ABSTRACT

The purpose of this study was to demonstrate that BASF Analytical Method D1513/01: " Validation of BASF Method D1513/01: Method for the Determination of Residues of BAS 750 F (Reg. No. 5834378) and its Metabolites, M750F003 (Reg. No. 5924326) and 1,2,4-Triazole (Reg. No. 87084) in Soil by LC-MS/MS using Micro-Extraction Procedure" and BASF Analytical Method L0214/01: "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", could be performed successfully at an outside facility with no prior experience with the method (Reference 1 and 2).

Principle of the method.

D1513/01: The residues of BAS 750 F, and its metabolites, M750F003, and 1,2,4-Triazole are extracted from 0.1 grams of soil by shaking twice, each with 0.8 mL of acetonitrile:water (70:30, v/v). For analysis of BAS 750 F and M750F003, an aliquot (0.1 mL) from the combined extract is diluted with 0.4 mL of acetonitrile:water (10:90, v/v) for anlaysis by LC-MS/MS. For analysis of 1,2,4-Triazole, an aliquot (0.2 mL (from the combined extract) is concentrated to 0.025 mL under nitrogen at 20 °C and reconstituted in water (0.475 mL) for analysis by LC-MS/MS.

L0214/01: The residues of BAS 750 F, and its metabolites, M750F003 and 1,2,4-Triazole are extracted from 5 grams of soil by shaking with a mixture of acetonitrile:water (70:30, v/v) for 30 minutes on a mechanical shaker at 225 rpm. After centrifugation (10 minutes at 4000 rpm) an aliquot (10 mL) is taken (extract 1) and the remaining supernatant is decanted and discarded. The same extraction procedure is repeated and after centrifugation a second 10-mL aliquot (extract 2) is combined with extract 1 and thoroughly mixed. Residues of BAS 750 F and M750F003 are analyzed by LC-MS/MS. For analysis of 1,2,4-Triazole, 5 mL of the combined extract 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 reconstituted to a volume of 1 mL with ultra-pure water and analyzed by LC-MS/MS.

Test conditions. For validation, untreated soil samples were fortified with BAS 750 F and its metabolites and analyzed according to the established method validation guidelines. The analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with each analyte at the method limit of quantitation (LOQ) and five replicates fortified at a higher level, corresponding to 10× the LOQ. The mass transitions described in the analytical methods were evaluated. In conjunction with the subject study, matrix- and solvent-matched standards were analyzed in a separate experiment to evaluate any potential matrix effects.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The LOQ was defined as the lowest fortification level tested. The LOQ for the three analytes (BAS 750 F or M750F003 or 1,2,4-triazole) in soil was 0.002 µg/g (ppm). The LOD for each analyte in soil was set at 0.0004 µg/g, which was 20% of the defined LOQ. The LOD for each analyte in soil was shown to be detectable as the absolute amount of analyte injected (0.0001 ng) into the LC-MS/MS when the lowest calibration standard was analyzed (0.005 ng/mL) with acceptable signal to noise ratio (S/N is >3:1).

Selectivity. The instrument method determines residues of BAS 750 F, M750F003 and 1,2,4-Triazole in soil matrices by LC-MS/MS. No interfering peaks were found at the retention times

for any analyte. The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each commodity had significant influence on analysis (matrix effects >20%) using method D1513/01 and L0214/01 and is presented in the results and discussion section; therefore, the validation samples using method D1513/01 were analyzed using matrix-matched calibration standard solutions for BAS 750 F, M750F003 (primary transition) and 1,2,4-Triazole (primary transition) and solvent-based calibration standard solutions for M750F003 (confirmatory transition) and 1,2,4-Triazole (confirmatory transition). The validation samples using method L0214/01 were analyzed using matrix-matched calibration standard solutions for all analytes.

Linearity (D1513/01). Acceptable linearity was observed for the standard range using 5–6 levels of standards and the two mass transitions tested: The method-detector response was linear over the 0.005–0.25 ng/mL range for BAS 750 F and M750F003, 0.01–0.50 ng/mL range for 1,2,4-Triazole (primary transition) and 0.01–1.0 ng/mL range for 1,2,4-Triazole (confirmatory transition) (r=≥0.990), for soil analyses.

Linearity (L0214/01). Acceptable linearity was observed for the standard range and the two mass transitions tested: The method-detector response was linear over the 0.025–3 ng/mL range for BAS 750 F and M750F003 and 0.125–15 ng/mL range for 1,2,4-Triazole (r = ≥0.990), for soil analyses.

Standard Stability. Standard solution stability was established during BASF study number 430689 (Reference 2). During the course of this study, the test/reference substance solutions were stored at an average temperature of 3 °C and all solutions were used within the demonstrated time period of stability. Standard solutions of BAS 750 F and M750F003 under refrigerated temperatures are considered to be stable for 43 days. Standard solutions of 1,2,4-Triazole under refrigerated temperatures are considered to be stable for 31 days.

Extract Stability (D1513/01). Extract stability was established during BASF method validation study number 784705 (Reference 1) and were used within the demonstrated time period of stability (11 to 13 days for all analytes).

Extract Stability (L0214/01). Extract stability was established during BASF method validation study number 430689 (Reference 2) and were used within the demonstrated time period of stability (7 to 10 days for all analytes).

Recovery and Repeatability (D1513/01). The independent laboratory validation (ILV) was performed successfully for soil and the LC-MS/MS ion transitions (primary and secondary) available for the method, using matrix-matched calibration standard solutions for BAS 750 F, M750F003 (primary transition) and 1,2,4-Triazole (primary transition) and solvent-based calibration standard solutions for M750F003 (confirmatory transition) and 1,2,4-Triazole (confirmatory transition).

Recovery and Repeatability (L0214/01). The independent laboratory validation (ILV) was performed successfully for soil and the LC-MS/MS ion transitions (primary and secondary) available for the method, using matrix-matched standards for all analytes.

Apparent residues of BAS 750 F and its metabolites were below the method limit of detection (<0.0004 ppm) which is 50% of LOQ in the control soil sample.

1. INTRODUCTION

1.1 Scope of the Method

BASF Analytical Method Number D1513/01 was developed to determine the residues of BAS 750 F and its metabolites M750F003 and 1,2,4-Triazole in soil matrices by LC-MS/MS using a micro-extraction procedure. BASF Analytical Method Number L0214/01 was developed to determine the residues of BAS 750 F and its metabolites M750F003 and 1,2,4-Triazole in soil matrices by LC-MS/MS. Both methods were developed at BASF Crop Protection (BASF) in Research Triangle Park, North Carolina. These methods were independently validated at ADPEN Laboratories, Inc (ADPEN).

The independent lab validation was conducted using two fortification levels limit of quantitation (0.002 ppm) and ten times of limit of quantitation (0.02 ppm) for one soil type per method. Soil (R1408640049) from BASF study no. 715267 was used for D1513/01 and soil (R1405960007) from BASF study no. 433578 was used for L0214/01. For each fortification level and matrix, five replicates were analyzed. Additionally, one reagent blank and two replicates of unfortified samples were examined.

1.2 Principle of the Method

D1513/01
The residues of BAS 750 F, and its metabolites, M750F003, and 1,2,4-Triazole are extracted from 0.1 grams of soil by shaking twice, each with 0.8 mL of acetonitrile:water (70:30, v/v). For analysis of BAS 750 F and M750F003, an aliquot (0.1 mL) from the combined extract is diluted with 0.4 mL of acetonitrile:water (10:90, v/v) for anlaysis by LC-MS/MS. For analysis of 1,2,4-Triazole, an aliquot (0.2 mL (from the combined extract is concentrated to 0.025 mL under nitrogen at 20 °C and reconstituted in water (0.475 mL) for analysis by LC-MS/MS. The mass transitions for BAS 750 F and it metabolites were monitored in positive mode.

L0214/01
The residues of BAS 750 F, and its metabolites, M750F003 and 1,2,4-Triazole are extracted from 5 grams of soil by shaking with a mixture of acetonitrile:water (70:30, v/v) for 30 minutes on a mechanical shaker at 225 rpm. After centrifugation (10 minutes at 4000 rpm) an aliquot (10 mL) is taken (extract 1) and the remaining supernatant is decanted and discarded. The same extraction procedure is repeated and after centrifugation a second 10-mL aliquot (extract 2) is combined with extract 1 and thoroughly mixed. Residues of BAS 750 F and M750F003 are analyzed by LC-MS/MS. For analysis of 1,2,4-Triazole, 5 mL of the combined extract 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 reconstituted to a volume of 1 mL with ultra-pure water and analyzed by LC-MS/MS. The mass transitions for BAS 750 F and it metabolites were monitored in positive mode.

1.3 Specificity

To demonstrate the specificity of the analytical method, an additional confirmatory mass transition was monitored simultaneous to the primary quantitation transition with the exception of analysis for M750F003 using Method D1513/01 and 1,2,4-Triazole using both methods.

Confirmation was then conducted using alternate chromatographic conditions as described in the respective method. Primary and confirmatory transitions for each analyte are listed below:

Analyte	Transition (*m/z*)			
	Method D1513/01		Method L0214/01	
	Primary	Confirmatory	Primary	Confirmatory
BAS 750 F	398 → 70	400 → 70	398 → 182	398 → 133
M750F003	288 → 70	288 → 70 [1]	288 → 159	288 → 103
1,2,4-Triazole	70 → 43	70 → 43 [2]	70 → 43	70 → 43 [3]

[1] Confirmatory analysis was conducted using the alternative chromatographic method, which uses the XBridge BEH Phenyl column (1.7 µm, 2.1 × 100 mm).
[2] Confirmatory analysis was conducted using the alternative chromatographic method, which uses the Unison UK-C18 column (3 µm, 3 × 75 mm) coupled to a Thermo Hypercarb column; (3 µm, 4.6 x 50 mm).
[3] Confirmatory analysis was conducted using the secondary chromatographic conditions, which uses the Synergi Hydro RP column (4 µm, 4.6 × 150 mm).

The methods were able to accurately determine residues of BAS 750 F and its metabolites. No interferences were observed at the retention time of the analyte peaks. Matrix enhancement was found to affect BAS 750 F, M750F003 (primary transition) and 1,2,4-Traizole (primary transition) in soil using analytical method D1513/01; therefore, matrix-matched standards were used. No matrix suppression or enhancement was found to affect M750F003 (confirmatory transition) and 1,2,4-Triazole (confirmatory transition) in soil using analytical method D1513/01; therefore, solvent-based standards were used. Matrix enhancement was found to affect all analytes in soil using analytical method L0214/01; therefore, matrix-matched standards were used.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test System

The test system considered in this study was soil.

The control soil sample was homogenized and provided by BASF, and sent from BASF on July 13, 2016. The control sample was received on July 14, 2016. Upon arrival at the laboratory, the sample was opened, inspected, and checked against enclosed shipping forms. The test system was received frozen and stored under frozen conditions at all times, unless necessary for laboratory analysis. The test system was characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267). A copy of the characterization data for the sample is provided in Appendix F.

A unique laboratory analysis code (e.g., 160729001-001) was provided by the Laboratory Information Management System (LIMS), which is cross-referenced to the BASF sample identification number on detailed analytical data reports.

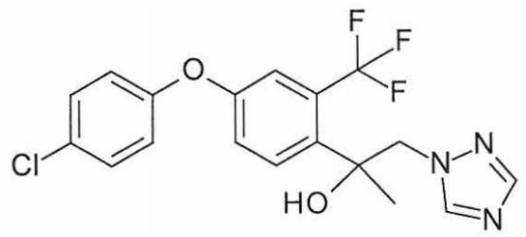
2.2 Test and Reference Substances

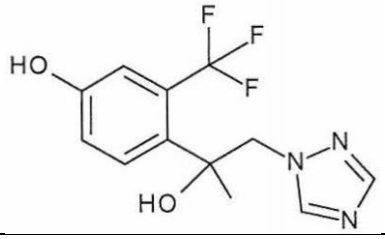
The reference substances of BAS 750 F, M750F003 and 1,2,4-Triazole were provided by the sponsor and received on July 14, 2016. Reference substances were stored in freezer E-119 (≤ -5°C) until use. BASF has retained a reserve sample of this chemical, and has

documentation specifying the location of the synthesis and characterization information available at BASF Crop Protection, Research Triangle Park, North Carolina.

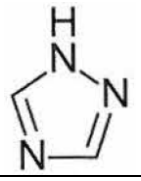
The certificates of analysis for all substances are presented in Appendix B. A summary of reference substance information is presented below.

Reference Substances:

BAS Code:	BAS 750 F
BASF Internal Code:	M750F000
Common Name:	Mefentrifluconazole
IUPAC Name:	(2RS)-2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
BASF Reg. Number:	5834378
CAS Number:	1417782-03-6
Batch Number:	L85-124
Molecular Formula:	$C_{18}H_{15}ClF_3N_3O_2$
Molecular Weight:	397.8 g/mol
Purity:	99.7%
Expiration Date:	July 1, 2017
Chemical Structure:	

BASF Internal Code:	M750F003
IUPAC Name:	4-[2-hydroxy-1-(1H-1,2,4-triazol-1-yl)propan-2-yl]-3-(trifluoromethyl) phenol
BASF Reg. Number:	5924326
Batch Number:	L84-250
Molecular Formula:	$C_{12}H_{12}F_3N_3O_2$
Molecular Weight:	287.2 g/mol
Purity:	99.6%
Expiration Date:	May 1, 2017
Chemical Structure:	

Reference Substances (Continued):

Common Name:	Triazole
IUPAC Name:	1,2,4-(1H)-triazole
BASF Reg. Number:	87084
CAS Number:	288-88-0
Batch Number:	AC10194-134
Molecular Formula:	$C_2H_3N_3$
Molecular Weight:	69.1 g/mol
Purity:	99.0%
Expiration Date:	April 1, 2022
Chemical Structure:	

3. ANALYTICAL METHODS

BASF Analytical Method D1513/01: "Validation of BASF Method D1513/01: Method for the Determination of Residues of BAS 750 F (Reg. No. 5834378) and its Metabolites, M750F003 (Reg. No. 5924326) and 1,2,4-Triazole (Reg. No. 87084) in Soil by LC-MS/MS using Micro-Extraction Procedure".

BASF Analytical Method L0214/01: "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" were used for the analysis of soil samples.

D1513/01
The residues of BAS 750 F and its metabolites, M750F003 and 1,2,4-Triazole, are extracted from 0.1 grams of soil by adding 0.8 mL of acetonitrile:water (70:30, v/v) twice. An aliquot (0.7 mL) is taken from the extract. Exactly 0.1 mL extract is added to an Alpha Tube with 0.4 mL of acetonitrile:water (10:90, v/v). The 0.5 mL final volume is vialed for LC-MS/MS determination.

L0214/01
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 cenlrifugation (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 BAS 750 F and metabolite M750F003 are directly analyzed by LC-MS/MS. For analysis of 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 reconstituted to a volume of 1 mL with ultra-pure water and analyzed by LC-MS/MS.

Instrument parameters for the methods are described in Tables 14 and 15 for BASF analytical methods D1513/01 and L0214/01.

The primary (quantitative) and secondary (confirmatory) transition ions monitored are presented below:

Method D1513/01					
Analyte	**Quantitation Method**	**Transition (*m/z*)**		**Ionization Mode**	**Retention Time (min)**
		Primary	**Confirmatory**		
BAS 750 F	Primary	398 → 70	400 → 70	Positive	3.0
M750F003	Primary	288 → 70	288 → 103[1]		2.1
	Alternate	288 → 70	--		2.0
1,2,4-Triazole	Primary	70 → 43	--		1.6
	Alternate	70 → 43	--		1.4

[1] Secondary transition did not have sufficient sensitivity for quantitation and was not validated.

Method L0214/01					
Analyte	**Quantitation Method**	**Transition (*m/z*)**		**Ionization Mode**	**Retention Time (min)**
		Primary	**Confirmatory**		
BAS 750 F	Primary	398 → 182	398 → 133	Positive	5.3
M750F003	Primary	288 → 159	288 → 103		4.2
1,2,4-Triazole	Primary	70 → 43	--		1.7
	Alternate	70 → 43	--		1.9

5. SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Tables 14 and 15.

Summary of Method

Type of Method	LC-MS/MS
Test Systems	Soil

Selected mass transitions (m/z)	**D1513/01** BAS 750 F 398 → 70* 400 → 70 M750F003 288 → 70* 288 → 70 1,2,4-Triazole 70 → 43* 70 → 43 **L0214/01** BAS 750 F 398 → 182* 398 → 133 M750F003 288 → 159* 288 → 103 1,2,4-Triazole 70 → 43* 70 → 43 *Primary quantification transition
Analytical Procedure	BASF Analytical Method D1513/01: " Validation of BASF Method D1513/01: Method for the Determination of Residues of BAS 750 F (Reg. No. 5834378) and its Metabolites, M750F003 (Reg. No. 5924326) and 1,2,4-Triazole (Reg. No. 87084) in Soil by LC-MS/MS using Micro-Extraction Procedure" (Reference 1) and BASF Analytical Method L0214/01: "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" (Reference 2)
Confirmatory Technique (D1513/01)	A secondary MRM transition for BAS 750 F (*m/z* 400 → *m/z* 70) and M750F003 (*m/z* 288 → *m/z* 70) and 1,2,4-Triazole (*m/z* 70 → *m/z* 43) was used for confirmation.
Confirmatory Technique (L0214/01)	A secondary MRM transition for BAS 750 F (*m/z* 398 → *m/z* 133) and M750F003 (*m/z* 288 → *m/z* 103) and 1,2,4-Triazole (*m/z* 70 → *m/z* 43) was used for confirmation.
Method of Quantitation	The quantitation is based on the monitoring of two mass transitions for BAS 750 F, M750F003 and 1,2,4-Triazole. Recovery data was reported for each mass transition considered.
LOD	0.0004 ppm (50% of LOQ)
LOQ	0.002 ppm (lowest fortification level)

Levels of Fortification	0.002 ppm and 0.02 ppm
Time Required	A set of 16 samples requires approximately 12 hours of work (calculation of the results included).
Justification of Ions	The ions used to conduct the ILV were determined in this validation and are shown in Appendix G.

6. DISCUSSION

Recovery Findings
Methods D1513/01 and L0214/01 proved to be suitable to determine residues of BAS 750 F, M750F003 and 1,2,4-Triazole in soil matrices to a LOQ of 0.002 ppm. The mean recovery values of the validation experiments were within 70–120%, which fulfills the guideline requirements for mean recovery values.

Linearity (D1513/01). Acceptable linearity was observed for the standard range using 5–6 levels of standards and the two mass transitions tested: The method-detector response was linear over the 0.005–0.25 ng/mL range for BAS 750 F and M750F003, 0.01–0.50 ng/mL range for 1,2,4-Triazole (primary transition) and 0.01–1.0 ng/mL range for 1,2,4-Triazole (confirmatory transition) (r=≥0.990), for soil analyses.

Linearity (L0214/01). Acceptable linearity was observed for the standard range and the two mass transitions tested: The method-detector response was linear over the 0.025–3 ng/mL range for BAS 750 F and M750F003 and 0.125–15 ng/mL range for 1,2,4-Triazole (r = ≥0.990), for soil analyses.

Specificity
Methods D1513/01 and L0214/01 determine residues of BAS 750 F, M750F003 and 1,2,4-Triazole in soil matrices. No interfering peaks were found at the retention time for BAS 750 F and its metabolites.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The limit of quantitation (LOQ) is defined as the lowest fortification level tested. For soil, the LOQ is 0.002 mg/kg (ppm). The limit of detection (LOD) is set at 0.0004 mg/kg, which is at 20% of the LOQ during this ILV.

Repeatability
The overall relative standard deviation (RSD, %) for all fortification levels were below 20%.

It was demonstrated that the methods D1513/01 and L0214/01 fulfill the requirements with regards to specificity, repeatability, limit of quantification, limit of detection, linearity and recoveries and are therefore applicable to correctly determine residues of BAS 750 F, M750F003 and 1,2,4-Triazole in soil matrices.

7. RECOMMENDATIONS/CONCLUSIONS FROM ILV

This independent laboratory validation was successfully completed on the first trial for all analytes at ADPEN Laboratories, Inc. Recovery results and statistical data demonstrate BASF Analytical Method D1513/01 and L0214/01 can be performed successfully for quantitation of BAS 750 F, M750F003 and 1,2,4-Triazole in soil.

Table 13 Example Standard Solutions Preparation and Dilution Data

Stock Standard Solutions

Standard ID#	Analyte	Parent Standard ID#	Adjusted Net Weight (mg)	Dilution Volume (mL)[1]	Final Conc. (ppm)	Prep. Date
C8366	BAS 750 F	P5566	9.85	10	985	7/25/2016
C8367	M750F003	P5567	9.26	10	922	7/25/2016

[1] Prepared in acetonitrile

Standard ID#	Analyte	Parent Standard ID#	Adjusted Net Weight (mg)	Dilution Volume (mL)[2]	Final Conc. (ppm)	Prep. Date
C8368	1,2,4-Triazole	P5568	10.66	10	1066	7/25/2016

[2] Prepared in water

Intermediate Standard Solution

Standard ID#	Analyte	Parent Standard ID#	Parent Conc. (ppm)	Aliquot Volume (mL)	Dilution Volume (mL)[3]	Final Conc. (ppm)	Prep. Date
I8959	BAS 750 F	C8366	985	1.02	10	100	7/25/2016
	M750F003	C8367	922	1.08			

[3] Prepared in acetonitrile:water (70:30, v/v)

Standard ID#	Analyte	Parent Standard ID#	Parent Conc. (ppm)	Aliquot Volume (mL)	Dilution Volume (mL)[4]	Final Conc. (ppm)	Prep. Date
I8960	1,2,4-Triazole	C8368	1066	0.938	10	100	7/25/2016

[4] Prepared in water

Calibration Standard Solutions

Standard ID#	Analyte	Parent Standard ID#	Parent Conc. (ppb)	Aliquot Volume (mL)	Dilution Volume (mL)[5]	Final Conc. (ppb)	Prep. Date
W13164	BAS 750 F, M750F003 (2-Mix)	W13163-4	10	3	10	3.0	7/25/2016
		W13163-4	10	1.25		1.25	
		W13163-4	10	0.5		0.5	
		W13163-4	10	0.25		0.25	
		W13164-2	1.25	1		0.125	
		W13164-3	0.5	1		0.05	
		W13164-4	0.25	1		0.025	

[5] Prepared in acetonitrile:water (70:30, v/v)

Table 13 Example Standard Solutions Preparation and Dilution Data (Continued)

Calibration Standard Solutions (Continued)

Standard ID#	Analyte	Parent Standard ID#	Parent Conc. (ppb)	Aliquot Volume (mL)	Dilution Volume (mL)[6]	Final Conc. (ppb)	Prep. Date
W13166	1,2,4-Triazole	W13165-3	100	1.5	10	15	7/25/2016
		W13165-3	100	1		10	
		W13165-3	100	0.625		6.25	
		W13165-3	100	0.25		2.5	
		W13166-2	10	1.25		1.25	
		W13166-2	10	1		1.0	
		W13166-2	10	0.5		0.5	
		W13166-3	6.25	1		0.625	
		W13166-4	2.5	1		0.25	
		W13166-5	1.25	1		0.125	
		W13166-10	0.5	1		0.05	
		W13166-7	0.25	1		0.025	
		W13166-8	0.125	0.8		0.01	

[6] Prepared in water

Table 14 Instrument Conditions and Parameters for BAS 750 F and M750F003 (D1513/01)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Acquity UPLC BEH C18; 1.7 µm, 2.1 × 50 mm; SN: 02433414615758		
Temperature:	50 °C		
Flow rate (µL/min):	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	95.0	5.0
	0.25	95.0	5.0
	1.50	60.0	40.0
	2.50	1.0	99.0
	3.45	1.0	99.0
	3.50	95.0	5.0
	4.00	95.0	5.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	40 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #28)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	35.00					
Internal Standard (IS):	4500.00					
Temperature (TEM):	550 °C					
Collision gas setting (CAD):	Medium					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
BAS 750 F Reg. No. 5834378	398 → 70	50.00	62.10	60.10	11.20	3.0
	400 → 70			68.30	11.60	
M750F003 Reg. No. 5924326	288 → 70	200.00	105.00	52.90	7.70	2.1

Table 15 Instrument Conditions and Parameters for M750F003 (D1513/01) used for Confirmation

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	XBridge BEH Phenyl; 1.7 µm, 2.1 × 100 mm; SN: 01403526518380		
Temperature:	50 °C		
Flow rate (µL/min):	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85.0	15.0
	0.05	85.0	15.0
	1.75	55.0	45.0
	2.50	1.0	99.0
	3.45	1.0	99.0
	3.50	85.0	15.0
	6.00	85.0	15.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	40 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5500.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
M750F003 Reg. No. 5924326	288 → 70	200.00	105.00	52.90	7.70	2.0

Table 16 Instrument Conditions and Parameters for 1,2,4-Triazole (Primary Transition) (D1513/01)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Hypercarb; 3 µm, 4.6 x 100 mm; SN:0790132T		
Temperature:	30 °C		
Flow rate (µL/min):	800		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	95.0	5.0
	2.50	90.0	10.0
	2.60	5.0	95.0
	3.60	5.0	95.0
	3.70	95.0	5.0
	5.00	95.0	5.0
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	50 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5000.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
1,2,4-Triazole Reg. No. 87084	70 → 43	200.00	148.00	28.00	12.00	1.6

Table 17 Instrument Conditions and Parameters for 1,2,4-Triazole (Confirmatory Transition) (D1513/01)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Unison UK-C18; 3 µm, 3 x 75 mm (SN:HK11A9F) couple to a Thermo Hypercarb; 3 µm, 4.6 x 50 mm (SN:0361529A)		
Temperature:	30 °C		
Flow rate (µL/min):	1000		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	99.0	1.0
	3.00	99.0	1.0
	3.50	20.0	80.0
	4.55	20.0	80.0
	5.55	99.0	1.0
	6.00	99.0	1.0
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	20 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5000.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
1,2,4-Triazole Reg. No. 87084	70 → 43	200.00	148.00	28.00	12.00	1.4

Table 18 Instrument Conditions and Parameters for BAS 750 F and M750F003 (L0214/01)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Aquasil C18; 3 µm, 3 × 150 mm; SN: 10334114		
Temperature:	25 °C		
Flow rate (µL/min):	800		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	95.0	5.0
	1.80	95.0	5.0
	1.90	70.0	30.0
	3.50	10.0	90.0
	3.60	1.0	99.0
	4.70	1.0	99.0
	6.40	1.0	99.0
	6.50	95.0	5.0
	8.00	95.0	5.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	40 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5000.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
BAS 750 F Reg. No. 5834378	398 → 182	200.00	62.10	44.00	8.00	5.3
	398 → 133			97.80	17.40	
M750F003 Reg. No. 5924326	288 → 159	200.00	105.00	41.10	8.10	4.2
	288 → 103			56.80	14.70	

Table 19 Instrument Conditions and Parameters for 1,2,4-Triazole (Primary Transition) (L0214/01)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Synergi Hydro RP; 4 µm, 3.9 x 150 mm; SN:H16-185400		
Temperature:	40 °C		
Flow rate (µL/min)	1000		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	99.0	1.0
	3.00	95.0	5.0
	3.10	1.0	99.0
	3.60	1.0	99.0
	3.70	99.0	1.0
	5.00	99.0	1.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	20 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5000.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
1,2,4-Triazole Reg. No. 87084	70 → 43	200.00	148.00	28.00	12.00	1.7

Table 18 Instrument Conditions and Parameters for 1,2,4-Triazole (Confirmatory Transition) (L0214/01) (Continued)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC System		
Column:	Hypercarb; 3 µm, 4.6 x 100 mm (SN:0790132T)		
Temperature:	30 °C		
Flow rate (µL/min)	1000		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	95.0	5.0
	2.50	90.0	10.0
	2.60	5.0	95.0
	3.60	5.0	95.0
	3.70	95.0	5.0
	5.00	95.0	5.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	20 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 5500 (Instrument #25)					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	45.00					
Internal Standard (IS):	5000.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	12.00					
GS1:	35.00					
GS2:	35.00					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	**Transition (m/z)**	**Dwell (msec)**	**DP**	**CE**	**CXP**	**Retention Time (min)**
1,2,4-Triazole Reg. No. 87084	70 → 43	200.00	148.00	28.00	12.00	1.8

Figure 29 Reside Calculations for Soil Matrices

Peak integration and quantitation were performed within Analyst® 1.6.2 software; using the calibration curve equation to determine amount of analyte found (ng) during sample analysis. Recovery results and concentration found (ppm) were calculated for each set of samples within LIMS and reported in Microsoft® Office Excel spreadsheet data reports, which are presented in Appendix C.

For the validation recoveries, the exact sample weight was used in calculating the final residues (ppm).

The following equations are used for residue and recovery calculations for BAS 750 F and its metabolites in soil.

a) Calibration curve: $y = mx + b$ Solving for x: $x = \frac{y-b}{m}$

Where, m = slope
b = y-intercept
x = Amount found (ng)
y = Peak area

b) Amount of sample injected (mg)= $\frac{(\text{injection size (mL)} \times \text{sample wt. (g)})}{\text{final sample volume (mL)}} \times \frac{1000 \text{ mg}}{1 \text{ g}}$

c) Residue found (ppm) = $\frac{\text{Amount found (ng)}}{\text{Amount of sample injected (mg)}}$

d) Recovery (%) = $\frac{\text{Residue in sample (ppm)}}{\text{Amount fortified (ppm)}} \times 100$

As an example, calculations to obtain BAS 750 F (primary transition) recovery results using 16072908-Recovery1-1 from work order WO-16072908 (soil) are shown below:

a) Calibration curve: $y = (4.05e+006)x + 313$

Solving for x: $x = \frac{21498 - 313}{4.05e+006} = 0.00522$ ng

b) Amount of sample injected (mg) = $\frac{0.04 \text{ mL} \times 5.01 \text{ g}}{80.0 \text{ mL}} \times \frac{1000 \text{ mg}}{1 \text{ g}} = 2.505$ mg

c) Residue found (ppm) = $\frac{0.00522 \text{ ng}}{2.505 \text{ mg}} = 0.0021$ ppm

d) Recovery (%) = $\frac{0.0021 \text{ ppm}}{0.002 \text{ ppm}} \times 100 = 104\%$

Statistical treatment of the data included calculation of means, standard deviations (SD), and percent relative standard deviations (%RSD). These calculations were performed using Microsoft® Excel and LIMS software. Results were rounded only for reporting purposes. No calculations were made with rounded numbers.

Appendix A. Recommendations for BASF Analytical Methods D1503/01 and L0214/01

The following are recommendations to BASF Analytical Method D1513/01:

1. A residual background of triazole was observed during the validation study, which could be greater than the two lowest calibration standards (0.01 ng/mL and 0.025 ng/mL). A portion of this residual background appears to result from the use of Thermo Scientific SepraSeal caps (Part # 4463) during extraction. Alterative caps should be explored to minimize residual background of triazole.
2. The technical procedure has a descrepancy for the particle size of the analytical column to be used for the confirmatory analysis of M750F003. The equipment list in section 2.3 states the partcile size should be 1.7 µm and the alternative chromatographic method in section 4.2.2 states it should be 2.5 µm. The correct Waters Corporation part number is listed in the equipment list for the column with 1.7 µm particle size (186002885).

No recommendations are necessary for BASF Analytical Method L0214/01/01.

ABSTRACT

BASF Method D1513/01 was developed to determine the residues of BAS 750 F (Reg No. 5834378) and Metabolites M750F003 (Reg No. 5924326) and 1,2,4-Triazole (Reg No. 87084) in soil using LC-MS/MS at BASF Corporation, Research Triangle Park, N.C.

A brief description of the method is provided below:

A 0.1 g soil sample aliquot is extracted shaking twice with a mixture of acetonitrile-water (70:30, v/v) using 0.8 mL for each extraction. For analysis of BAS 750 F and M750F003, an aliquot (0.1 mL) from the combined extract is diluted with acetonitrile-water (10:90, v/v, 0.4 mL) for analysis by LC-MS/MS.

For analysis of 1,2,4-Triazole, an 0.2 mL aliquot is concentrated to 0.025 mL under nitrogen at 20ºC and reconstituted again in water (0.475 mL) for the LC-MS/MS for analysis.

The method has a limit of quantitation of 0.002 mg/kg in soil for each analyte and is defined as the lowest fortification level tested. The limit of detection in soil is set at 0.0004 mg/kg which is at 20% of LOQ. The LOD is defined as the absolute amount (0.05 pg) of analyte injected into the LC-MS/MS parameters using lowest standard of the calibration.

DEFINITIONS AND ACRONYMS

Sample Set:	A group of samples that are extracted and cleaned up at the same time using the same method represented.
Untreated Sample:	A sample that has not been treated with the test substance.
Control Sample:	Usually an untreated sample used for fortification experiments (can be acquired from same study or from a different source).
Unknown Sample:	The samples with unknown residues.
Treated Sample:	A sample that has been treated with the test substance.
Blank:	Solvent, solution or mobile phase injected together with a sample set.
Reagent Blank:	A complete analysis conducted using solvents and reagents only in absence of any sample. Also known as blank of reagents or procedural blank. This sample is analyzed within the sample set in order to evaluate possible contamination on chemicals/reagents.
Procedural Recovery:	A control sample to which a known amount of analyte has been added before sample work up. This sample is then carried through the method and analyzed with the unknown samples in order to determine the reliability of the method.
Instrument Recovery:	A control sample which is carried through the method and to which a known amount of analyte has been added before injection. This sample is analyzed within the sample set in order to evaluate the matrix effect in the instrument.
Analytical Run:	A group of samples that undergo a determinative measurement on an analytical instrument (such as GC, HPLC, CE, GC/MS, or LC/MS/MS) in a defined and continuous sequence under identical instrumental conditions.
Limit of Quantitation (LOQ):	Lowest tested concentration of the analyte in a sample that can be determined with acceptable accuracy and precision according to the method.
Limit of Detection (LOD):	Concentration of analyte equivalent to a defined percentage of the limit of quantitation of the method (e.g. 20% of LOQ). At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3-5 x baseline noise).

1. INTRODUCTION

Method D1513/01 was successfully tested during method development in different soil types.

BAS 750 F is a fungicide used in many crops. A residue analytical method (D1513/01), for the analysis of the BAS 750 F (Reg No. 5834378) and metabolites Reg No. 5924326 and 1,2,4-Triazole (Reg. No. 87084) in soil was developed using micro-extraction followed by LC-MS/MS determination at BASF RTP, NC.

The method limit of quantitation (LOQ) is 0.002 mg/kg and the limit of detection (LOD) is set at 0.0004 mg/kg which is 20 % of LOQ.

2. MATERIALS

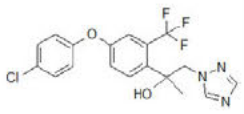
2.1 Safety

The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Store work clothing separately. Keep away from food, drink and animal feed stuffs. No eating, drinking, smoking or tobacco use at the place of work. Hands and/or face should be washed before breaks and at the end of the shift. Details are given in the Materials Safety Data Sheets (MSDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood.
Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

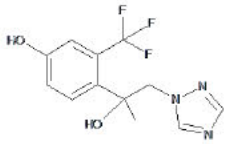
2.2 Test and Reference Item

Test and reference items should be stored according to the information provided in the certificate of analysis.

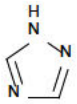
BAS-Code	750 F
Common Name	-/-
IUPAC Name	2-[4-(4-Chlorophenoxy)-2-(Trifluoromethyl)Phenyl]-1-(1H-1,2,4-Triazol-1-yl)Propan-2-ol
BASF Reg. No.	5834378
CAS-No.	-/-
Molecular Formula	$C_{18}H_{15}ClF_3N_3O_2$
Molecular Weight	397.8



BAS-Code	M750F003
Common Name	-/-
IUPAC Name	4-[2-Hydroxy-1-(1H-1,2,4 triazol-1-yl)Propan-2-yl]-3-(Trifluoromethyl)Phenol
BASF Reg. No.	5924326
CAS-No.	-/-
Molecular Formula	$C_{12}H_{12}F_3N_3O_2$
Molecular Weight	287.2



BAS-Code	M750F001
Common Name	Triazole
IUPAC Name	1,2,4-(1H)-Triazole
BASF Reg. No.	87084
CAS-No.	288-88-0
Molecular Formula	$C_2H_3N_3$
Molecular Weight	69.1



2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Balance, Top Loader	Model PJ3600	Mettler DeltaRange	----
Beakers	Various sizes	PYREX Brand, VWR Scientific Products	13922-029
Bottle, Amber glass	Qorpak , 4 oz, Teflon® cap	VWR	89042-908
Centrifuge	Allegra 6	Bechman Coulter	----
Cylinder, Graduated	Various sizes	Various	----
Flask, Erlenmeyer, 24/40	1000 mL	Various	----
LC Vials	2 mL	Waters	600000669CV
Repeater Pipette	1000 µL, 250 µL, 25 µL	Gilson Microman	F148506G
Ultrasonic Bath	Branson 1210	Branson	----
Volumetric flask	10 mL, 25 mL, 50 mL	VWR – Class A	89041-924
Volumetric pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL, 10mL, 20 mL, 25 mL	VWR – Class A	13-650-2A
Vortex	Genie 2	VWR Scientific Products	14216-184
Plastic Micro Tubes	1.4 mL Alphanum tubes	Thermo Scientific	4253
Glass Micro Tubes	1.0 mL glass inserts	Waters	186001436
Quadra 3® NS	Model 300-110/112	TomTec	----
Tips	Non-sterilized polypropylene tips, 0.036 orifice	TomTec	196-205
Cap Mats	Cap mat for 96 well plates	Waters	186000856
Alpha Tube Caps	Alpha Tube Caps	Thermo Scientific	4463
Auto-vortexer	VX-2500 Multi-tube vortexer	VWR	58816-115
96-Well Nitrogen Evaporator	SPE Dry 96	Biotage	SD-9600-DHS-NA
UPLC	Acquity UPLC Classic System	Waters	----
Mass Spectrometer	Sciex 5500 Mass Spectrometer	AB Sciex	----
HPLC Column	Acquity UPLC BEH C18, 2.1 x 50 mm, 1.7 µm	Waters	186002350
HPLC Column	Hypercarb 100 x 4.6mm, 3 µm	Thermo Scientific	35003-104630
HPLC Column	Xbridge Phenyl, 2.1 x 100 mm, 2.5 µm	Waters	186002885
HPLC Column	Unison UK-C18, 75 x 3 mm, 3µm	Imtakt	UK033
HPLC Column	Hypercarb 50 x 4.6mm, 3 µm	Thermo Scientific	35003-054630

Note: The equipment and instrumentation listed above may be subs ituted by hat of similar specifications. The applicability is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

2.4 Reagents

2.4.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Formic acid (LC Mobile Phase Use)	Reagent Grade ≥95%	Sigma Aldrich	F0507-100 mL
Water	HPLC Grade	BDH Aristar Plus	87003-652
Acetonitrile	HPLC Grade	EMD	AX0145P-1

Note: Equivalent reagents and chemicals from o her suppliers may be substituted.

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solution	S1	Acetonitrile-Water (70:30, v/v) Add 700 mL of acetonitrile and 300 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Dilution solution	S2	Acetonitrile-Water (10:90, v/v) Add 100 mL of acetonitrile and 900 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution
Final Volume solution	S3	Acetonitrile-water (20:80, v/v) Add 200 mL of acetonitrile and 800 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution
HPLC mobile phase A	LC1	Formic Acid in Water (1000/1,v/v) Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	Formic Acid in Water (1000/10,v/v) Add 1000 mL of water and 10 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase C	LC3	Formic Acid in Acetonitrile (1000/1,v/v) Add 1000 mL of acetonitrile and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

Note: If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified.

2.4.3 Standard Solutions

Stock Solutions

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of analyte into a flask and add the required volume of solvent.

For example, weigh 10 mg BAS 750 F or M750F003 into a 10 mL volumetric flask. Dissolve and dilute to mark with **acetonitrile**. This creates a solution containing 1 mg/mL of BAS 750 F or M750F003 in **acetonitrile**. Ensure a complete homogeneous solution (e.g. by sonication and/or vortexing). The stock solutions for all other analytes are made in a similar fashion.

Alternatively, weigh 10 mg 1,2,4-Triazole into a 10 mL volumetric flask. Dissolve and dilute to mark with **water**. This creates a solution containing 1 mg/mL of 1,2,4-Triazole in **water**. Ensure a complete homogeneous solution (e.g. by sonication and/or vortexing).

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example using one of the following approaches:

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

A correction for purity is done if the purity is ≤ 95%. If the purity is > 95 % correction is optional.

Fortification Solutions

Prepare mixed standard solutions for fortification by combining stock solutions of each analyte (see above) in a flask. Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of Mixed Fortification Solutions

Take stock solution	Volume (mL)	Dilute with Acetonitrile to a final volume of (mL)	Concentration (µg/mL)
Reg. No. 5834378 Reg. No. 5924326	0.1 0.1	10	10
Take solution (µg/mL)	**Volume (mL)**	**Dilute with Acetonitrile to a final volume of (mL)**	**Concentration (µg/mL)**
10	2	10	2
2	1	10	0.2
10	0.1	10	0.1
0.2	1	10	0.02

Take stock solution	Volume (mL)	Dilute with Water to a final volume of (mL)	Concentration (µg/mL)
1,2,4-Triazole	0.1	10	10
Take solution (µg/mL)	**Volume (mL)**	**Dilute with Water to a final volume of (mL)**	**Concentration (µg/mL)**
10	2	10	2
2	1	10	0.2
10	0.1	10	0.1
0.2	1	10	0.02

Note: A different concentration scheme may be used, if other fortifica ion levels are needed for the analysis.
If necessary, the volume of solution prepared may be changed as long as the propor ions are not modified.

Calibration Standard Solutions

Prepare mixed standard calibration solutions for LC / MS-MS analysis by using the solutions that were prepared in Section "Fortification Solutions" in flasks. Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of Standard Solutions for Calibration (BAS 750 F and M750F003)

Take solution (ng/mL)	Volume (mL)	Dilute with S3[1] to a final volume of (mL)	Concentration (ng/mL)
100 (in ACN)[2]	0.5	50	1
1	12.5	50	0.25
1	5	50	0.1
1	1.25	50	0.025
1	0.5	50	0.01
1	0.25	50	0.005

[1] S3: acetonitrile-water (20:80, v/v)
[2] Solution is from “Fortification Solutions” above

Preparation of Standard Solutions for Calibration (1-2-4-Triazole)

Take solution (ng/mL)	Volume (mL)	Dilute with Water to a final volume of (mL)	Concentration (ng/mL)
100[1]	0.5	50	1
1	25	50	0.5
1	12.5	50	0.25
1	2.5	50	0.05
1	1.25	50	0.025
1	0.5	50	0.01

[1] Solution is from “Fortification Solutions” above

Note: A different concentration scheme may be used and addi ional standards may be prepared as needed.
If necessary, the volume of solution prepared may be changed as long as the propor ions are not modified.

Additional Information:

- Use amber bottles with Teflon-lined screw caps as storage containers for all standard solutions.

2.4.4 Stability of Standard Solutions

During method development BAS 750 F and M750F003 stock and fortification solutions in acetonitrile were shown to be stable (less than 10% decline) for 3 months. Additionally 1,2,4-Triazole solutions in water were shown to be stable (less than 10% decline) for 3 months.

During method development, it was shown that calibration solutions for BAS 750 F and M750F003 in acetonitrile-water (20:80 v/v) were stable (less than 10% decline) for at least 30 days.

An official evaluation of standard solution storage stability will be conducted in the validation of this method

BASF recommends that stock solutions (1 mg/mL) in acetonitrile be made fresh every three months. Dilution of stock solutions should be stored refrigerated no longer than one month or according to their established storage stability in a particular solvent.

3. ANALYTICAL PROCEDURE

3.1 Sample Preparation and Storage

Bulk soil samples are subjected to cryogenic homogenization using a Crusher Mill with liquid nitrogen and stored frozen (≤5°C) before analysis. Samples have to be adequately homogenized beforehand to assure that the aliquot taken for sample analysis with smaller sample size (0.1 g), is representative of the whole bulk sample received from the field.

3.2 Weighing and Fortification

For treated samples and control samples, weigh 0.1 ± 0.01 g of soil sample into a 1.4 mL Alpha Tube.

For fortified samples, weigh at this stage 0.1 ± 0.01 g of control soil sample into a 1.4 mL Alpha Tube and add fortification solution on the matrix.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	0.1 g	-	-	0.00 mg/kg
Fortification (LOQ)	0.1 g	0.02 µg/mL	10 µL	0.002 mg/kg *
Fortification (10xLOQ)	0.1 g	0.2 µg/mL	10 µL	0.02 mg/kg
Fortification (100xLOQ)	0.1 g	2 µg/mL	10 µL	0.2 mg/kg
Treated	0.1 g	-	-	-

* limit of quantification (LOQ)

Note: Volume of spiking solution added to generate the fortified sample should not exceed 10% of sample weight or volume.

3.3 Extraction of Sample Material

Following procedure is described for automation using Quadra 3® NS. A single or multi-channel automatic pipette could be used alternatively for solvent delivery. See Appendix A for an example of the automated liquid handling system program.

a. Add 0.8 mL of extraction solvent S1 (acetonitrile-water, 70:30, v/v) to the 1.4 mL Alpha Tube containing soil (Section 3.2).
b. Firmly cap the 1.4 mL Alpha Tube with a Matrix SepraSeal cap.
c. Vortex the 1.4 mL Alpha Tubes containing the soil upside down using a multi-tube vortexer for 2 minutes. Flip the 1.4 mL Alpha Tubes to an upright position and vortex using multi-tube vortexer for another 2 minutes.
d. Centrifuge the samples for 5 min at 4000 rpm.
e. Detach the SepraSeal cap from the 1.4 mL Alpha Tubes containing the soil.
f. Aliquot exactly **0.284 mL** of extract to a separate 1.4 mL Alpha Tube, cap this tube, and set it aside.
g. Remove exactly **0.366 mL** from the original extract in 1.4 mL Alpha tube in Step 3.3[e], and discard, leaving behind exactly **0.15 mL** on the soil marc.
h. Add 0.8 mL of extraction solvent S1 (acetonitrile-water, 70:30, v/v) to the 1.4 mL Alpha Tube containing soil (Step 3.3[e]).
i. Firmly cap the 1.4 mL Alpha Tube with a Matrix SepraSeal cap.

j. Vortex the 1.4 mL Alpha Tubes containing the soil upside down using a multi-tube vortexer for 2 minutes. Flip the 1.4 mL Alpha Tubes to an upright position and vortex using multi-tube vortexer for another 2 minutes.
k. Centrifuge the samples for 5 min at 4000 rpm.
l. Detach the SepraSeal cap from the 1.4 mL Alpha Tubes containing the soil in Step 3.3[h]**.**
m. Aliquot exactly **0.416 mL** from this extract and transfer it to the 1.4 mL Alpha Tube that was set aside in Step 3.3[f]. The 1.4 mL Alpha Tube with the soil can now be discarded.
n. Firmly cap the 1.4 mL Alpha Tube with a Matrix SepraSeal cap and vortex the combined extracts for 10 seconds. Proceed to Section 3.4 to prepare the samples for measurement.

Note: An explanation for the exact aliquots taken from each extract can be found in the section 4.3.

This could be method stopping point

3.4 Preparation for Measurement

Following procedure is described for automation using Quadra 3® NS. A single or multi-channel automatic pipette could be used alternatively for solvent delivery. See Appendix A for an example of the automated liquid handling system program.

3.4.1 For BAS 750 F and M750F003

a) Transfer exactly 0.1 mL of extract in step 3.3[n] to a 1.4 mL Alpha Tube and add exactly 0.4 mL of S2 (Acetonitrile-water, 10:90, v/v).
b) Firmly cap the 1.4 mL Alpha Tube with a Matrix SepraSeal cap and vortex the tubes for 10 seconds.
c) Transfer the contents of the 1.4 mL Alpha Tube to a 1 mL Waters Glass Insert for analysis on LC-MS/MS.
d) The final volume of 0.5 mL is true for residues of BAS 750 F and M750F003 at LOQ. Samples are ready for analysis.

In case of higher residues dilute with appropriate amounts of final volume solvent S3 (Acetonitrile-water, 20:80, v/v).

Note: Mixing he extract solvent with S2 requires significant agitation that can only be achieved through vortexing. Because he 1 mL Waters Glass inserts do not have individual caps, he solutions must be vortexed in 1.4 mL Alpha Tubes and then transferred to he 1 mL Waters Glass Inserts for analysis.

3.4.2 For 1,2,4-Triazole

a) Transfer exactly 0.2 mL from the extract in step 3.3[n] into a 1.4 mL Alpha Tube
b) Place the tube in a 96-well SPE Dry. Set the temperature of nitrogen under the samples to 40ºC and the temperature of nitrogen on top of the samples to 20ºC. Set the flow of both nitrogen streams to 70 mL/min. Dry for approximately 60 minutes to a volume of 0.025 mL, making sure not to go to dryness. Use a 1.4 mL Alpha Tube with exactly 0.025 mL of water as a reference.

Note: It may be necessary to check samples at 55 minutes, and at 5-7 minute increments after that, to remove them individually from the SPE Dry as hey get to the desired volume.

c) Add exactly 0.475 mL of water to the 1.4 mL Alpha Tubes.
d) Firmly cap the 1.4 mL Alpha Tube with a Matrix SepraSeal cap, sonicate the samples for 2 minute, then vortex the samples for 10 seconds.
e) Transfer the contents of the 1.4 mL Alpha Tube to a 1 mL Waters Glass Insert for analysis on LC-MS/MS.

All samples are now prepared at the limit of quantitation level (0.002 mg/kg). The final volume of 0.5 mL is true for residues of 1,2,4-Triazole at LOQ. Samples are ready for analysis.

In case of higher residues dilute with appropriate amounts of water to fit in calibration curve.

Note: Sonication and vortexing are needed to homogenize the samples. Because the 1 mL Waters Glass inserts do not have individual caps, the solutions must be sonicated and vortexed in 1.4 mL Alpha Tubes and then transferred to the 1 mL Waters Glass Inserts for analysis.

3.5 Influence of matrix effects on analysis

During method development no significant matrix effects were observed for soil matrices. If significant suppression occurs, matrix-matched standards may be utilized. Matrix-matched calibration standards are used for quantitation when signal suppression or enhancement is >20% compared to the response for standards prepared in calibration solution alone. Use the following tables below to prepare matrix matched standards if necessary.

1. Prepare precursor standards for matrix matched calibration standards in the following manner from the respective fortification solutions found in Section 2.4.3:

BAS 750 F and M750F003 Mixed Precursor Solutions

Take solution in Acetonitrile (ng/mL)	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (ng/mL)
100*	2.5	10	25
25	4	10	10
10	2.5	10	2.5
2.5	4	10	1
1	5	10	0.5

*This solution is prepared in Section 2.4.3 under the header “Fortification Solutions”

1,2,4-Triazole Precursor Solutions

Take solution (ng/mL)	Volume (mL)	Dilute with water to a final volume of (mL)	Concentration (ng/mL)
100*	5	10	50
50	5	10	25
25	2	10	5
5	5	10	2.5
2.5	4	10	1

*This solution is prepared in Section 2.4.3 under the header “Fortification Solutions”

2. When preparing five matrix match calibration standards, prepare at least five extra control samples by completing all steps through Section 3.4 (additional control matrix may need to be prepared to dilute samples with residues higher than LOQ).
3. Combine all the extracts from Section 3.5[2] above into one culture tube and vortex to ensure homogeneity. Keep extracts to be used for BAS 750 F and M750F003 separate from 1-24-Triazole extracts.
4. Prepare the matrix matched calibration standards according to the tables below, using the combined control extract from Section 3.5[3] and the precursor standards from Section 3.5[1]:

BAS 750 F and M750F003 Matrix Matched Standards

Take Precursor Solution (ng/mL)	Volume (mL)	Volume of Control Extract (mL)	Concentration (ng/mL)
25	0.01	0.99	0.25
10	0.01	0.99	0.1
2.5	0.01	0.99	0.025
1	0.01	0.99	0.01
0.5	0.01	0.99	0.005

1,2,4-Triazole Matrix Matched Standards

Take Precursor Solution (ng/mL)	Volume (mL)	Volume of Control Extract (mL)	Concentration (ng/mL)
50	0.01	0.99	0.5
25	0.01	0.99	0.25
5	0.01	0.99	0.05
2.5	0.01	0.99	0.025
1	0.01	0.99	0.01

3.6 Stability of Extracts and Final Volumes

Details are provided in **Appendix G.**

3.7 Moisture Determination

The procedural recoveries will not be corrected for moisture content of the sample. Results of soil analysis are reported on a “dry weight” basis for residue determination. Therefore field treated soil sample weights must be corrected for moisture content by any method the laboratory customarily uses. The moisture determination will be conducted for the treated samples with residue value above LOD. An example of a moisture determination procedure is provided below:

The percent moisture is determined using an automated moisture determination equipment (Mettler Toledo LP16) using the formula below:

Moisture content [%] = ((Weight moist soil - Weight dry soil)/Weight moist soil) x 100

4. QUANTIFICATION AND CALCULATION

4.1 Set-up of the analytical run

A sequence for measurement generally consists of:
- Calibration standards
- Control samples
- Procedural recovery samples
- Unknown samples
- Instrument recovery sample

Reagent Blanks or blanks can also be injected if necessary. Each injection set should begin and end with an injection of a calibration standard. Standards should be interspersed with samples. Each calibration standard should be at least injected twice. At least 5 calibration levels need to be injected.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions

BAS 750 F and M750F003 Primary Quantitation Method	**Parameter**		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Acquity UPLC BEH C18, 2.1 x 50 mm, 1.7 µm		
Column Temperature	50°C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	0.6 mL/min		
Gradient (including wash and equil bration)	Time (min)	Phase A	Phase B
	0.00	95	5
	0.25	95	5
	1.50	60	40
	2.50	1	99
	3.45	1	99
	3.5	95	5
	4.00	95	5
Detection System	AB Sciex API 5500 Mass Spectrometer		
Ionization	Turbo Spray (ESI)		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BAS 750 F	398 --> 70 400 --> 70*	positive	approx. 2.5 min
M750F003	288 --> 70* 288 --> 103**	positive	approx. 1.6 min.

*Proposed as the primary quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time

**Based on the sensitivity of the instrument, the secondary transitions for M750F003 may not be strong enough for quantitation. See section 4.2.2 for alternative chromatographic conditions for M750F003.

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of he fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on he detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the for ification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

4.2.2 Instrumentation and Conditions

M750F003 Alternative Chromatographic Method	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Xbridge Phenyl, 2.1 x 100 mm, 2.5 µm		
Column Temperature	50°C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	0.6 mL//min		
Gradient (including wash and equil bration)	Time (min)	Phase A	Phase B
	0.00	85	15
	0.05	85	15
	1.75	55	45
	2.50	1	99
	3.45	1	99
	3.50	85	15
	4.00	85	15
Detection System	AB Sciex API 5500 Mass Spectrometer		
Ionization	Turbo Spray (ESI)		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
M750F003	288 --> 70*	positive	approx. 2.1 min.

*Proposed as the primary quantification transition.

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of he fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on he detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the for ification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

4.2.3 Instrumentation and Conditions

1,2,4-Triazole Primary Quantitation Method	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Hypercarb, 100 x 4.6 mm, 3 µm		
Column Temperature	30°C		
Injection Volume	50-100 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 990/10, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	0.8 mL/min		
Gradient (including wash and equil bration)	Time (min)	Phase A	Phase B
	0.00	99	1
	1.50	97	3
	3.50	50	50
	4.50	50	50
	4.55	99	1
	5.00	99	1
Detection System	AB Sciex API 5500 Mass Spectrometer		
Ionization	Turbo Spray (ESI)		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
Reg. No. 87084	70 --> 43*	positive	approx. 2.9 min.

*Proposed as the primary quantification transition. Because there are no other quantitative transitions for 1,2,4-Triazole, see section 4.2.4 for alternative chromatographic conditions.

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of he fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on he detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the for ification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

4.2.4 Instrumentation and Conditions

1,2,4-Triazole Alternative Chromatographic Method	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Unison UK-C18 75 x 3 mm, 3 µm couple to a Thermo Hypercarb 50 x 4.6, 3 µm		
Column Temperature	30°C		
Injection Volume	50-100 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/10, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	1.0 mL/min		
Gradient (including wash and equil bration)	Time (min)	Phase A	Phase B
	0.00	99	1
	3.00	99	1
	3.50	20	80
	4.55	20	80
	5.55	99	1
	6.00	99	1
Detection System	AB Sciex API 5500 Mass Spectrometer		
Ionization	Turbo Spray (ESI)		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
Reg. No. 87084	70 --> 43*	positive	approx. 2.8 min.

*Proposed as the primary quantification transition.

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of he fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on he detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the for ification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

4.2.5 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected (i.e. required for enforcement) in duplicate. The calibration curve is obtained by direct injection of mixed standards of BAS 750 F and M750F003 at a range of 0.005 ng/mL to 0.25 ng/mL. A separate calibration curve is obtained by the direct injection of 1,2,4-Triazole standards at a range of 0.01 ng/mL to 0.5 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

Linear calibration functions are preferred for evaluation. If other functions are used (e.g. quadratic), this should be fully justified.

4.3 Calculation of Residues and Recoveries

4.3.1 Calculation of Residues

Calculation of results is based on area measurements. For the procedural recoveries, the sample weight will be considered 0.1 g in the final calculation of residues [mg/kg]. The method requires that the sample weight to be 0.1 ± 0.01 g for fortification samples. The recovery is the percentage of the fortified amount (µg or ng), which is recovered through the method and the weights cancels out, as shown in the equation below, during the final calculation step.

The residues of BAS 750 F in mg/kg are calculated as shown in equations **I** and **II**:

I. **Concentration [ng/mL]** $= \frac{\text{Response} - \textit{Intercept}}{\textit{Slope}} = C_A$

II. **Residue [mg/kg]** $= \frac{V_{end} \times C_A}{G \times A_F \times 1000}$

V_{end} = Final volume of the extract after all dilution steps [mL] (see Section 4.3.2)
C_A = Concentration of analyte as read from the calibration curve [ng/mL]
G = Weight of the sample extracted [g]
A_F = Aliquotation factor
= Aliquot taken from combined extract (Step 3.4.1)/Total volume of extract soln added
1000 = Factor remaining after all unit conversions

The recoveries of spiked compounds are calculated according to equation **III**:

III. **Recovery %** $= \frac{(\text{Residue in fortified sample} - \text{Residue in control}) \times 100}{\text{Amount of analyte fortified}}$

IV. **Soil residues based on soil dry weight**

Residue [mg/kg] (Dry residue) = $= \frac{\text{Wet Sample Residue [mg/kg]} \times 100}{(100 - \text{"moisture content [\%]"})}$

4.3.2 Calculation of Correction of Factor in the extraction step using Quadra3® NS

An automated solvent delivery system is limited to transfer the entire extract volume (0.8 mL) added from the extraction tube containing the soil marc, therefore, an Correction Factor ($CF_{Extract}$) is applied to the equal aliquots from both extraction steps before combining them. In this case, as each extraction volume is the same volume, equal aliquots from both extraction steps can be taken and combined to be called the combined extract. The effective extract volume will still be considered the sum of both extract volumes, not the volume of the combined extract.

The $CF_{Extract}$ in extraction steps is shown below:

$$CF_{Extract} = \frac{Volume\ Left\ After\ First\ Extraction\ Step\ (mL)}{Volume\ in\ First\ Extraction\ Step\ (mL)}$$

$$= \frac{0.15\ mL}{0.8\ mL} = 0.1875$$

Note: 0.15 mL is left behind after the first extraction because that is the volume that can accurately and consistency be left behind by the Quadra 3® NS.

0.8 mL is the total extract volume for each extraction step.

The $CF_{Extract}$ is applied to the equal aliquots from each extraction step as shown below:

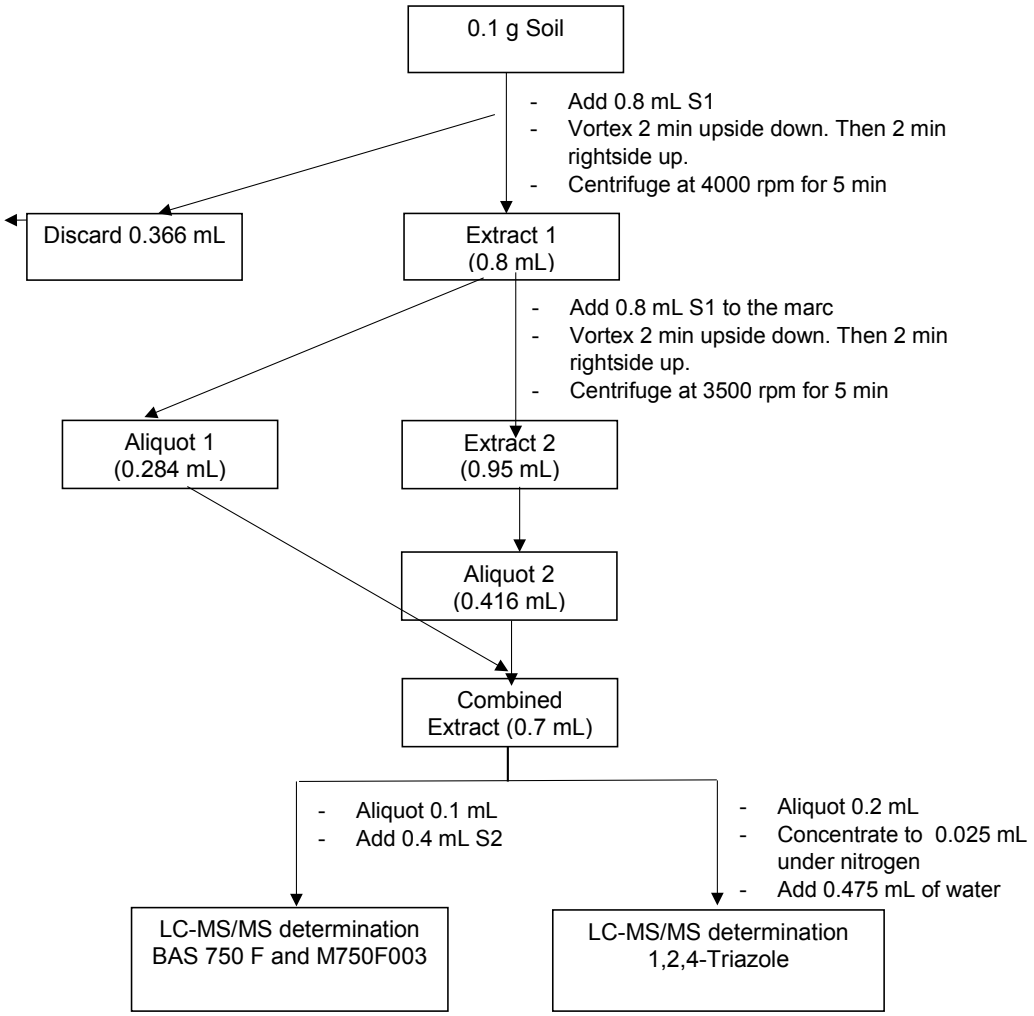
Aliquot taken from Extraction Step 1 = Equal Aliquot (mL) X (1 - $CF_{Extract}$)
Aliquot taken from Extraction Step 2 = Equal Aliquot (mL) X (1 + $CF_{Extract}$)

Aliquot taken from Extraction Step 1 = 0.35 mL X (1 – 0.1875)
Aliquot taken from Extraction Step 2 = 0.35 mL X (1 + 0.1875)

Note: The $CF_{Extract}$ represents the volume left behind from the first extraction. Since it is left behind in the first extraction step, 1-$CF_{Extract}$ is applied to the first aliquot. Because he volume left behind is incorporated into the second extraction step, 1+$CF_{Extract}$ is applied to the second aliquot. This ensures that over both aliquots, an equal portion of each extraction step is present.

0.35 mL is chosen as an equal aliquot for the calculations because it yields a final volume 0.7 mL for the combined extract; this is enough extract to make re-dilu ions from the extract if necessary.

5. FLOWCHART



S1 = Acetonitrile-Water (70:30, v/v)
S2 = Acetonitrile-Water (10:90, v/v)

8.2 Example Quadra 3® NS Method

Shuttle Layout

Position 1	Position 2	Position 3
Steps 1-48: PP Plate (Solvent) Steps 48-66: ACN-H_2O (10:90) Steps 66-End: 1 mL Glass Inserts (BAS 750 F and M750F003)	Steps 1-48: ACN-H_2O (70:30, v/v) Steps 48-End: Water	All Steps: Plastic Tips
Position 6	Position 5	Position 4
Steps 1-48: PP Plate (Discard) Steps 48-End: 1.4 mL Alpha Tubes (BAS 750 F and M750F003 mixing)	Steps 1-77: 1.4 mL Alpha Tubes (Combined Extracts) Steps 77-End: 1 mL Glass Inserts (Triazole FV)	Steps 1-48: 1.4 mL Alpha Tubes (Weighed Samples) Steps 48-End: 1.4 mL Alpha Tubes (Triazole mixing)

Program Steps

(1) Load Tips from Pos. 3
(2) Mix 400.0 ul @1355, 4 times, at ACN:Water (70:30, v/v) on 2
(3) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(4) Aspirate 400.0 ul @1345 from ACN:Water (70:30, v/v) on 2
(5) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(6) Empty Sample @950 to PP Plate (Solvent) on 1
(7) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(8) Aspirate 400.0 ul @1345 from ACN:Water (70:30, v/v) on 2
(9) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(10) Empty Sample @950 to PP Plate (Solvent) on 1
(11) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(12) Aspirate 200.0 ul @1345 from ACN:Water (70:30, v/v) on 2
(13) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(14) Empty Sample @950 to PP Plate (Solvent) on 1
(15) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(16) Aspirate 400.0 ul @1345 from ACN:Water (70:30, v/v) on 2
(17) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(18) Empty Sample @800 to 1.4 mL Alpha Tubes (Weighed Samples) on 4
(19) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(20) Aspirate 400.0 ul @1345 from ACN:Water (70:30, v/v) on 2
(21) Aspirate 10.0 ul @5 from ACN:Water (70:30, v/v) on 2
(22) Empty Sample @800 to 1.4 mL Alpha Tubes (Weighed Samples) on 4
(23) Pause Program at Pos. 3 (Preform steps 3.3[b-e])

(24) Mix 350.0 ul @1200, 1 times, at PP Plate (Solvent) on 1
(25) Aspirate 10.0 ul @0 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(26) Aspirate 284.0 ul @1260 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(27) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(28) Empty Sample @1100 to 1.4 mL Alpha Tubes (Combined Extracts)
(29) Aspirate 10.0 ul @0 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(30) Aspirate 366.0 ul @1300 from 1.4 mL Alpha Tubes (Weighed Samples) on 4

(31) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(32) Empty Sample @1100 to PP Plate (Discard) on 6
(33) Aspirate 10.0 ul @0 from PP Plate (Solvent) on 1
(34) Aspirate 400.0 ul @1300 from PP Plate (Solvent) on 1
(35) Aspirate 10.0 ul @35 from PP Plate (Solvent) on 1
(36) Empty Sample @800 to 1.4 mL Alpha Tubes (Weighed Samples) on 4
(37) Aspirate 10.0 ul @0 from PP Plate (Solvent) on 1
(38) Aspirate 400.0 ul @1350 from PP Plate (Solvent) on 1
(39) Aspirate 10.0 ul @35 from PP Plate (Solvent) on 1
(40) Empty Sample @750 to 1.4 mL Alpha Tubes (Weighed Samples) on 4
(41) Pause Program at Pos. 3 (Preform steps 3.3[i-l])
(42) Mix 100.0 ul @1400, 2 times, at PP Plate (Solvent) on 1
(43) Aspirate 10.0 ul @5 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(44) Aspirate 416.0 ul @1280 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(45) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Weighed Samples) on 4
(46) Empty Sample @1100 to 1.4 mL Alpha Tubes (Combined Extracts)
(47) Mix 300.0 ul @1350, 5 times, at 1.4 mL Alpha Tubes (Combined Extracts)
(48) Pause Program at Pos. 3 (Replace trays 1, 2, 4, and 6. See shuttle positions at the top of this section).

(49) Aspirate 10.0 ul @5 from 1.4 mL Alpha Tubes (Combined Extracts)
(50) Aspirate 100.0 ul @1300 from 1.4 mL Alpha Tubes (Combined Extracts)
(51) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Combined Extracts)
(52) Empty Sample @1000 to 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(53) Aspirate 10.0 ul @5 from 1.4 mL Alpha Tubes (Combined Extracts)
(54) Aspirate 200.0 ul @1375 from 1.4 mL Alpha Tubes (Combined Extracts)
(55) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Combined Extracts)
(56) Empty Sample @1000 to 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(57) Timed Dispense for 3 cycles @800 to 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(58) Shuck Tips to Pos. 3
(59) Pause Program at Pos. 1 (Replace tips)
(60) Load Tips from Pos. 3
(61) Mix 400.0 ul @1350, 4 times, at ACN-H2O (10:90, v/v) on 1, 50.0 ul Air Gap
(62) Aspirate 10.0 ul @35 from ACN-H2O (10:90, v/v) on 1
(63) Aspirate 400.0 ul @1350 from ACN-H2O (10:90, v/v) on 1
(64) Aspirate 10.0 ul @35 from ACN-H2O (10:90, v/v) on 1
(65) Empty Sample @800 to 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(66) Pause Program at Pos. 1 (Preform steps 3.4.1[b] and replace tray 1 (see shuttle positions at the top of this section)).

(67) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(68) Aspirate 300.0 ul @1380 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(69) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(70) Empty Sample @795 to 1 mL Glass Inserts (750 and M03) on 1
(71) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(72) Aspirate 250.0 ul @1380 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(73) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(74) Empty Sample @795 to 1 mL Glass Inserts (750 and M03) on 1
(75) Timed Dispense for 6 cycles @800 to 1.4 mL Alpha Tubes (750 and M03 Homogenization) on 6
(76) Shuck Tips to Pos. 3
(77) Pause Program at Pos. 1 (Preform step 3.4.2[b] and replace the tips and tray 5 (see shuttle positions at the top of this section)).

(78) Load Tips from Pos. 3
(79) Mix 400.0 ul @1355, 5 times, at Water on 2, 50.0 ul Air Gap
(80) Aspirate 10.0 ul @5 from Water on 2
(81) Aspirate 250.0 ul @1345 from Water on 2, 50.0 ul Air Gap
(82) Aspirate 10.0 ul @5 from Water on 2
(83) Empty Sample @800 to 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(84) Aspirate 10.0 ul @5 from Water on 2

(85) Aspirate 230.0 ul @1345 from Water on 2, 50.0 ul Air Gap
(86) Aspirate 10.0 ul @5 from Water on 2
(87) Empty Sample @800 to 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(88) Pause Program at Pos. 1 (Perform step 3.4.2[c])

(89) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(90) Aspirate 400.0 ul @1380 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(91) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(92) Empty Sample @800 to 1 mL Glass Inserts (Triazole FV)
(93) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(94) Aspirate 120.0 ul @1380 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(95) Aspirate 10.0 ul @40 from 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(96) Empty Sample @800 to 1 mL Glass Inserts (Triazole FV)
(97) Timed Dispense for 6 cycles @800 to 1.4 mL Alpha Tubes (Triazole Homogenization) on 4
(98) Shuck Tips to Pos. 3
(99) Quit Program at Pos. 5

Appendix E. BASF Analytical Method L0214/01 (Excerpt Pages Taken from the Validation Report Illustrating the Working Procedure)

Instrumental Analysis

Analytical Procedure

Weighing and Fortification

For preparation of untreated control samples, 5.0 g of soil was weighed into a 50 mL PP-tube. For preparation of fortified samples 5.0 g of soil was weighed into 50 mL PP-tube. Then, fortification solutions were added to the soil as shown in the following table:

Sample Type	Sample Weight	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [µL]	Level of Fortification [mg/kg]
Control	5 g	-	-	0.00
Fortification (LOQ)	5 g	0.1	100 µL	0.002 [1)]
Fortification (10xLOQ)	5 g	1	100 µL	0.02
Treated	5 g	-	-	-

[1)] Limit of quantification

Extraction of Sample Material

5.0 g soil aliquots were extracted with 40 mL of extraction solvent S1 (acetonitrile/water, 70/30, v/v) and shaken on a mechanical shaker for 30 min at 225 rpm at ambient room temperature. After centrifugation for 10 min at 4000 rpm at 20°C an aliquot of 10 mL (extract 1) was taken and the remaining supernatant was decanted and discarded. The extraction procedure was repeated and after centrifugation another aliquot of 10 mL (extract 2) was combined with extract 1 and thoroughly mixed to obtain a homogenous extract.

Preparation for Measurement

For Reg. No. 5834378 and Reg. No. 5924326:
For residues of Reg. No. 5834378 and Reg. No. 5924326 around the LOQ an aliquot of the combined extract (V_{end} = 80 mL, V_{end} = final volume) was transferred into a HPLC vial and analysed by LC-MS/MS. For samples containing higher residues, dilutions with appropriate amounts of S1 were performed.

For Reg. No. 87084:
5 mL of the combined extracts (extract 1 and extract 2, see section 2.4.1.2) were transferred into a tared culture tube and the volume was reduced in a nitrogen evaporator to a volume less than 1 mL, which was confirmed by weighing, assuming a density of 1 g/cm^3. The reduced extract was then filled up to a volume of 1 mL with ultra-pure water. The final volume of 16 mL was used for residues of Reg. No. 87084 at LOQ. For samples containing higher residues, dilutions with appropriate amounts of ultra-pure water were performed.
An aliquot of the reconstituted extract was transferred into HPLC vials and analysed by LC-MS/MS.

Instrumentation and Conditions

The chromatographic system and conditions used for analysis of Reg. No. 5834378 and Reg. No. 5924326 are shown in the table below.

Reg. No. 5834378 Reg. No. 5924326	Parameter		
Chromatographic System	Waters Acquity LC System		
Analytical-column	Aquasil C18, 150 x 3 mm, 3 µm particle size		
Column Temperature	25°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	800 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A [%]	Phase B [%]
	0.0	95	5
	1.8	95	5
	1.9	70	30
	3.5	10	90
	3.6	1	99
	4.7	1	99
	6.4	1	99
	6.5	95	5
	8.0	95	5
Detection System	AB Sciex API 5000 Mass Spectrometer		
Ionisation	Turbo Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 5834378	398 → 182 [1)] 398 → 133	positive	approx. 4.5 min
Reg. No. 5924326	288 → 159 [1)] 288 → 103	positive	approx. 3.5 min

[1)] Proposed as quantification transition for further studies based on the validated method described in this report. Any of these transitions could be used for quantification in case interference is observed at the same retention time, but proposal is based on the higher signal-to-noise ratio and/or higher overall response. During method validation, both mass transitions were used for quantification to confirm validity.

For analysis of Reg. No. 87084 two different analytical columns were used as only one mass transition was investigated. The conditions for both analytical conditions are shown in the tables below.

Reg. No. 87084	Parameter		
Chromatographic System	Waters Acquity LC System		
Analytical-column	Hypercarb™ 100 x 4.6 mm, 5 µm particle size		
Column Temperature	30°C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	1.0 mL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A [%]	Phase B [%]
	0.0	95	5
	2.5	90	10
	2.6	5	95
	3.6	5	95
	3.7	95	5
	5.0	95	5
Detection System	AB Sciex API 5000 Mass Spectrometer		
Ionisation	Turbo Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 87084	70 → 43	positive	approx. 1.80 min

Reg. No. 87084	Parameter		
Chromatographic System	Waters Acquity LC System		
Analytical-column	Synergi Hydro RP 150 x 4.6 mm, 4 µm particle size		
Column Temperature	40°C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	1.0 mL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A [%]	Phase B [%]
	0.0	99	1
	3.0	95	5
	3.1	1	99
	3.6	1	99
	3.7	99	1
	5.0	99	1
Detection System	AB Sciex API 5000 Mass Spectrometer		
Ionisation	Turbo Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 87084	70 → 43	positive	approx. 2.25 min

Calibration Procedure

Calculation of results was based on the peak area measured using a calibration curve. Seven calibration levels were injected per calibration curve. Calibration curves were obtained by direct injection of Reg. No. 5834378 and Reg. No. 5924326 mix standards covering a concentration range of 0.025 ng/mL to 3 ng/mL and Reg. No. 87084 standards over a range of 0.125 ng/mL to 15 ng/mL. The same volume was injected for all samples and standards.

Influence of Matrix Effects on the Analysis

In order to assess the influence of the matrix effects on the analysis, the response of each analyte in the presence of matrix was compared to standards prepared in pure solvent or aqueous mixtures of such (see Table 24 to Table 45). Calibration standard solutions prepared in S1 solvent (Reg. No. 5834378 and Reg. No. 5924326) or pure water (Reg. No. 87084)) were compared to their respective calibration standards prepared in blank matrix extracts. For preparation of matrix-matched standards, refer to chapter 2.3.2.3.

Quality Control Samples QCS

Quality control samples (QCS) were prepared at a concentration of 0.125 ng/mL for Reg. No. 5834378 and Reg. No. 5924326 and 0.625 ng/mL for Reg. No. 87084, corresponding to the limit of quantification (LOQ = 0.002 mg/kg) of each analyte. Quality control samples were analysed together within each set of samples in order to generally assess the impact of the matrix on the overall performance of the instrument without addressing any influences arising during sample preparation.

Calculation of Residues and Recoveries

For the procedural recoveries, the sample aliquot was considered 5 g in the final calculation of residues [mg/kg]. The method requires that the sample aliquot was 5.0 g for fortification samples. The residues in [mg/kg] are calculated as shown in equations I and II.

I. Concentration Final Volume [ng/mL]

$$= \frac{Response - Intercept}{Slope} = C_A$$

II. Residues in the Sample Matrix [mg/kg]

$$= \frac{V_{end} \times C_A}{G \times A_F \times 1000}$$

V_{end}	=	Final volume of the extract after all dilution steps [mL]
C_A	=	Concentration of analyte obtained from the calibration curve [ng/mL]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquotation factor = 1.0 (= 100%)
1000	=	Factor remaining after all unit conversions

Recovery is the percentage of the fortified amount of the analyte, which is recovered through the method. The recoveries of spiked compounds are calculated according to equation III:

III. **Recovery corrected [%]** $= \frac{(Residue\ in\ fortified\ sample - Residue\ in\ control) \times 100}{Amount\ of\ analyte\ fortified}$

IV. **Soil residues based on soil dry weight**

Residue [mg/kg] (Dry residue) $= \frac{Wet\ Sample\ Residue\ [mg/kg] \times 100}{(100 - "moisture\ content\ [\%]")}$

Example of Calculation:

Reg. No. 5834378 (Mass transition 398→182) in soil fortified at 0.002 mg/kg:

The following values were used in this calculation:

Worklist no.	*2014ldc0108*
Peak area of fortified sample (ForL0083)	*13148.7*
Peak area of control sample [1] (ConL0029, ConL0030)	*0.0*
Slope	*102000*
Intercept	*793*
Sample Aliquot	*5 g*
Final Volume (V_{end})	*80 mL*
Aliquotation Factor (A_F)	*1.0 (= 100%)*

[1] Mean area of two control samples in the same worklist

Concentration of fortified sample (C_A) $= \frac{13148.7 - 793}{102000} = 0.121\ ng/mL$

Residue (fortified sample) $= \frac{80\ mL \times 0.121\ ng/mL}{5\ g \times 1.0 \times 1000} = 0.001936\ mg/kg$

Residue (untreated sample) $= \frac{80\ mL \times 0.0\ ng/mL}{5\ g \times 1.0 \times 1000} = 0.0\ mg/kg$

Recovery [%] $= \frac{0.001936\ mg/kg}{0.002\ mg/kg} \times 100\ \% = 96.8\ \%$

Recovery corrected [%] $= \frac{(0.001936\ mg/kg - 0.0\ mg/kg) \times 100\%}{0.002\ mg/kg} = 96.8\%$

Since the control value (untreated samples) is less than the limit of detection, the corrected recovery is only calculated exemplarily here.

Remark: Calculations in this example were performed with rounded numbers.