.188 and 0.250 µg dimethoate/bee.

Test conditions: Temperature: 24.0°C – 26.5°C; relative humidity: 45% - 67%; photoperiod: 24 h darkness; food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics; Multiple sequentially-rejective Fisher Test after Bonferroni-Holm for mortality data (one-sided greater, $\alpha = 0.05$); Probit maximum likelihood regression for calculation of the LD_{50} value of the reference item.

II. RESULTS AND DISCUSSION

After 48 hours of contact exposure, no mortality occurred in the control groups treated with either deionized water, tween solution or acetone.
In the test item treatment, no mortality occurred after thoracic consumption of 6.2, 12.5, 25.0, 50.0 and 100.0 µg a.s./bee, after 48 hours. No test item induced behavioral effects were observed. The results are summarized in Table 8.3.1.1.2-1.

Table 8.3.1.1.2-1: Toxicity of BAS 750 F to *Apis mellifera* L. (honeybee) in a contact toxicity test

Treatment	Dosage [applied]	Mortality [%]		
		4 h	24 h	48 h
Control	Water	0.0	0.0	0.0
	Tween	0.0	0.0	0.0
	Acetone	0.0	0.0	0.0
BAS 750 F [µg a.s./bee]	6.2	0.0	0.0	0.0
	12.5	0.0	0.0	0.0
	25.0	0.0	0.0	0.0
	50.0	0.0	0.0	0.0
	100.0	0.0	0.0	0.0
Endpoint [µg a.s./bee]				
LD_{50} (48 h)	> 100.0			

The LD_{50} value (24 h) for the reference item was determined to be 0.195 µg dimethoate/bee (95% confidence limits: 0.180- 0.212 µg dimethoate/bee) in the contact toxicity test.

III. CONCLUSION

In an acute contact toxicity study with BAS 750 F on honeybees the LD_{50} value (48 h) was determined to be > 100.0 µg a.s./bee.

Report:	CA 8.3.1.1.2/2 Amsel K., 2015a Acute toxicity of BAS 750 F to the bumblebee Bombus terrestris L. under laboratory conditions 2014/1275250
Guidelines:	OECD 213 (1998), OECD 214 (1998), Hanewald et al. (2013), Van der Steen (1996), Van der Steen (2001)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an acute contact toxicity test, bumblebees (young adult worker bumblebees (*Bombus terrestris*) were exposed to BAS 750 F. The toxicity of the test item was determined at analyzed doses of 12.5, 25.0, 50.0, 100.0 and 200.0 µg BAS 750 F/bumblebee. Additionally, bumblebees were treated with Dimethoate EC 400 as a reference item at dose rates ranging from 2.5 to 10.0 µg dimethoate/bumblebee (nominal) or with deionized water, TritonX solution, and acetone controls.

After 96 hours of contact exposure, no mortality occurred in the control groups treated neither with deionized water, TritonX solution nor with acetone. In the test item treatment, no statistical significant mortality occurred after thoracic application of 200.0, 100.0, 50.0, 25.0 and 12.5 µg BAS 750 F/bumblebee, after 96 hours. The dose rate of 50.0 µg BAS 750 F/bumblebee revealed a slight mortality of 3.3%, which is not statistically significant when compared with the acetone control. Furthermore, no behavioral abnormalities of surviving bumblebees occurred throughout the contact toxicity test.

In an acute contact toxicity study with BAS 750 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 200.0 µg BAS 750 F/bumblebee.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378); batch no.: COD-001740; analyzed purity: 98.8%.

B. STUDY DESIGN

Test species: *Bombus terrestris* L. (bumblebee), young adult worker bumblebees derived from healthy and queen-right hives; source: Biobest Belgium N.V., Westerlo, Belgium; collected on the morning prior to use.

Test design: In a 96-hour test, adults of *Bombus terrestris* were exposed to 5 doses of BAS 750 F in an appropriate carrier (acetone) placed on the dorsal bumblebee thorax. In total, 3 treatment groups were set up: 5 dose rates of the test item, 3 control groups and 4 dose rates of the reference item with 30 replicates per dose and 1 bumblebee per replicate, respectively. Assessments of bumblebee mortality and behavioral effects were done after 4, 24, 48, 72 and 96 hours.

Endpoints: Mortality, behavioral impairments.

Reference item: BAS 152 11 I (dimethoate, nominal 400.0 g/L).

Test doses: Water control (deionized water), TritonX control (1% (v/v) TritonX solution), acetone control (pure acetone); reference item at dose rates of 2.5, 4.0, 6.3 and 10.0 µg dimethoate/bumblebee; test item at dose rates of 12.5, 25.0, 50.0, 100.0 and 200.0 µg BAS 750 F/bumblebee.

Test conditions: Temperature: 24.6 °C – 25.2 °C, relative humidity: 58.8% – 60.9%, photoperiod: 24 h darkness; food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics. Fisher's Exact Binominal Test with Bonferroni Correction for mortality data (one-sided greater, $\alpha = 0.05$). Logit analysis using linear weight regression for calculation of the LD_{50} values for the reference item.

II. RESULTS AND DISCUSSION

After 96 hours of contact exposure, no mortality occurred in the control groups treated neither with deionized water, TritonX solution nor with acetone. In the test item treatment, no statistical significant mortality occurred after thoracic application of 12.5, 25.0, 50.0, 100.0 and 200.0 µg BAS 750 F/bumblebee, after 96 hours. The dose rate of 50.0 µg BAS 750 F/bumblebee revealed a slight mortality of 3.3%, which is not statistically significant when compared with the acetone control. Furthermore, no behavioral abnormalities of surviving bumblebees occurred throughout the contact toxicity test. The results are summarized in Table 8.3.1.1.2-2.

Table 8.3.1.1.2-2: Toxicity of BAS 750 F to *Bombus terrestris* (bumblebee) in a contact toxicity test

Treatment	Dosage	Mortality [%]			
		24 h	48 h	72 h	96 h
Control	Water control	0.0	0.0	0.0	0.0
	1% TritonX *	0.0	0.0	0.0	0.0
	Acetone	0.0	0.0	0.0	0.0
BAS 750 F [µg a.s./bumblebee]	12.5	0.0	0.0	0.0	0.0
	25.0	0.0	0.0	0.0	0.0
	50.0	3.3	3.3	3.3	3.3
	100.0	0.0	0.0	0.0	0.0
	200.0	0.0	0.0	0.0	0.0
Endpoint [µg a.s./bumblebee]					
LD_{50} (96 h)	> 200.0				

* 1% TritonX control belongs to reference item.

III. CONCLUSION

In an acute contact toxicity study with BAS 750 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 200.0 µg BAS 750 F/bumblebee.

CA 8.3.1.2 Chronic toxicity to bees

Report:	CA 8.3.1.2/1 Kleebaum K., 2015a Chronic toxicity of BAS 750 F (Reg.No. 5834378) to the honeybee Apis mellifera L. under laboratory conditions 2013/1235086
Guidelines:	Decourty et al. (2005), Suchail et al. (2001), CEB No. 230 (2012), Current ring test protocol of the AG-Bienenschutz (2014)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In a 10-day chronic oral toxicity test, 1 - 4 day old worker honeybees (*Apis mellifera*) were exposed to a daily application of BAS 750 F diluted in the bee food (50% (w/v) aqueous sucrose solution). The chronic toxicity of the test item was determined at nominal doses of 6.2, 12.5, 24.9, 49.9 and 99.8 µg a.s./bee/day (effective doses were 8.3, 13.3, 26.9, 48.2 and 110.5 µg a.s./bee/day), corresponding to concentrations of 0.160, 0.320, 0.641, 1.281 and 2.562 g a.s./kg food, respectively. Additionally, honeybees were treated with Dimethoate EC 400 as a reference item at nominal doses ranging from 5.9 to 27.3 ng a.s./bee/day. Untreated diet served as a control, and untreated diet with 1% v/v Tween20 served as a detergent control.

After 10 days of continuous exposure, a mean mortality of 1.7% in the detergent control and 1.7% in the control were observed. In the test item group mortalities between 0.0 and 8.3% occurred, which were not statistically significant increased compared to the control groups.

During the testing period no behavioral abnormalities could be observed in any test item group.

In a 10 day chronic toxicity feeding test with BAS 750 F (Reg. No. 5 834 378) the NOED was determined to be ≥ 110.5 µg consumed a.s./bee/day, and the NOEC ≥ 2.562 g a.s./kg food, respectively. The LD_{50} and LC_{50} were determined to be > 110.5 µg consumed a.s./bee/day and > 2.562 g a.s./kg food.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378); batch no.: COD-001740; analyzed purity: 98.8% (tolerance ± 1.0%).

B. STUDY DESIGN

Test species: *Apis mellifera iberica* L. (honeybee); 1 - 4 day old bees; derived from a healthy and queen-right colony; source: Beekeeper Joaquin Cordero, Cazalla, Spain.

Test design: In a 10-day test, young adults of *Apis mellifera* L. were exposed daily to 5 doses of BAS 750 F in treated food (50% w/v aqueous sucrose solution). In total, 4 treatment groups were set up: 5 doses of the test item, 2 untreated controls and 4 doses of the reference item with 3 replicates per dose and 20 bees per replicate. Assessments of bee mortality and behavioral effects were done daily during the study.

Endpoints: Mortality, behavioral impairments.

Reference item: Dimethoate 400 EC (analyzed content of a.s.: 400.9 g/L).

Test doses: Control 1: untreated diet (50% (w/v) aqueous sucrose solution)
Control 2: untreated diet (50% (w/v) aqueous sucrose solution with 1% Tween20)

Test item treatments:

Nominal dose/concentration	
Doses [µg a.s./bee]	**Concentrations [g a.s./kg food]**
6.2	0.160
12.5	0.320
24.9	0.641
49.9	1.281
99.8	2.562

Reference item treatments: 1.1, 2.2, 4.4 and 8.8 µg dimethoate/bee/day.

Test conditions: Temperature: 33.3° C - 35.0° C ; relative humidity: 46% - 60%, photoperiod: 24 h darkness; food: 50% (w/v) aqueous sucrose solution.

Statistics: Descriptive statistics; for mortality data Fisher's Exact Binomial Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$). The median lethal doses/concentrations of test and reference item were calculated with Probit analysis using linear maximum likelihood regression.

II. RESULTS AND DISCUSSION

After 10 days of continuous exposure, a mean mortality of 1.7% in the detergent control and 1.7% in the control were observed. In the test item group mortalities between 0.0 and 8.3% occurred, which were not statistically significantly increased compared to the control groups (Fishers Exact Binomial Test with Bonferroni Correction, one-sided greater, $\alpha = 0.05$). As no effects were observed, all LC_X (LD_X) values are greater than the highest test concentration (dose).

During the testing period no behavioral abnormalities could be observed in any test item group. The results are summarized in Table 8.3.1.2-1.

Table 8.3.1.2-1: Cumulative mortality and toxicity endpoints of honeybees (*Apis mellifera* L.) exposed to BAS 750 F in a chronic oral toxicity test

Treatment [BAS 750 F]			Mortality after 10 days	
Actual daily mean doses [µg consumed a.s./bee/day]	**Overall doses [µg a.s./bee/day]**	**Concentration [g a.s./kg food]**	**Cumulative mortality [%]**	**Corrected cumulative mortality [%]**
Control	Control	Control	1.7	--
Tween control	Tween control	Tween control	1.7	--
8.3	6.2	0.160	6.7	5.1
13.3	12.5	0.320	8.3	6.7
26.9	24.9	0.641	1.7	0.0
48.2	49.9	1.281	3.3	1.6
110.5	99.8	2.562	0.0	0.0

Endpoints		10 days
Test item doses [µg consumed a.s./bee/day]	**LD_{50}**	> 110.5
	NOED [1)]	≥ 110.5
Test item concentrations [g a.s./kg food]	**LC_{50}**	> 2.562
	NOEC [1)]	≥ 2.562

1) Fisher's Exact Binominal Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$).

In the reference item treatment, the LD_{50} was determined to be 12.7 ng consumed dimethoate/bee/day, which corresponds to an LC_{50} of 0.423 mg dimethoate/kg food.

III. CONCLUSION

In a 10 day chronic toxicity feeding test with BAS 750 F (Reg. No. 5 834 378) the NOED was determined to be ≥ 110.5 µg consumed a.s./bee/day, and the NOEC ≥ 2.562 g a.s./kg food, respectively. The LD_{50} and LC_{50} were determined to be > 110.5 µg consumed a.s./bee/day and > 2.562 g a.s./kg food.

CA 8.3.1.3 Effects on honeybee development and other honeybee life stages

Report:	CA 8.3.1.3/1 Kleebaum K., 2015b Acute toxicity of BAS 750 F to honeybee larvae (Apis mellifera L.) under laboratory conditions (in vitro) 2013/1235087
Guidelines:	OECD 237 (2013) Honey bee (Apis mellifera) larval toxicity test single exposure
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an acute oral larval toxicity test, four day old honeybee larvae (*Apis mellifera*) were exposed to one application of BAS 750 F diluted in the larvae food. The toxicity of the test item was determined with concentrations of 7.4, 14.8, 29.7, 59.3 and 118.7 µg a.s./larva, corresponding to 0.2, 0.4, 0.9, 1.8 and 3.5 g a.s./kg food). Additionally, honeybee larvae were treated with dimethoate as the reference item. Untreated diet served as a control, in addition to a solvent control with Tween20 and acetone (each 1% v/v).

Control mortality was 2.8% and 13.9% after 72 hours and 96 hours of exposure, respectively (corresponding to a test duration of 7 and 8 days) was seen. The solvent control showed a mortality of 13.9% after 72 h which did not increase at 96 h of exposure. After 72 hours, larvae fed with 59.3 and 118.7 µg a.s./larva revealed a mortality of 91.7% and 83.3%, respectively, which was statistically significant in comparison to the solvent control group. The increase in mortality compared to the solvent control was statistically significant after 96 hours in this test item groups as well (97.2% and 94.4% in the 59.3 and 118.7 µg a.s./larva treatment groups, respectively).

After 72 h of exposure (7 d test duration), deviations from the normal food consuming behavior occurred in 11.1%, 18.8%, 100.0% and 100.0% of the remaining individuals treated with 14.8, 29.7, 59.3 and 118.7 µg a.s./larva. After 96 hours of exposure (8 d test duration), deviations to the normal food consuming behavior and correspondingly, to developing into an average sized larva were present in 3.3, 4.2, 100.0 and 50.0% of the remaining larvae, which were treated with 14.8, 29.7, 59.3 and 118.7 µg a.s., respectively.

In an acute oral larval toxicity study with BAS 750 F on honeybee larvae, the LD_{50} value (96 h exposure or 8 d test duration) was determined to be 43.9 µg a.s./larva (equivalent to LC_{50} = 1.295 g a.s./kg food). The NOED was determined to be 29.7 µg a.s./larva (equivalent to NOEC = 0.875 g a.s./kg food).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378); batch no.: COD-001740; analyzed purity: 98.8% (tolerance ± 1.0%).

B. STUDY DESIGN

Test species: *Apis mellifera* L. subspecies *carnica* P. (honeybee); synchronized first instar larvae (one day old); derived from three healthy and queen-right colonies; source: Bienenfarm Kern GmbH, Leipzig, Germany.

Test design: One day old honeybee larvae of *Apis mellifera* were transferred from brood combs to polystyrene grafting cells in 48-well cell culture plates 3 days before start of the treatment. After the 4 day old larvae were exposed to a single application of BAS 750 F diluted in the larvae food (aqueous sugar solution mixed with royal jelly) for 96 hours (days test duration). In total, 4 treatment groups were set up: 5 doses of the test item, 2 untreated control groups and 4 doses of the reference item with 3 replicates per dose and 12 larvae per replicate. After the day of application, additional feeding of the larvae took place 24 and 48 hours later. Assessments of larval mortality were done after 24, 48, 72 and 96 hours. Additionally, other observations such as small body size or large quantities of remaining food after 72 (7 d test duration) and 96 hours (8 d test duration) were noted. In an analytical phase of the study, the concentration of the active substance in the test item stock solution was determined.

Endpoints: Mortality (NOED, LD_{50}), quantitative observations: body size, remaining food.

Reference item: Dimethoate technical (analyzed purity: 99.8%).

Test doses: Control 1: untreated diet (50% aqueous sugar solution with 50% royal jelly)
Control 2: untreated diet with Tween20 and acetone (each 1% v/v)

Test item treatments:

Nominal dose/concentration of BAS 750 F	
Doses [µg a.s./larva]	**Concentrations [g a.s./kg food]**
7.4	0.219
14.8	0.438
29.7	0.875
59.3	1.751
118.7	3.501

Reference item treatments: 1.1, 2.2, 4.4 and 8.8 µg dimethoate/larva.

Test conditions: Temperature: 34.0° C – 34.5° C; relative humidity: 93% - 97%, photoperiod: 24 h darkness; food: 50% aqueous sugar solution with 50% royal jelly.

Statistics: Descriptive statistics; Fisher's Exact Binomial Test with Bonferroni Correction for mortality data (one-sided greater, $\alpha = 0.05$) and No Observed Effect Level. The median lethal doses/concentrations of test and reference item were calculated with Probit analysis.

II. RESULTS AND DISCUSSION

Control mortality was 2.8% and 13.9% after 72 hours and 96 hours of exposure, respectively (corresponding to a test duration of 7 and 8 days) was seen. The solvent control showed a mortality of 13.9% after 72 h which did not increase at 96 h of exposure. After 72 hours, larvae fed with 59.3 and 118.7 µg a.s./larva revealed a mortality of 91.7% and 83.3%, respectively, which was statistically significant in comparison to the solvent control group (Fisher's Exact Binomial Test with Bonferroni Correction, $\alpha = 0.05$). The increase in mortality compared to the solvent control was statistically significant after 96 hours in this test item groups as well (97.2% and 94.4% in the 59.3 and 118.7 µg a.s./larva treatment groups, respectively; Fisher's Exact Binomial Test with Bonferroni Correction, $\alpha = 0.05$). The 96h (8d) NOED is 29.7 µg a.s./larva, the calculated LD_{10} and LD_{50} are 22.1 and 43.9 µg a.s./larva, respectively.

After 72 hours of exposure (7 d test duration), deviations from the normal food consuming behavior occurred in 11.1%, 18.8%, 100.0% and 100.0% of the remaining individuals treated with 14.8, 29.7, 59.3 and 118.7 µg a.s./larva. After 96 hours (8 d test duration) of exposure, deviations to the normal food consuming behavior and correspondingly, to developing into an average sized larva were still present in 3.3, 4.2, 100.0 and 50.0% of the remaining larvae, which were treated with 14.8, 29.7, 59.3 and 118.7 µg a.s., respectively. The results are summarized in Table 8.3.1.3-1.

Table 8.3.1.3-1: Toxicity of BAS 750 F to *Apis mellifera* (honeybee) in an acute oral larval toxicity test after exposure of 72 and 96 hours (7d and 8d test duration, respectively)

Dosage [µg a.s./larva]	Concentration [g a.s./kg food]	72 h mortality [%]		96 h mortality [%]	
		absolute	corrected [1)]	absolute	corrected [1)]
Control	Control	2.8	--	13.9	--
Tween control	Tween control	13.9	--	13.9	--
7.4	0.219	5.6	0.0	13.9	0.0
14.8	0.438	0.0	0.0	16.7	3.2
29.7	0.875	11.1	0.0	25.0	12.9
59.3	1.751	91.7 *	90.3	97.2 *	96.8
118.7	3.501	83.3 *	80.6	94.4 *	93.5

	Endpoints [µg BAS 750 F/bee]	
	72 h	96 h
LD_{50} [µg a.s./larva] (95% confidence limits)	n.d.	43.9 (24.0 – 80.2)
NOED [µg a.s./larva]	29.7	29.7
LC_{50} [g a.s./kg food] (95% confidence limits)	n.d.	1.295 (0.710 – 2.363)
NOEC [µg a.s./kg food]	0.875	0.875

* Statistically significantly different compared to the control (Fisher's Exact Binomial Test with Bonferroni Correction, one-sided greater; $\alpha = 0.05$).

1) According to Schneider-Orelli (1947).

In the reference item treatment group, larvae fed with 8.8 µg a.s./larva resulted in a mortality of 61.1% (corrected for control mortality: 60.0%), 72 hours after application. The LD_{50} value was determined to be 5.2 µg dimethoate/larva.

III. CONCLUSION

In an acute oral larval toxicity study with BAS 750 F on honeybee larvae, the LD_{50} value (96 h exposure = 8 d test duration) was determined to be 43.9 µg a.s./larva (equivalent to LC_{50} = 1.295 g a.s./kg food). The NOED was determined to be 29.7 µg a.s./larva (equivalent to NOEC = 0.875 g a.s./kg food).

Report: CA 8.3.1.3/2
Royer S., 2015 a
BAS 750 F (Reg.No. 5834378) - Honey bee larvae test (repeated exposure, observation 21 days) under laboratory conditions (in vitro) - Non-GLP
2014/1327676
Guidelines: OECD 237 (2013) Honey bee (Apis mellifera) larval toxicity test single exposure
GLP: no

EXECUTIVE SUMMARY

The effects of the test item BAS 750 F (Reg. No. 5834378) on survival of the honeybee larvae *Apis mellifera* were investigated in a laboratory test with chronic exposure over a time period of 21 days. Synchronized 1st larval stage (L1) honeybee larvae (*Apis mellifera carnica*) were fed with artificial diet for 6 days. On days 3, 4, 5 and 6, larvae were fed with diet containing four different concentrations of BAS 750 F (Reg. No. 5 834 378) resulting in concentrations of 40.63, 81.25, 162.5 and 325.0 mg a.s./kg food, corresponding to total doses of 6.3, 12.5, 25.0 and 50.1 µg a.s./larva. Untreated diet served as a control, in addition to a solvent control with acetone equivalent to the dose used in the treatment groups. All treatment groups and controls contained larvae from three different bee colonies. Survival of the larvae and pupae was recorded on rearing days 4, 5, 6, 7, 8, 14 and 21. Successful adult emergence was recorded on rearing day 21.

After 21 days, feeding of BAS 750 F in concentrations of 40.63, 81.25, 162.5 and 325 mg a.s./kg food (corresponding to total doses of 6.3, 12.5, 25.0 and 50.1 µg a.s./larva) caused mean mortalities of 14.6%, 22.0%, 14.6% and 43.9%, respectively. These resulted in mean corrected mortalities of -20.7%, -10.3%, -20.7% and 20.7%, respectively. The mortality in the different treatments did not show statistically significant effects compared to the solvent control.

At the end of 21 days, 75.6% and 70.7% of larvae honeybees emerged as adults in the untreated control and solvent control, respectively. In the test item treatment groups, 85.4%, 78.0%, 85.4% and 56.1% emerged in the 6.3, 12.5, 25.0 and 50.1 µg a.s./larva test item treatment groups, respectively.

In a chronic oral larval toxicity study with BAS 750 F on honeybee larvae, the NOEC value (21 d) was determined to be ≥ 325 mg a.s./kg food (equivalent to LC_{50} (21 d) > 325 mg a.s./kg food). The NOED was determined to be ≥ 50.1 µg a.s./larva (equivalent to LD_{50} > 50.1 µg a.s./larva).

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378); batch no.: COD-001740; analyzed purity: 98.8%.

B. STUDY DESIGN

Test species: Larvae of *Apis mellifera* L. subspecies *carnica* P. (honeybee); synchronized first larval stage (L1); derived from three healthy and queen-right colonies; source: in-house colonies.

Test design: Chronic feeding test according to OECD Draft "Guidance document for honeybees larval toxicity test, repeated exposure" (25 Feb 2014) and OECD Guidelines for the testing of chemicals No. 237: "Honey bee (*Apis mellifera*) larval toxicity test, single exposure" (adopted: 26 July 2013). L1 honeybee larvae of *Apis mellifera* were transferred from brood combs to polystyrene grafting cells in 48-well cell culture plates. After this, in a 21 day chronic test, the larvae were fed during larval development with artificial diet, containing the test item on rearing days 3, 4, 5 and 6. In total, 3 treatment groups were set up: 4 doses of the test item, 1 untreated control group and 1 solvent control, each with 41 larvae from 3 different bee colonies. Survival was assessed over a time period of 21 days. Successful adult emergence was assessed on rearing day 21.

Endpoints: Mortality (21 day NOEC/NOED, 21 day LC_{50}/LD_{50}).

Test doses: Control 1: untreated diet (50% aqueous sugar solution with 50% royal jelly)
Control 2: untreated diet with acetone (0.5% w/w)
Test item treatments:

Nominal dose/concentration of BAS 750 F	
Doses [µg a.s./larva]	**Concentrations [mg a.s./kg food]**
6.3	40.63
12.5	81.25
25.0	162.5
50.1	325.0

Test conditions: Measured mean temperature and humidity:
34.7°C and 94.1% (days 1 – 8)
34.9°C and 78.3% (days 8 – 14)
35.7°C and 52.8% (days 14 – 21)

Statistics: Descriptive statistics; Fisher's Exact Binomial Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$) and No Observed Effect Level.

II. RESULTS AND DISCUSSION

After 21 days, feeding of BAS 750 F in concentrations of 40.63, 81.25, 162.5 and 325 mg a.s./kg diet (corresponding to total doses of 6.3, 12.5, 25.0 and 50.1 µg a.s./larva) caused mean mortalities of 14.6%, 22.0%, 14.6% and 43.9%, respectively. These resulted in mean corrected mortalities of -20.7%, -10.3%, -20.7% and 20.7%, respectively. The mortality in the different treatments did not show statistically significant effects compared to the solvent control (Fisher's Exact Binomial Test with Bonferroni Correction, one-sided greater, $\alpha = 0.05$).

At the end of 21 days, 75.6% and 70.7% of larvae honeybees emerged as adults in the untreated control and solvent control, respectively. In the test item treatment groups, 85.4%, 78.0%, 85.4% and 56.1% emerged in the 6.3, 12.5, 25.0 and 50.1 µg a.s./larva test item treatment groups, respectively. The results were not statistically significant compared to the solvent control (Fisher's Exact Binomial Test with Bonferroni Correction≥, one-sided greater, $\alpha = 0.05$). As no effects were observed, all LC_X (LD_X) values are greater than the highest test concentration (dose). The results are summarized in Table 8.3.1.3-2.

Table 8.3.1.3-2: Toxicity of BAS 750 F to *Apis mellifera* (honeybee) in a chronic oral larval toxicity test after 21 days

Dosage [µg a.s./larva]	**Concentration [mg a.s./kg food]**	**21 day mortality [%]**		**21 day adult emergence [%]** [2)]
		absolute	**corrected** [1)]	
Control	Control	24.4	--	75.6
Acetone solvent control	Acetone solvent control	29.3	--	70.7
6.3	40.63	14.6	-20.7	85.4
12.5	81.25	22.0	-10.3	78.0
25.0	162.5	14.6	-20.7	85.4
50.1	325.0	43.9	20.7	56.1
Endpoints [21 d]				
LD_{50} [µg a.s./larva]		> 50.1		
NOED [µg a.s./larva]		≥ 50.1		
LC_{50} [mg a.s./kg food]		> 325		
NOEC [mg a.s./kg food]		≥ 325		

1) Corrected for solvent control mortality according to Schneider-Orelli (1947).
2) Adult emergence is calculated as the reverse of the pupae mortality on day 21: adult emergence=100%-mortality (day21).

III. CONCLUSION

In a chronic oral larval toxicity study with BAS 750 F on honeybee larvae, the NOEC value (21 d) was determined to be ≥ 325 mg a.s./kg food (equivalent to LC_{50} (21 d) > 325 mg a.s./kg food). The NOED was determined to be ≥ 50.1 µg a.s./larva (equivalent to LD_{50} > 50.1 µg a.s./larva).

CA 8.3.1.4 Sub-lethal effects

According to regulation 283/2013 no studies are required. Furthermore, the results of the above mentioned studies did not indicate any concern that should be addressed further.

CA 8.3.2 Effects on non-target arthropods other than bees

Effects on non-target arthropods other than bees are investigated with the formulated product due to technical reasons and representing the actual exposure situation. Therefore, no studies with the active substance are available. All data available for addressing the potential risk of BAS 750 F for non-target arthropods are presented in MCP 10.3.2.

CA 8.3.2.1 Effects on Aphidius rhopalosiphi

Studies have been conducted with the representative formulations and are described in M-CP 10.3.2.

CA 8.3.2.2 Effects on Typhlodromus pyri

Studies have been conducted with the representative formulations and are described in M-CP 10.3.2.

CA 8.4 Effects on non-target soil meso- and macrofauna

For the active substance BAS 750 F, new studies on soil macro-organisms have been performed, which are considered in the soil macro-organism risk assessment. Summaries of these studies are provided below and an overview on studies and endpoints is given in Table 8.4-1.

Table 8.4-1 Toxicity of BAS 750 F to non-target soil meso- and macrofauna

Substance	Species	Endpoint	Value [mg/kg dry soil]	Reference (BASF DocID)	EU agreed
BAS 750 F	*Eisenia fetida*	LC_{50} $LC_{50\ CORR}$	1000 > 500*	2015/1003342°	no
		NOEC $NOEC_{CORR}$	8.0 4.0*	2013/1235075	no
1,2,4-triazole M750F001		NOEC	≥ 1.0	2004/1041154	yes#
BAS 750 F	*Folsomia candida*	NOEC	≥ 400	2013/1235081	no
1,2,4-triazole M750F001		NOEC	1.8	2002/1007851	yes#
BAS 750 F	*Hypoaspis aculeifer*	NOEC	≥ 1000	2013/1235082	no
1,2,4-triazole M750F001		NOEC	171	2014/1326895	no

* Toxicity endpoint is adjusted using a soil factor of 2 to address the organic content of the soil (peat 10%), since the log P_{ow} of the substance is > 2.

° Study is presented as additional information in chapter M-CA 8.7.

studies with the metabolite 1,2,4-triazole have already been evaluated and EU agreed e.g. Tebuconazole: EFSA Journal 2014; 12(1):3485; 88 pp.

CA 8.4.1 Earthworms – sub-lethal effects

Report: CA 8.4.1/1
Friedrich S., 2013a
Sublethal toxicity of Reg.No. 5834378 (BAS 750 F) to the earthworm Eisenia fetida in artificial soil
2013/1235075
Guidelines: OECD 222 (2004)
GLP: yes
(certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In a chronic toxicity study, adults of *Eisenia fetida* (Annelida: Oligochaeta) were exposed to BAS 750 F (Reg. No. 5 834 378). The test item was mixed into artificial soil at concentrations of 1, 2, 4, 8 and 16 mg BAS 750 F/kg dry soil. For the control treatment, the soil was left untreated. The artificial test soil had an organic content of 10% (as sphagnum peat). Assessment of mortality of the adults was carried out after 28 days and reproduction (number of juveniles) was assessed after 56 days.

BAS 750 F did not show any statistically significant effects on survival and body weight. The mortality of adult worms was between 0.0% and 5.0% in the test item treatments and 2.5% in the control group. The weight change of adult worms was between 30.1% and 37.5% in the test item treatments and 36.3% in the control group. In the control, a mean of 108.3 juveniles was counted. In the test item treatment groups, mean numbers of juveniles between 81.0 and 119.0 were counted. The reproduction rate was significantly different compared to the control at 16 mg a.s./kg dry soil, the highest treatment rate tested. No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all test item treated groups was comparable to the control.

In a 56-day reproduction study with BAS 750 F (Reg. No. 5 834 378) no adverse effects on survival and biomass development were determined at concentrations up to and including 16 mg a.s./kg dry soil. Statistically significant effects on the number of juveniles of *Eisenia fetida* were determined at 16 mg a.s./kg dry soil. Therefore, the NOEC for mortality and biomass was ≥ 16 mg a.s./kg dry soil, whereas the NOEC for reproduction was 8 mg a.s./kg dry soil.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8% (analyzed, ± 1.0%).

B. STUDY DESIGN

Test species: *Eisenia fetida*; adult worms with clitellum and weight of 389 - 539 mg, approximately 4 months old; source: W. Neudorff GmbH KG followed by in-house culture.

Test design: In a 56-day test, adults of *Eisenia fetida* were exposed to five concentrations of BAS 750 F in treated artificial soil according to OECD 222 (10% peat). In total, 6 treatment groups were set up (5 concentrations of the test item and untreated control group) with 4 replicates for the test item treatments and 8 replicates for the control, 10 adult worms per replicate. The artificial soil was treated and filled into vessels, before the earthworms were introduced on the top of the soil.
Assessment of worm mortality, behavioral effects and weight change was done after 28 days of exposure, after additional 28 days (56 days after application) reproduction (number of juveniles) was assessed.

Endpoints: Mortality, weight change, feeding activity, reproduction rate.

Reference item: Nutdazim 50 Flow (Carbendazim SC 500).

Test concentrations: Control, 1, 2, 4, 8 and 16 mg BAS 750 F/kg dry soil.

Test conditions: Artificial soil according to OECD 222 with 10% peat; pH 6.13 - pH 6.20 at test initiation, pH 5.74 – pH 5.83 at test termination; water content 55.9% - 56.3% of its maximum water holding capacity (WHC) at test initiation and 55.3% - 55.9% of WHC at test termination, temperature: 18.0°C – 20.6°C; photoperiod: 16 hours light : 8 hours dark, light intensity: 530 lux, feeding with horse manure.

Statistics: Descriptive statistics; Fisher’s Exact Binominal test for mortality ($\alpha = 0.05$, one-sided greater).Williams-t-test for weight change and reproduction data ($\alpha = 0.05$, one-sided smaller), Probit analysis (Finney 1971).

II. RESULTS AND DISCUSSION

BAS 750 F did not show any statistically significant effects on mortality and body weight. The mortality of adult worms was between 0.0% and 5.0% in the test item treatments and 2.5% in the control group. The weight change of adult worms was between 30.1% and 37.5% in the test item treatments and 36.3% in the control group.
In the control, a mean of 108.3 juveniles was counted. In the test item treatment groups, mean numbers of juveniles between 81.0 and 119.0 were counted. The reproduction rate was significantly different compared to the control at 16 mg a.s./kg dry soil, the highest treatment rate tested (Williams-t-test, $\alpha = 0.05$, one-sided smaller). No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all test item treated groups was comparable to the control. The EC_{10} for reproduction was determined to be 5.3 mg a.s./kg. Main results are summarized Table 8.4.1-1.

Table 8.4.1-1: Effects of BAS 750 F on *Eisenia fetida* in a 56-day reproduction study

BAS 750 F [mg a.s./kg dry soil]	**Control**	**1**	**2**	**4**	**8**	**16**
Mortality (28 d) [%]	2.5	0.0	2.5	2.5	2.5	5.0
Weight change (28 d) [%]	36.3	34.6	37.5	36.5	34.1	30.1
Number of juveniles (56 d)	108.3	112.5	119.0	97.5	91.8	81.0*
Reproduction (56 d) [% of control]	100	103.9	109.9	90.1	84.8	74.8
Endpoints [mg a.s./kg dry soil]						
NOEC (day 28)	≥ 16					
NOEC (day 56)	8					
EC_{10} (day 56)	5.3					
EC_{50} (day 56)	> 16					

* Statistically significantly different compared to the control (Williams-t-test, $\alpha = 0.05$, one-sided smaller).

In a separate study the reference item Nutdazim 50 Flow (carbendazim, SC 500) caused a statistically significant lower biomass increase of and reproduction of *Eisenia fetida*. The reproduction rate was clearly inhibited by 39% and 100% compared to the control at the test concentrations of 5 and 10 mg product/kg dry soil.

III. CONCLUSION

In a 56-day reproduction study with BAS 750 F (Reg. No. 5 834 378) no adverse effects on survival and biomass development were determined at concentrations up to and including 16 mg a.s./kg dry soil. Statistically significant effects on number of juveniles of *Eisenia fetida* were determined at 16 mg a.s./kg dry soil. Therefore the NOEC for mortality and biomass was ≥ 16 mg a.s./kg dry soil, whereas the NOEC for reproduction was 8 mg a.s./kg dry soil.

CA 8.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

CA 8.4.2.1 Species level testing

Report:	CA 8.4.2.1/1 Friedrich S., 2013b Effects of BAS 750 F on the reproduction of the collembolan Folsomia candida 2013/1235081
Guidelines:	OECD 232 (2009), ISO 11267 (1999)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

The effects of BAS 750 F (Reg. No. 5 834 378) on survival and reproduction of the collembolan *Folsomia candida* were investigated in a laboratory study over 28 days. Five test concentrations of 25, 50, 100, 200 and 400 mg a.s./kg dry soil were incorporated into the soil with 4 replicates per treatment (each containing 10 juvenile collembolans). An untreated control and a solvent control with 8 replicates each were included. Assessment of adult collembolan mortality, behavioral effects and reproduction rate (number of juveniles) was carried out after 28 days.

In the test item treatments mortality rates of 0% to 5% were observed, compared to 6.3% in the control and 5.0% in the solvent control. No statistically significant mortality compared to the control was observed in any of the test item concentrations. In the control a mean of 468 juveniles and in the solvent control 467 juveniles was counted. In the treatment groups a mean number of juveniles of 452 to 485 were counted. No statistically significant effects on the number of juveniles compared to the solvent control were recorded at any concentration tested. Differences between the behavior of the collembolans in the control groups and the test item groups could not be observed.

In a 28-day collembolan reproduction study with BAS 750 F (Reg. No. 5 834 378) the EC_{50} was > 400 mg a.s./kg dry soil. The NOEC based on reproduction and mortality was ≥ 400 mg a.s./kg dry soil, the highest concentration tested.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8% (analyzed, ± 1.0%).

B. STUDY DESIGN

Test species: Collembola (*Folsomia candida),* juveniles (9 - 12 days old); source: in-house culture.

Test design: 28-day test in treated artificial soil according to ISO 11267 and OECD 232; artificial soil filled in glass vessels was treated with different concentrations of the test item before collembolans were introduced on top of soil; 7 treatment groups (5 test item concentrations, control, solvent control); 4 replicates for the test item treatments, 8 replicates for the control and solvent control, each containing 10 juvenile collembolans.
Assessment of adult mortality, behavioral effects and reproduction rate (number of juveniles) after 28 days.

Endpoints: Mortality, behavioral effects, reproduction rate.

Reference item: Boric acid (100% analyzed). The effects of the reference item were investigated in a separate study.

Test rates: Control, 25, 50, 100, 200 and 400 mg a.s./kg dry soil.

Test conditions: Artificial soil according to OECD 232 with a peat content of 5%; pH 6.03 to pH 6.09 at test initiation, pH 5.76 – pH 5.84 at test termination; water content 56.8% - 57.3% of maximum water holding capacity (WHC) at study initiation and 55.7% - 56.6% of WHC at test termination; temperature: 9.6°C - 20.7°C; photoperiod: 16 h light : 8 h dark, light intensity: 510 lux; food: dry yeast supplied twice a week.

Statistics: Descriptive statistics, Fisher’s Exact Binominal Test for mortality data ($\alpha = 0.05$, one-sided greater), Williams-t-test for reproduction ($\alpha = 0.05$, one-sided smaller).

II. RESULTS AND DISCUSSION

In the test item treatments mortality rates of 0% to 5% were observed, compared to 6.3% in the control and 5.0% in the solvent control. No statistically significant mortality compared to the control was observed in any of the test item concentrations (Fishers Exact Binominal Test, $\alpha = 0.05$). In the control a mean of 468 juveniles and in the solvent control 467 juveniles was counted. In the treatment groups a mean number of juveniles of 452 to 485 were counted. No statistically significant effects on the number of juveniles compared to the solvent control were recorded at any concentration tested (Williams-t-test, $\alpha = 0.05$, one-sided smaller). Differences between the behavior of the collembolans in the control groups and the test item groups could not be observed. No EC_X value is presented as no clear dose response could be determined and no effects were observed at the highest rate. The results are summarized in Table 8.4.2.1-1.

Table 8.4.2.1-1: Effect of BAS 750 F on collembolans (*Folsomia candida*) in a 28-day reproduction study

BAS 750 F [mg a.s./kg dry soil]	**Control**	**Solvent control**	**25**	**50**	**100**	**200**	**400**
Mortality (day 28) [%]	6.3	5.0	5.0	5.0	5.0	5.0	0.0
No. of juveniles (day 28)	468	467	470	455	472	485	452
Reproduction (day 28) [% of control]	--	100	101	98	101	104	97
Endpoints [mg a.s./kg dry soil]							
$NOEC_{mortality, reproduction}$	≥ 400						
LC_{50}	> 400						
EC_{50}	> 400						

In a separate study the EC_{50} (reproduction) of the reference item boric acid was calculated to be 108 mg a.s./kg dry soil.

III. CONCLUSION

In a 28-day collembolan reproduction study with BAS 750 F (Reg. No. 5 834 378) the EC_{50} was > 400 mg a.s./kg dry soil. The NOEC based on reproduction and mortality was ≥ 400 mg a.s./kg dry soil, the highest concentration tested.

Report: CA 8.4.2.1/2
Schulz L., 2014a
Effects of BAS 750 F on the reproduction of the predatory mite Hypoaspis aculeifer
2013/1235082
Guidelines: OECD 226 (2008)
GLP: yes
(certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

The effects of BAS 750 F (Reg. No. 5 834 378) on survival and reproduction of the soil mite *Hypoaspis aculeifer* were investigated in a chronic laboratory study over 14 days. The test item was mixed into artificial soil at rates of 62.5, 125, 250, 500 and 1000 mg BAS 750 F/kg dry soil. Test item treatments were replicated four times each. As control treatments, one untreated control and one prepared with acetone as solvent control, each with 8 replicates, was included. Each treatment contained 10 adult female soil mites. Assessments of mortality and reproduction were carried out after 14 days of exposure. No differences in behavior and morphology between mites in the solvent control and the test item treatments could be observed.

Test item treatment groups had mortality rates of between 0.0% - 7.5%. In the untreated control and the solvent control the mortality rate was 5.0% and 2.5%, respectively. The observed mortality rates for adult mites in the test item treatment groups compared to the solvent control were not statistically significant.

In the untreated and the solvent control group, mean numbers of 332.6 and 309.4 juveniles were counted, respectively. In the test item treatment groups the mean number of juveniles was between 293.0 and 330.8. BAS 750 F showed no statistically significantly adverse effects on reproduction at all test concentrations.

In a 14-day reproduction study with BAS 750 F (Reg. No. 5 834 378) on predatory soil mites (*Hypoaspis aculeifer*), the LC_{50} and EC_{50} values were determined to be > 1000 mg a.s./kg dry soil. The NOEC for mortality and reproduction was determined to be ≥ 1000 mg a.s./kg dry soil.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8% (analyzed, ± 1.0%).

B. STUDY DESIGN

Test species: *Hypoaspis aculeifer* (CANESTRINI), adult female predatory mites (age difference 3 days); source: in-house culture originally purchased from Katz Biotech AG, Baruth, Germany.

Test design: 14-day chronic laboratory test (according to OECD 226) on effects of BAS 750 F on mortality and reproduction of soil mites. Different concentrations of the test item were homogenously mixed into artificial soil (5% peat) which was then filled in glass vessels before the soil mites were introduced on top of the soil; 7 treatment groups (control, solvent control, 5 test item concentrations); 8 replicates for the control treatments and 4 replicates for test item treatments, each with 10 soil mites; assessment of adult mortality and reproduction effects (number of juveniles) after 14 days.

Endpoints: Mortality and reproduction rate after 14 days.

Reference item: Dimethoate EC 400 (411.7 g/L analyzed). The effects of the reference item were investigated in a separate study.

Test rates: Untreated and solvent control (acetone), 62.5, 125, 250, 500 and 1000 mg test item/kg dry soil.

Test conditions: Artificial soil according to OECD 226; pH 5.5 – pH 5.7 at test initiation, pH 5.7 – pH 5.8 at test termination; water content at test initiation 49.69% – 58.24% of maximum water holding capacity (WHC) and 48.28% – 57.30% of maximum WHC at test termination; temperature: 19.5°C – 21.1°C; photoperiod: 16 h light : 8 h dark; light intensity: 522 lux; food: cheese mites (*Tyrophagus putrescentiae*) supplied twice to three times a week.

Statistics: Descriptive statistics; Fisher's Exact Binominal Test with Bonferroni Correction for mortality ($\alpha = 0.05$, one-sided greater), Dunnett-t-test for reproduction ($\alpha = 0.05$, one-sided smaller).

II. RESULTS AND DISCUSSION

Test item treatment groups had mortality rates of between 0.0% - 7.5%. In the untreated control and the solvent control the mortality rate was 5.0% and 2.5%, respectively. The observed mortality rates for adult mites in the test item treatment groups compared to the solvent control were not statistically significant (Fisher's Exact Binominal Test with Bonferroni Correction, $\alpha = 0.05$, one-sided greater). Differences between the behavior and the morphology of the mites in the solvent control and the test item treatment groups could not be observed.

In the untreated and the solvent control group, mean numbers of 332.6 and 309.4 juveniles were counted, respectively. In the test item treatment groups the mean number of juveniles was between 293.0 and 330.8. BAS 750 F showed no statistically significantly adverse effects on reproduction at all test concentrations (Dunnett-t-test, $\alpha = 0.05$, one-sided smaller). No EC_X value is presented as no clear dose response could be determined and no effects were observed at the highest rate. The results are summarized in Table 8.4.2.1-2.

Table 8.4.2.1-2: Effects of BAS 750 F on predatory mite (*Hypoaspis aculeifer*) mortality and reproduction (day 14)

BAS 750 F [mg a.s./kg dry soil]	**Control**	**Solvent control**	**62.5**	**125**	**250**	**500**	**1000**
Mortality [%]	5.0	2.5	7.5	2.5	0.0	0.0	0.0
No. of juveniles (day 14)	332.6	309.4	296.5	322.5	293.0	330.8	298.3
Reproduction (day 14) [% of solvent control]	--	100	96	104	95	107	96
Endpoints [mg a.s./kg dry soil]							
$NOEC_{mortality + reproduction}$	≥ 1000						
LC_{50}	> 1000						
EC_{50}	> 1000						

The reference item was tested in a separate study at concentrations of 4.10, 5.12, 6.40, 8.00 and 10.00 mg dimethoate/kg dry soil. The EC_{50} (reproduction) was calculated to be 6.64 mg dimethoate/kg dry soil. The results of the reference test demonstrate the sensitivity of the test system.

III. CONCLUSION

In a 14-day reproduction study with BAS 750 F (Reg. No. 5 834 378) on predatory soil mites (*Hypoaspis aculeifer*), the LC_{50} and EC_{50} values were determined to be > 1000 mg a.s./kg dry soil. The NOEC for mortality and reproduction was determined to be ≥ 1000 mg a.s./kg dry soil.

Report:	CA 8.4.2.1/3 Schulz L., 2014 b 1,2,4-triazole - CGA71019 - Effects on the reproduction of the predatory mite Hypoaspis aculeifer 2014/1326895
Guidelines:	OECD 226 (2008)
GLP:	yes (certified by Pflanzenschutzamt der Landwirtschaftskammer Hannover, Hannover, Germany)

EXECUTIVE SUMMARY

The effects of the test item CGA71019 (=1,2,4-triazole, referred to as "test item" below) on survival and reproduction of the soil mite *Hypoaspis aculeifer* were investigated in a laboratory study over 14 days. The test item was mixed into artificial soil according to OECD 226 (5% peat) at concentrations of 9.07, 16.3, 29.4, 52.9, 95.3, 171, 309, 556 and 1000 mg test item/kg dry soil. For the control treatment, the soil was left untreated. 8 replicates for the control and 4 replicates for the test item treatments were prepared, respectively; each containing 10 adult soil mites. Assessment of mortality and reproduction was carried out after 14 days.

Adult soil mite mortality rates of 0.0% to 10.0% were recorded in the test item treatment groups, compared to 3.8% mortality in the control group. This resulted in corrected mortality rates ranging from -1.3% to 6.5% in the treatment groups. The observed mortality rates in the test item treatment groups compared to control were not statistically significant.

The mean number of juveniles was 187.0 in the control and 212.8, 200.3, 203.5, 239.0, 244.8, 184.0, 116.3, 41.8 and 20.0 at concentrations of 9.07, 16.3, 29.4, 52.9, 95.3, 171, 309, 556 and 1000 mg test item/kg dry soil, respectively. The test item caused no statistically significantly adverse effects on reproduction up to and including a test concentration of 171 mg test item/kg dry soil. Statistically significant effects on reproduction were observed at 309, 556 and 1000 mg test item/kg dry soil.

In a 14-day reproduction study with the test item CGA71019 (=1,2,4-triazole) on soil mites (*Hypoaspis aculeifer*), the LC_{50} was estimated to be > 1000 mg test item/kg dry soil. The NOEC for mortality was determined to be ≥ 1000 mg test item/kg dry soil, while the NOEC for reproduction was determined to be 171 mg test item/kg dry soil.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: CGA71019 (=1,2,4-triazole); batch no. R 200; analyzed purity: 99.0% (tolerance ± 2.0%).

B. STUDY DESIGN

Test species: Soil mites: *Hypoaspis aculeifer* (CANESTRINI); age: adults with an age difference of 2 days.

Test design: The effects of the test item on mortality and reproduction of the soil mite *Hypoaspis aculeifer* were investigated in a chronic laboratory experiment over a time period of 14 days according to OECD 226. Different concentrations of the test item were homogeneously mixed into the artificial soil (5% peat) which was then filled into glass vessels after which the soil mites were introduced on top of the soil; 10 treatment groups (9 test item concentrations, control); 8 replicates/control group and 4 replicates/test item treatment group each with 10 soil mites. Assessment of adult mortality and reproduction effects was carried out after 14 days.

Endpoints: Mortality and reproduction rate (no. juveniles) after 14 days.

Reference item: Dimethoate EC 400 (content of a.s. dimethoate: 400 g/L nominal). The effects of the reference item were investigated in a separate study.

Test concentrations: Control, 9.07, 16.3, 29.4, 52.9, 95.3, 171, 309, 556 and 1000 mg test item/kg dry soil.

Test conditions: Artificial soil according to OECD 226 (5% peat), pH 6.0 at test initiation, pH 5.7 - pH 6.0 at test termination; water content at test initiation 49.47% - 51.83% of maximum water holding capacity (WHC) and 48.5% - 51.52% of maximum WHC at test termination; temperature 19.5 °C – 21.2 °C; photoperiod: 16 h light : 8 h dark; light intensity: 511 lux. Feeding of mites with *Tyrophagus putrescentiae*.

Statistics: Descriptive statistics; Fisher’s Exact Binomial Test with Bonferroni Correction for mortality ($\alpha = 0.05$) and Williams-t-test for reproduction ($\alpha = 0.05$), Probit Analysis for EC-values.

II. RESULTS AND DISCUSSION

Adult soil mite mortality rates of 0.0% to 10.0% were recorded in the test item treatment groups, compared to 3.8% mortality in the control group. This resulted in corrected mortality rates ranging from -1.3% to 6.5% in the treatment groups. The observed mortality rates in the test item treatment groups compared to control were not statistically significant (Fisher's Exact Binomial Test with Bonferroni Correction, $\alpha = 0.05$).

The mean number of juveniles was 187.0 in the control and 212.8, 200.3, 203.5, 239.0, 244.8, 184.0, 116.3, 41.8 and 20.0 at concentrations of 9.07, 16.3, 29.4, 52.9, 95.3, 171, 309, 556 and 1000 mg test item/kg dry soil, respectively. The test item caused no statistically significantly adverse effects on reproduction up to and including a test concentration of 171 mg test item/kg dry soil. Statistically significant effects on reproduction could be observed at 309, 556 and 1000 mg test item/kg dry soil (Williams-t-test, $\alpha = 0.05$). The results are summarized in Table 8.4.2.1-3.

Table 8.4.2.1-3: Effects of the test item CGA71019 (=1,2,4-triazole) on *Hypoaspis aculeifer* in a 14-day reproduction study

CGA71019 [mg/kg dry soil]	**Control**	**9.07**	**16.3**	**29.4**	**52.9**	**95.3**	**171**	**309**	**556**	**1000**
Mortality (day 14) [%]	3.8	10.0	2.5	0.0	2.5	2.5	0.0	0.0	2.5	5.0
Mean no. of juveniles (day 14)	187.0	212.8	200.3	203.5	239.0	244.8	184.0	116.3 *	41.8 *	20.0 *
Reproduction (day 14) [% of control]	--	-13.8	-7.1	-8.8	-27.8	-30.9	1.6	37.8	77.7	89.3
Endpoints [mg CGA71019/kg dry soil]										
$NOEC_{mortality}$	≥ 1000									
$NOEC_{reproduction}$	171.0									
LC_{50}	> 1000									

* Statistically significantly different compared to the control (William's t-test, $\alpha = 0.05$).

III. CONCLUSION

In a 14-day reproduction study with the test item CGA71019 (=1,2,4-triazole) on soil mites (*Hypoaspis aculeifer*), the LC_{50} was estimated to be > 1000 mg test item/kg dry soil. The NOEC for mortality was determined to be ≥ 1000 mg test item/kg dry soil, while the NOEC for reproduction was determined to be 171 mg test item/kg dry soil.

CA 8.5 Effects on nitrogen transformation

For the active substance BAS 750 F, studies on the effects on soil microbial activity have been performed. The nitrogen transformation study summary is provided below; the carbon transformation study summary can be found in chapter M-CA 8.7. An overview of the studies and endpoints are given in Table 8.5-1.

Table 8.5-1 Toxicity of BAS 750 F to non-target soil micro-organisms

Test substance	Test design *	Endpoints used in risk assessment	Reference (BASF DocID)	EU agreed
BAS 750 F	C	< 25% effects after 28 days at 2.53 mg/kg dry soil	2015/1108621	no
	N	< 25% effects after 28 days at 2.53 mg/kg dry soil	2015/1108623	no
1,2,4-triazole M750F001	N	< 25% effects after 28 days up to and including 0.333 mg/kg dry soil	2000/1021861	yes#

* C = Carbon transformation, N = Nitrogen transformation.

** Carbon transformation studies are listed for reference, but not used in the following risk assessment as carbon transformation studies are no longer required according to EU Commission Regulation No.283/2013. Study is presented as additional information (see M-CA 8.7).

the study has already been evaluated and EU agreed, e.g. Epoxiconazole, EFSA Scientific Report, 138, 2008, DAR, Vol. 3, B.9, 2006

Report: CA 8.5/1
Schulz L., 2015
Effects of BAS 750 F (Reg.No. 5834378) on the activity of soil microflora (Nitrogen transformation test)
2015/1108623
Guidelines: OECD 216 (2000)
GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

EXECUTIVE SUMMARY

In a soil microbial activity study, the effects of BAS 750 F (Reg. No. 5 834 378) on the nitrogen transformation were investigated in a loamy sand soil. BAS 750 F (Reg. No. 5 834 378) was applied to samples of the soil at nominal test concentrations of 0.51 mg/kg and 2.53 mg/kg dry soil. BAS 750 F (Reg. No. 5 834 378) treated soils and controls were incubated at approx. 20 °C in the dark. Triplicate samples of each treatment were removed for analysis of mineral nitrogen 0, 7, 14 and 28 days after application.

There were no adverse effects on nitrogen transformation at any application rate at the end of the 28-day incubation period.

Based on the results of this study, in accordance with OECD guideline 216, BAS 750 F (Reg. No. 5 834 378) caused no adverse effects (< 25% deviation from control) on the soil nitrogen transformation (measured as NO_3-N production) up to a concentration of 2.53 mg BAS 750 F/kg dry soil, after a 28-day incubation period.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch No. COD-001740, analysed purity: 98.8 % (tolerance ± 1.0%).

B. STUDY DESIGN

Test species: Biologically active agricultural soil: loamy sand (DIN 4220) / sandy loam (USDA), pH 6.4, 1.44 % C_{org}, WHC: 35.59 g/100 g dry soil.

Test design: Determination of the N-transformation (NO_3-N-production) in soil enriched with lucerne meal (concentration in the soil 0.5%). Comparison of test item treated soil with a non-treated soil. NH_4-nitrogen formed from organically bound nitrogen and NO_3-nitrogen formed from the nitrification process was determined using an Autoanalyzer (BRAN and LUEBBE). Sampling scheme: 0, 7, 14 and 28 days after treatment. Sub-samples (3 replicates per treatment) were withdrawn from the bulk batches and subjected to the measurement.

Endpoints: Effects on the NO_3-N production 0, 7, 14 and 28 days after application.

Test concentrations: Control, 0.51 mg and 2.53 mg BAS 750 F (Reg.No. 5 834 378)/kg dry soil.

Reference item: Dinoterb (purity: 98.0% ± 0.5% analysed). The reference item was tested in a separate study at rates of 6.80, 16.00 and 27.00 mg/kg.

Test conditions: Soil moisture: approx. 45% of maximum water holding capacity; measured water content: 16.19 - 17.19 g/100 g dry soil; pH 6.1 - 6.2. Soil samples were incubated at 19.4 - 20.8°C while stored in glass flasks in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse effects of BAS 750 F (Reg.No. 5 834 378) on nitrogen transformation in soil could be observed at both test concentrations (0.51 mg/kg dry soil and 2.53 mg/kg dry soil) after 28 days (time interval 0-28). Only negligible deviations from the control of -3.7% (test concentration 0.51 mg/kg dry soil) and +2.1% (test concentration 2.53 mg/kg dry soil) were measured at the end of the 28-day incubation period.

The results are summarized in Table 8.5-2.

Table 8.5-2: Effects of BAS 750 F on soil micro-organisms (nitrogen transformation) on days 0, 7, 14 and 28 of incubation

Time interval (days)	Control	0.51 mg BAS 750 F (Reg. No. 5834378) per kg dry soil		2.53 mg BAS 750 F (Reg. No. 5834378) per kg dry soil	
	NO_3-N [mg/kg dry soil]	NO_3-N [mg/kg dry soil]	% Deviation from control [1)]	NO_3-N [mg/kg dry soil]	% Deviation from control [1)]
0-7	30.67	31.13	+1.5	31.67	+3.3
0-14	44.53	44.20	-0.7	45.03	+1.1
0-28	64.27	61.87	-3.7	65.60	+2.1

1) Based on NO_3-N production; - = inhibition, + = stimulation.

In a separate study the reference item Dinoterb produced a stimulation of nitrogen transformation of +33.2% and +46.9% at 16.00 mg and 27.00 mg/kg dry soil, respectively, determined 28 days after application.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 216, BAS 750 F caused no adverse effects (< 25% deviation from control) on the soil nitrogen transformation (measured as NO_3-N production) up to a concentration of 2.53 mg BAS 750 F/kg dry soil, after a 28-day incubation period.

CA 8.6 Effects on terrestrial non-target higher plants

CA 8.6.1 Summary of screening data

Non-target plant GLP studies with the representative formulations are available. Further screening data are not required.

CA 8.6.2 Testing on non-target plants

Effects on non-target plants are usually investigated with the formulated product due to technical reasons and representing the actual exposure situation. Therefore, no data for the active substance are available. Studies were performed with the representative formulations and are described in M-CP 10.6.2 together with a risk assessment.

CA 8.7 Effects on other terrestrial organisms (flora and fauna)

Report: CA 8.7/1
Friedrich S., 2015 a
Acute toxicity of BAS 750 F to the earthworm Eisenia fetida in artificial soil with 10% peat
2015/1003342
Guidelines: OECD 207 (1984), ISO 11268-1 (1993)
GLP: yes
(certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

EXECUTIVE SUMMARY

In an acute toxicity study, adults of *Eisenia fetida* (Annelida: Oligochaeta), were exposed to BAS 750 F (Reg. No. 5 834 378). The test item was mixed into artificial soil (10% peat) at concentrations of 62.5, 125, 250, 500 and 1000 mg BAS 750 F/kg dry soil. For the control treatment, the soil was left untreated. Assessment of mortality of the adults was carried out 7 and 14 days after treatment. Assessment of behavior and biomass development as sub-lethal parameters was carried out after 14 days.

After 14 days of exposure, 0% mortality was observed in all test item concentrations and in the control group. The biomass development was not statistically significantly different compared to the control at 62.5 mg BAS 750 F/kg dry soil, but was statistically significantly different at all other test concentrations. No abnormal behavior of the worms were observed in the test item treatment groups or control group during the test.

In a 14-day acute toxicity study with earthworms (*Eisenia fetida*), exposure to BAS 750 F (Reg. No. 5 834 378) resulted in an LC_{50} was estimated to be greater than 1000 mg BAS 750 F/kg dry soil. The NOEC was determined to be 62.5 mg BAS 750 F/kg dry soil.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8% (analyzed, ± 1.0%).

B. STUDY DESIGN

Test species: *Eisenia fetida*; adult worms with clitellum and weight of 300 – 600 mg, age: less than one year; source: in-house culture.

Test design: In a 14-day acute test, adults of *Eisenia fetida* were exposed to five concentrations of BAS 750 F in treated artificial soil according to OECD 207 (10% peat). In total, 6 treatment groups were set up (5 concentrations of the test item and untreated control group) with 4 replicates, 10 adult worms per replicate. The artificial soil was treated and filled into glass vessels, before the earthworms were introduced on the top of the soil.
Assessment of worm mortality was done 7 and 14 days after exposure, and biomass development and behavioral effects 14 days after exposure at test termination.

Endpoints: Mortality, biomass development.

Reference item: 2-Chloroacetamide. The effects of the reference item were investigated in a separate study.

Test concentrations: Control, 62.5, 125, 250, 500, 1000 mg BAS 750 F/kg dry soil.

Test conditions: Artificial soil according to OECD 207 with 10% peat; pH 5.97 - pH 6.12 at test initiation, pH 5.74 – pH 5.85 at test termination; water content 56.4% - 56.7% of its maximum water holding capacity (WHC) at test initiation and 55.7% - 56.1% of WHC at test termination, temperature: 19.1°C – 22.0°C; photoperiod: continuous illumination, light intensity: 530 lux, feeding with horse manure.

Statistics: Descriptive statistics; Williams-t-test for biomass data ($\alpha = 0.05$, one-sided greater).

II. RESULTS AND DISCUSSION

After 14 days of exposure, 0% mortality was observed in all test item concentrations and in the control group. The biomass development was not statistically significantly different compared to the control at 62.5 mg BAS 750 F/kg dry soil, but was statistically significantly different at all other test concentrations (William's t-test, $\alpha = 0.05$, one-sided greater). No abnormal behavior of the worms were observed in the test item treatment groups or control group during the test. The results are summarized in Table 8.7-1.

Table 8.7-1: Effects of BAS 750 F on *Eisenia fetida* in a 14-day acute study

BAS 750 F [mg/kg dry soil]	**Control**	**62.5**	**125**	**250**	**500**	**1000**
Mortality (28 d) [%]	0	0	0	0	0	0
Biomass development (14 d) [%]	-6.1	-9.0	-11.6 *	-16.9 *	-21.5 *	-26.2 *
Endpoints [mg BAS 750/kg dry soil]						
LC_{50}	> 1000					
NOEC	62.5					

* Statistically significantly different compared to the control (Williams-t-test, $\alpha = 0.05$, one-sided greater).

In a separate study with the reference item 2-Chloroacetamide, the 14-day LC_{50} was calculated as 21.3 mg a.s./kg dry soil with 95% confidence limits ranging from 20.3 to 22.4 mg a.s./kg dry soil

III. CONCLUSION

In a 14-day acute toxicity study with earthworms (*Eisenia fetida*), exposure to BAS 750 F (Reg. No. 5 834 378) resulted in an LC_{50} was estimated to be greater than 1000 mg BAS 750 F/kg dry soil. The NOEC was determined to be 62.5 mg BAS 750 F/kg dry soil.

Report:	CA 8.7/2 Schulz L., 2015 Effects of BAS 750 F (Reg.No. 5834378) on the activity of soil microflora (Carbon transformation test) 2015/1108621
Guidelines:	OECD 217 (2000)
GLP:	yes (certified by sächsiches Staatsministerium für Umwelt und Landwirtschaft, Dresden)

EXECUTIVE SUMMARY

In a soil microbial activity study, the effects of BAS 750 F (Reg. No. 5 834 378) on the carbon transformation were investigated in a loamy sand. BAS 750 F (Reg. No. 5 834 378) was applied to samples of the soil at nominal test concentrations of 0.51 mg/kg and 2.53 mg/kg dry soil. BAS 750 F (Reg. No. 5 834 378) treated soils and controls were incubated at approx. 20 °C in the dark. Triplicate samples of each treatment were removed for determination of carbon transformation (measured as O_2-consumption) 0, 7, 14 and 28 days after application.

There were no adverse effects on the carbon transformation at any application rate at the end of the 28-day incubation period.

Exposure of BAS 750 F (Reg. No. 5 834 378) in a field soil up to a test concentration of 2.53 mg BAS 750 F (Reg. No. 5 834 378)/kg dry soil caused no adverse effects (deviation from control < 25%, OECD 217) on the soil carbon transformation (measured as O_2-consumption) at the end of the 28-day incubation period.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 750 F (Reg. No. 5 834 378), batch No. COD-001740, analyzed purity: 98.8 % (tolerance ± 1.0%).

B. STUDY DESIGN

Test species: Biologically active agricultural soil: loamy sand (DIN 4220) / sandy loam (USDA), pH 6.4, 1.44% C_{org}, WHC: 35.59 g/100 g dry soil.

Test design: Determination of carbon-transformation in soil after addition of glucose. Comparison of test item treated soil with a non-treated and a reference item treated soil. 3 replicates per concentration. A "BSB-digi" respirometer system was used to measure the O2-consumption over a period of 12 hours at different sampling intervals. Sampling scheme: 0, 7, 14 and 28 days after treatment. Sub-samples were withdrawn from the bulk batches and subjected to measurement.

Test concentrations: Control, 0.51 mg and 2.53 mg BAS 750 F (Reg. No. 5 834 378)/kg dry soil.

Endpoints: Effects on O_2 consumption after 28 day of exposure.

Reference item: Dinoterb (purity: 98.0% ± 0.5% analyzed). The reference item was tested in a separate study at rates of 6.80, 16.00 and 27.00 mg/kg.

Test conditions: Soil moisture: 45% of its maximum water holding capacity: measured water content: 16.86 - 17.77 g/100 g dry soil; pH 6.1 - 6.3. Soil samples were incubated at 19.4 - 20.8 °C while stored in steel vessels in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse effects of BAS 750 F (Reg. No. 5 834 378) on carbon transformation in soil could be observed at both test concentrations (0.51 mg/kg dry soil and 2.53 mg/kg dry soil) after 28 days. Only negligible deviations from the control of +1.1% (test concentration 0.51 mg/kg dry soil) and -1.1% (test concentration 2.53 mg/kg dry soil) were measured at the end of the 28-day incubation period. The results are summarized in Table 8.7-2.

Table 8.7-2: Effects of BAS 750 F on soil micro-organisms (carbon transformation) on days 0, 7, 14 and 28 of incubation

Soil (days) (days)	Control	0.51 mg BAS 750 F/kg dry soil		2.53 mg BAS 750 F/kg dry soil	
	O_2 consumption [mg/h/kg dry soil]	O_2 consumption [mg/h/kg dry soil]	% Deviation from control [1)]	O_2 consumption [mg/h/kg dry soil]	% Deviation from control [1)]
Loamy sand soil (0 d)	17.03	16.91	-0.7	17.22	+1.1
Loamy sand soil (7 d)	16.60	17.30	+4.2	16.57	-0.2
Loamy sand soil (14 d)	15.50	15.61	+0.7	15.37	-0.8
Loamy sand soil (28 d)	14.32	14.47	+1.1	14.16	-1.1

[1)] Based on O_2-consumption; - = inhibition; + = stimulation

In a separate study the reference item Dinoterb caused an inhibition of carbon transformation of -0.1% and -39.6% at 16.00 mg and 27.00 mg a.s./kg dry soil, respectively, determined 28 days after application.

III. CONCLUSION

Exposure of BAS 750 F in a field soil up to a test concentration of 2.53 mg BAS 750 F/kg dry soil caused no adverse effects (deviation from control < 25%, OECD 217) on the soil carbon transformation (measured as O_2-consumption) at the end of the 28-day incubation period.

CA 8.8 Effects on biological methods for sewage treatment

Report: CA 8.8/1
Hammer S., 2014a
BAS 750 F - Determination of the inhibition of oxygen consumption in the activated sludge respiration inhibition test
2014/1049095

Guidelines: OECD 209, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part C.11

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

EXECUTIVE SUMMARY

The effect of BAS 750 F on the respiration rate of activated sludge collected from a wastewater treatment plant was determined. BAS 750 F was tested at nominal concentrations of 1000, 500, 250, 125 and 62.5 mg a.s./L. Oxygen consumption rate of aerobic micro-organisms was assessed after a contact period of 3 hours under aeration.

The biological results are based on nominal concentrations. No significant inhibition of respiration was measured up to the highest tested concentration of 1000 mg a.s./L (nominal).

The EC_{20} and EC_{50} values of BAS 750 F in the activated sludge respiration inhibition test are both > 1000 mg a.s./L. Disturbances in the bio-degradation process of activated sludge are not to be expected if the test item is correctly introduced into adapted wastewater treatment plants at low concentrations.

I. MATERIAL AND METHODS

Test item:	BAS 750 F (Reg. No. 5 834 378), batch no. COD-001740, purity: 98.8% (± 1.0% tolerance).
Test species:	Activated sludge from wastewater plants in Mannheim (Germany) treating municipal sewage.
Test design:	Assessment of the inhibitory effect of the test item on the oxygen consumption rate of aerobic micro-organisms (activated sludge) after short-term exposure of 3 hours; the inoculum was aerated during the contact period; 3 replicates for the test item, 2 replicates for the reference item and 6 replicates for the control, sludge concentration: 1.5 g/L Dw.
Test concentrations:	Control, 1000, 500, 250, 125 and 62.5 mg a.s./L (nominal).
Reference item:	3.5-dichlorophenol. The reference item was applied at 1, 10 and 100 mg/L.
Test conditions:	Temperature: 21.1 - 21.3 °C; pH 7.5 - 8.1; 1 L glass beakers, 500 mL of test mixture per vessel.
Analytics:	Not applicable.
Statistics:	Descriptive statistics, probit method for calculation of EC_x values.

II. RESULTS AND DISCUSSION

No significant inhibition of respiration was measured up to the highest tested concentration of 1000 mg a.s./L (nominal).

III. CONCLUSION

The EC_{20} and EC_{50} values of BAS 750 F in the activated sludge respiration inhibition test are both > 1000 mg a.s./L. Disturbances in the bio-degradation process of activated sludge are not to be expected if the test item is correctly introduced into adapted wastewater treatment plants at low concentrations.

CA 8.9 Monitoring data

No monitoring data concerning potential adverse effects of the active substance BAS 750 F to non-target organisms are available.

We create chemistry

BAS 750 F

Document M-CA, Section 9

LITERATURE DATA

Compiled by:

[redacted]

Telephone: [redacted]
E-mail:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 9 LITERATURE DATA

BAS 750 F

A literature search on BAS 750 F was performed by the BASF Group Information Center. The Literature Search Report on BAS 750 F describes the general search and evaluation process as well as details on search profiles, search histories and summary tables.

The complete search report is provided in K-CA 9/1 (BASF DocID 2016/1052687).
In addition, a search report with confidential information is provided in K-CA 9/4 (BASF DocID 2015/1277150).

The first step of the search result processing based on summary records was done by the Information Center and involved the separation into "hits" and "ballast" (obviously irrelevant records). The “ballast” was not further processed.

The “hits” were further evaluated by the scientific experts and categorized into “not relevant”, “not reliable”, and “used for dossier”. This is documented in EXCEL files which are attached to the search report in K-CA 9 with the file names as listed below (alphabetical order):

Analytics:	BAS 750 F Literature Search - Analytics
Consumer Safety:	BAS 750 F Literature Search – Consumer Safety
Ecotoxicology:	BAS 750 F Literature Search – Ecotox
Environmental Fate:	BAS 750 F Literature Search – Efate
Product Chemistry:	BAS 750 F Literature Search – Product Chemistry
Toxicology:	BAS 750 F Literature Search – Toxicology
	BAS 750 F Literature Search – Toxicology impurities confidential

The hits in Analytics, Ecotox Terrestric and Ecotox Wildlife, Environmental Fate, Metabolism and Residues in Animals and Plants, Product Chemistry as well as Toxicology did not contribute to the risk assessment and were therefore not further discussed in the dossier.

Triazole Derivative Metabolites (TDMs)

A literature search on TDMs was performed (BASF Doc ID 2015/1277467) for the TDMG . The detailed methodology employed and the results obtained for Toxicology, Consumer Safety, Ecotoxicology and Environmental Fate are provided in the four other documents of M-CA 9. (BASF DocID 2015/1228510; BASF DocID 2015/1228511, BASF DocID 2015/1276731 and BASF DocID 2015/1228512).

The hits in Toxicology, Environmental Fate, Consumer Safety and Ecotoxicology did not contribute to the risk assessment and were therefore not further discussed in the dossier.