ABSTRACT

The purpose of this study is to validate BASF Analytical Method Number. D1513/01 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.

Principle of the method: 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, a 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 transitions for BAS 750F were at *m/z* 398 → *m/z* 70 and at *m/z* 400 → *m/z* 70 for primary and confirmation quantitation. The transition for M750F003 was at *m/z* 288 → *m/z* 70 for primary quantitation. The transition for 1,2,4-triazole was at *m/z* 70 → *m/z* 43 for primary quantitation. Secondary chromatographic methods using transitions at *m/z* 288 → *m/z* 70 and at *m/z* 70 → *m/z* 43 were used for M750F003 and 1, 2, 4-Triazole, respectively for confirmation. All transitions were monitored in positive mode for primary and confirmation quantification, respectively. The results are calculated by direct comparison of the sample peak responses to those of external standards.

Test conditions: The method was validated at two fortification levels (0.002 and 0.02 µg/g) for each analyte (BAS 750 F or M750F003 or 1,2,4-triazole) in a sandy loam soil and a sand soil. For validation, untreated soil samples (a sandy loam soil and a sand soil) were fortified with either BAS 750 F or M750F003 or 1,2,4-triazole 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 analyte at the method limit of quantitation, 0.002 µg/g (ppm), and five replicates fortified at a higher level, corresponding to ten times of the limit of quantitation, 0.02 µg/g (ppm). For each level, the mass transitions described above were evaluated.

In conjunction with the subject study, matrix- and solvent-matched standards were analysed in a separate experiment to evaluate any potential matrix effects. The stability of each analyte in extract solutions for each matrix of interest was also tested.

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 method determines BAS 750 F or M750F003 or 1,2,4-triazole residues in soil matrices by using LC-MS/MS. No interfering peaks were found at the retention times for these analytes. The MRM transitions used to identify the three analytes were determined by product ion spectra.

ABSTRACT (continued)

The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from the each commodity had no significant influence on analysis (matrix effects <20%); therefore, the validation samples were analysed only using solvent-based calibration standard solutions.

Linearity.
BAS 750 F: Acceptable linearity was observed for the standard range using five calibration levels and two mass transitions tested for BAS 750 F (*m/z* 398 → *m/z* 70 and at *m/z* 400 → *m/z* 70) and for the two independent analytical sets tested for BAS 750 F. The method-detector response was linear over the 0.005-0.25 ng/mL range (r = ≥0.999).

M750F003: Acceptable linearity was observed for the standard range using the mass transition tested for M750F003 (*m/z* 288 → *m/z* 70) and for the two independent analytical sets tested for M750F003. Secondary chromatographic method using transition at *m/z* 288 → *m/z* 70 was also linear. The method-detector response was linear over the 0.005-0.25 ng/mL range (r = ≥0.998) for both primary and confirmatory chromatographic system.

1,2,4-Triazole: Acceptable linearity was observed for the standard range and the mass transition tested for 1,2,4-Triazole (*m/z* 70 → *m/z* 43) Acceptable linearity was observed for the standard range and the two independent runs tested for 1,2,4-Triazole. Secondary chromatographic method using transition *m/z* 70 → *m/z* 43 for 1, 2, 4-Triazole was also linear. The method-detector response was linear over the 0.01-0.5 ng/mL range (r = ≥0.988) for both primary and confirmatory chromatographic system

Standard stability. Stock and intermediate (fortification) standards solutions of BAS 750 F and M750F003 were prepared in acetonitrile and exhibited stability up to 92 days in acetonitrile.

The calibration solutions of BAS 750 F or M750F003 were prepared in every month by serial dilution of the fortification standards solutions of BAS 750 F or M750F003 with acetonitrile-water (20-80, v/v). The calibration solutions showed stability up to 31 days.

Stock, intermediate (fortification) and calibration standards solutions of 1,2,4 triazole were prepared in water and exhibited stability up to 92 days in water.

During the course of this study, the test/reference substance solutions were stored in a refrigerator at an average temperature of 3°C and all solutions were used within the demonstrated time period of stability.

Extract stability. The method validation fortification sample extracts were stored at refrigerator (if needed) prior to analysis and were analysed within 0 to 7 days of extraction. The acceptable method recoveries obtained during analysis demonstrate the storage stability of residues of BAS 750 F and its metabolites, M750F003 and 1,2,4-Triazole in the extracts prior to analysis. In addition, the recoveries from stored solutions generated during extract stability experiments performed in conjunction with this study, which included tests on initial extracts and extracts at the final volume stage that was stored at room temperature, indicated that residues of BAS 750 F and its metabolites, M750F003 and 1,2,4-Triazole are stable in extracts for at least the time period tested, 11-12 days, sufficient to support the storage intervals and conditions incurred by sample extracts in the study.

1. INTRODUCTION

1.1 Background and Purpose of Study

BAS 750 F is a fungicide used in several crops. A residue analytical method (D1513/01), for the analysis of BAS 750 F and metabolites, M750F003 and 1,2,4-Triazole, in soil was validated at BASF Crop Protection in Research Triangle Park, North Carolina.

The purpose of this study is to validate BASF Analytical Method Number. D1513/01 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.

1.2 Principle of the Method

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, a 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.

All transitions were monitored in positive mode for primary and confirmation quantification, respectively. The results are calculated by direct comparison of the sample peak responses to those of external standards.

1.3 Specificity

To demonstrate the specificity of the analytical method, one additional mass transition (*m/z* 400 → *m/z* 70) was monitored simultaneous to the primary quantitation transition (*m/z* 398 → *m/z* 70) for analysis of BAS 750 F. Similarly, for metabolite M750F003 and 1,2,4-Triazole , the specificity of the analytical method, was demonstrated using secondary chromatographic method.

The method was able to accurately determine residues of BAS 750 F and no interference was observed at the retention time of the analyte peak. No matrix suppression or enhancement was found to affect the analyte. For M750F003 and 1,2,4-Triazole, secondary mass transitions were not reliable for quantitation, therefore two independent chromatographic method using the primary quantitation transition were used.

2. MATERIALS AND METHODS

2.1 Test Systems

The test systems considered in this study were the top 3 inches of soil from BASF study 715267 (**Reference 1**); trial R140864 (sandy loam) and trial R140865 (sand) was used.

The test systems were characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267). A copy of these characterization data for both soil types is provided in the **Appendix A.**

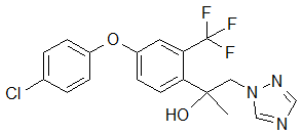
Test System (continued)

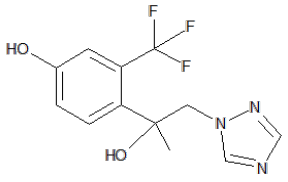
Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 784705-1). The test system samples were assigned unique numbers according to SOP 10.04.XX and these were recorded in each analytical set or "Master Sheet" [e.g., Sample matrix (soil, 784705-03-4, from Master Sheet No. 784705-03]. The actual sample numbers used for the analysis were identified in the raw data and in this final report.

2.2 Test and Reference Substances

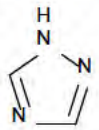
The test/reference standards shown below were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference items were maintained frozen until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

The certificate of analysis is presented in **Appendix B**. A detailed summary of the reference substances are presented below

Code Name	BAS 750 F	Chemical structure: 
BASF Reg. No.	5834378	
CAS No.	1417782-03-6	
Molecular Formula	$C_{18}H_{15}ClF_3N_3O_2$	
Molecular Weight	397.8 g/mol	
IUPAC Name	(2RS) 2-[4-(4-Chlorophenoxy)-2-(Trifluoromethyl)Phenyl]-1-(1H-1,2,4-Triazol-1-yl)Propan-2-ol	
Lot Number	L85-124	
Purity	99.7%	
Storage	Keep at Room Temperature or Cooler	
Expiration Date	July 01, 2017	

Code Name	M750F003	Chemical structure: 
BASF Reg. No.	5924326	
CAS No.	NA	
Molecular Formula	$C_{12}H_{12}F_3N_3O_2$	
Molecular Weight	287.2	
IUPAC Name	4-[2-Hydroxy-1-(1H-1,2,4-Triazol-1-yl)Propan-2-yl]-3-(Trifluoromethyl)Phenol	
Lot Number	L84-250	
Purity	99.6%	
Storage	Keep at Room Temperature or Cooler	
Expiration Date	May 01, 2017	

Test and Reference Substance (continued)

Code Name	480M52 (old code BF 480-16)	Chemical structure: 
BASF Reg. No.	87084	
CAS No.	288-88-0	
Molecular Formula	$C_2H_3N_3$	
Molecular Weight	69.0667	
IUPAC Name	1,2,4-(1H)- Triazole	
Lot Number	AC10194-134	
Purity	99.0%	
Storage	Keep in refrigerator	
Expiration Date	April 1, 2022	

The test/reference items in solution were used in the study to generate data for both instrument and method performance. Quantitation of residues in all samples was achieved using calibration curves calculated by linear regression of instrument responses for the reference items. The performance of the instrument was evaluated during each injection set. The solution stability detail is provided in **Section 4.3**.

2.3 Route of Administration

In this method validation study, the test items were applied to the test system as analytical standard solutions (BAS 750 F and M750F003 in acetonitrile and Triazole in water) by micropipette to ensure precise delivery of a small amount of the test items.

3. ANALYTICAL METHOD

3.1 Principle of the Method

Using BASF Analytical Method No. D1513/01, residues of BAS 750 F and metabolites, M750F003 and 1,2,4-Triazole, in soil matrices are determined using LC-MS/MS. The working method validated in this study is provided in **Appendix C**. A brief description of the methodogy as follows.

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, a 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 20°C and reconstituted again in water (0.475 mL) for the LC-MS/MS for analysis.

The transitions for BAS 750F were at *m/z* 398 → *m/z* 70 and at *m/z* 400 → *m/z* 70 for primary and confirmation quantitation. The transition for M750F003 was at *m/z* 288 → *m/z* 70 for primary quantitation. The transition for 1,2,4-triazole was at *m/z* 70 → *m/z* 43 for primary quantitation. Secondary chromatographic methods using transitions at *m/z* 288 → *m/z* 70 and at *m/z* 70 → *m/z* 43 were used for M750F003 and 1, 2, 4-Triazole, respectively for confirmation. All transitions were monitored in positive mode for primary and confirmation quantification, respectively. The results are calculated by direct comparison of the sample peak responses to those of external standards

Analytical Method (continued)

3.2 Specificity/Selectivity

The residues of BAS 750 F are determined by LC-MS/MS, monitoring (in the positive mode) ion transitions at *m/z* 398 → *m/z* 70 (proposed as the primary transition for quantitation) and *m/z* 400 → *m/z* 70 (typically for confirmatory purposes). The results are calculated by direct comparison of the sample peak responses to those of external standards. The MRM transitions used to identify BAS 750 F were determined by product ion spectra (**Appendix K**). As LC-MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary.

The residues of M750F003 and 1,2,4 triazole are determined by LC-MS/MS, monitoring ion transitions at *m/z* 288 → *m/z* 70 and *m/z* 70 → *m/z* 43 (proposed as the primary transition for quantitation; positive mode), respectively. Since a secondary transition was not reliable for quantitation for these two metabolites, a second independent chromatographic method using the primary transition for each compound was used for confirmation.

3.3 Validation of Method

For validation, untreated soil samples were fortified with residues of BAS 750 F and metabolites, M750F003 and 1,2,4-Triazole then analyzed according to the established method validation guidelines. To test the repeatability of the method, the analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.002 μg/g (ppm), and five replicates fortified at a higher level, corresponding to 10 X the limit of quantitation, 0.02 μg/g (ppm), The example of recovery calculation is provided in **Appendix D.** The validation data including the detail analytical data for each matrix types are provided in **Appendix E.**

3.4 Influence of Matrix Effects on Analysis

In conjunction with the subject study, matrix-matched standards and solvent-based standards were analysed in a separate experiment to evaluate any potential matrix effects on LC-MS/MS analysis. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with only solvent. The matrix-matched standards were made by adding an aliquot each of three solvent-based standards with control soil matrix to the desired matrix-matched standards concentration. Each set of matrix-matched standards (for each soil matrix) was bracketed by a block of calibration standards and was to have an additional single injection of each of the tested standard levels occur during the run. Only the standards which immediately bracket a matrix set, and all standard injections within that matrix set, were used in calculations involving matrix effects.

The data generated were evaluated by comparing the average area response of the standards for three injections without matrix and three injection with matrix, for each of the three standard concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) required a difference in area of <20%, calculated as the "Mean Area Change (%)". For each analyte/matrix/ion transition, an overall average "Mean Area Change (%)" across the three tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects. The detail data from matrix effect evaluation is provided in **Appendix F.**

5. CALCULATIONS AND RAW DATA

An example calculation is included in **Appendix D.** Detailed analytical data such as supporting raw data necessary for re-calculations, standards and calibration curve data are provided in **Appendix E.** The graph showing the reliability of the calibration is provided in **Appendix I.** Example standard curves and chromatographs are provided in **Appendix J**.

6. STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or relative standard deviation (RSD) for the procedural recoveries and area counts and calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards. The statistical calculations throughout this report were performed using an automated computer spreadsheet (Microsoft Excel®) and were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These are due to rounding and have no effect on the scientific conclusions presented in this report. The detailed analytical data may be consulted for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at BASF inspected the analytical procedures for compliance with Good Laboratory Practices that included adherence to the protocol. The dates inspected are detailed in the quality assurance unit statement. Study samples and test and reference items were maintained in secured (i.e. pad-locked) storage with limited access. Freezer temperatures were continuously monitored by electronic means.

7. SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Section 9 on pages 22-23.

8. COMMENTS FROM INDEPENDENT LABORATORY VALIDATION

This independent laboratory validation was successfully completed on the first trial for all analytes at ADPEN Laboratories, Inc. (**Reference 2**). Recovery results and statistical data demonstrate BASF Analytical Method D1513/01 can be performed successfully for quantitation of BAS 750 F, M750F003 and 1,2,4-Triazole in soil. The method is well-written and contains a fair amount of comments to guide the analyst through the procedure for the first time.

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. 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.

COMMENTS FROM INDEPENDENT LABORATORY VALIDATION (continued)

Recommendations for improvement of the BASF Analytical Method D1513/01 are presented below:

1. A residual background of 1,2,4-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 discrepancy 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 particle 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).

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.

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

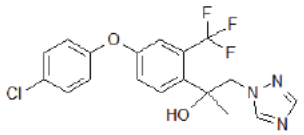
2.1 Safety

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.

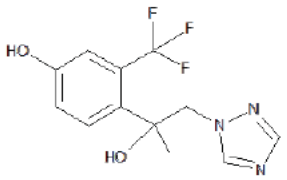
2.2 Test and Reference Item

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

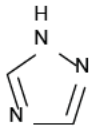
BAS-Code	750 F
Common Name	-/-
IUPAC Name	2-[4-(4-Chlorophenoxy)-2-(Trifluoromethyl)Phenyl]-1-(1H-1,2,4-Triazol-1-yl)Propan-2-ol
BASF Reg. No.	5834378
CAS-No.	-/-
Molecular Formula	$C_{18}H_{15}ClF_3N_3O_2$
Molecular Weight	397.8



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 substituted by that 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 other 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 fortification levels are needed for the analysis.
If necessary, the volume of solution prepared may be changed as long as the proportions 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 additional standards may be prepared as needed.
If necessary, the volume of solution prepared may be changed as long as the proportions 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 the extract solvent with S2 requires significant agitation that can only be achieved through vortexing. Because the 1 mL Waters Glass inserts do not have individual caps, the solutions must be vortexed in 1.4 mL Alpha Tubes and then transferred to the 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 they 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:
- o Calibration standards
- o Control samples
- o Procedural recovery samples
- o Unknown samples
- o 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 equilibration)	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 the fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on the 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 fortification 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 equilibration)	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 the fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on the 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 fortification 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 equilibration)	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 the fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on the 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 fortification 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 equilibration)	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 the fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on the 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 fortification 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]** $= \dfrac{\text{Response} - \mathit{Intercept}}{\mathit{Slope}} = C_A$

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

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

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

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

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:

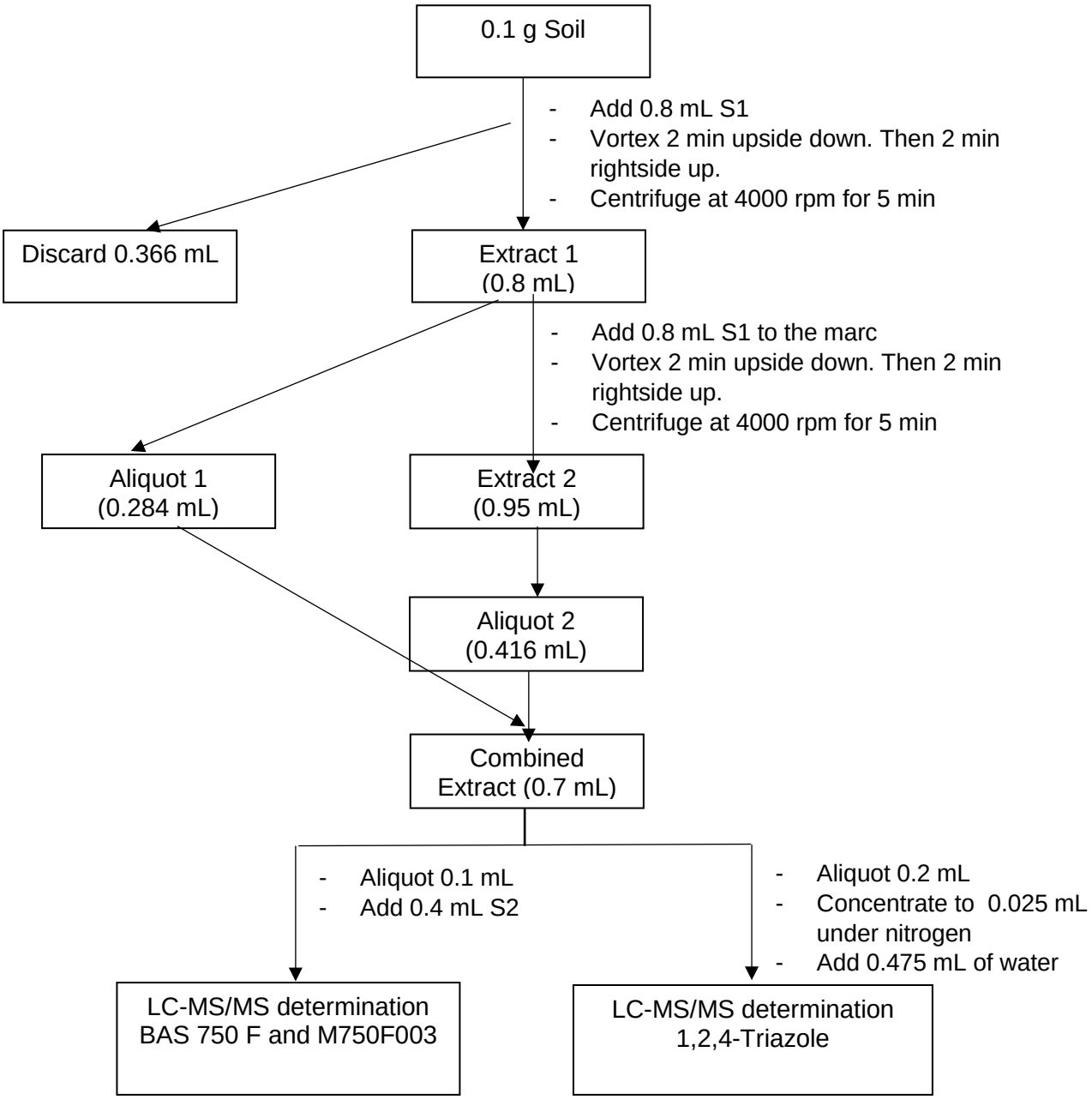
Aliquot taken from Extraction Step 1 = Equal Aliquot (mL) X (1 - $CF_{Extract}$)
Aliquot taken from Extraction Step 2 = Equal Aliquot (mL) X (1 + $CF_{Extract}$)

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 the 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-dilutions from the extract if necessary.

5. FLOWCHART



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

6. METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (= 60 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) requires 1 working days (8 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7. CONCLUSION AND METHOD CAPABILITIES

Recoveries, Chromatograms, and Calibration Curves
Recovery data will be provided in the validation part of the analytical method D1513/01.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
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.

The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
The tested untreated soil samples showed no significant interferences (<20 or 30%) at the retention time of the analytes.

Confirmatory Techniques
For BAS 750 F the quantitation is possible with two different transitions. Therefore, no additional confirmatory technique is required.

For M750F003 and 1,2,4-Triazole (Reg. No. 87084) specificity of the method is confirmed by separate chromatographic separations for each analyte. See sections 4.2.2 and 4.2.4, respectively, for specific parameters.

Potential Problems:

A residual background of triazole was observed during the independent laboratory validation study, which was 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.

8. APPENDIX

8.1 Example of Calculation

The detail calculation is provided in **Appendix H.**

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

Typical Recovery Calculation for LC/MS/MS Quantitation

Example: BAS 750F, *m/z* 398 → 70; soil sample fortified at 0.002 mg/kg (sample 784705-2-4):

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

Aliquot Factor (A_F) $= \frac{\textit{Sample\_Volume}}{\textit{Total\_Extract\_Volume}}$

Residue [mg/kg] $= \frac{V_{end} \times C_A}{G \times A_F \times 100}$

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

The following values were used in this calculation:

Response of fortified sample	27884
Response of control sample	0
Slope:	1004438.2776
Intercept:	2322.0471
Sample mass (G):	0.1 g
Final Volume (V_{end}):	0.5 mL
Aliquotation factor A_F (0.1/1.6):	0.0625 (= 6.25%)
Dilution Factor	1
Injection Volume	20
Conversion Factor	1000

Residues in the final volume [ng] (C_A) $= \frac{27884 - 2322.0471}{1004438.2776} = 0.0254 \text{ ng/mL}$

Residue [mg/kg] $= \frac{0.5 \text{ mL} \times 0.0254 \text{ ng/mL}}{0.1 \text{ g} \times 0.0625 \times 1000} = 0.00204 \frac{\text{mg}}{\text{kg}}$

Recovery % $= \frac{0.00204 \text{ ppm} - 0 \text{ ppm}}{0.002 \text{ ppm}} \times 100 = 102\%$

ABSTRACT

The purpose of this study was the validation of analytical method L0214/01 for the determination of Reg. No. 5834378 (BAS 750 F) and its metabolites Reg. No. 5924326 and Reg. No. 87084 (1,2,4-Triazole) in soil by LC-MS/MS.

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/cm^3). The concentrated extract is filled up to a volume of 1 mL with ultra-pure water and analysed by LC-MS/MS.

Test conditions. The method was validated at two fortification levels (0.002 and 0.02 mg/kg) for soil. For each fortification level and matrix type, five replicates were analysed. Additionally, at least two replicates of unfortified samples were analysed (control samples). Two mass transitions were evaluated for quantification and confirmation of Reg. No. 5834378 and Reg. No. 5924326. One mass transition of Reg. No. 87084 was evaluated, but on two different analytical columns, Hypercarb™ and Synergi Hydro RP.
Solvent- as well as matrix-matched standards were analysed to assess potential matrix effects. Matrix-matched standards were required for analysis of Reg. No. 5834378 (BAS 750 F).

Limit if Quantification (LOQ) and Limit of Detection (LOD). The limit of quantification (LOQ) was defined by the lowest fortification level successfully tested, hence 0.002 mg/kg. The limit of detection was estimated as 20% of the limit of quantification, equivalent to 0.0004 mg/kg.

Selectivity. 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.

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.

INTRODUCTION

Scope of the Study

The purpose of the study was to validate the analytical method L0214/01 for the determination of Reg. No. 5834378 (BAS 750 F) and its metabolites Reg. No. 5924326 and Reg. No. 87084 (1,2,4-Triazole) in soil by LC-MS/MS.

A residue analytical method for Reg. No. 5834378 and its metabolites Reg. No. 5924326 and Reg. No. 87084 in soil with a limit of quantification (LOQ) of 0.002 mg/kg was required. The described BASF method L0214/01 allows the determination of the analytes with the required limit of quantification in soil.

This method was developed at BASF SE, located in Limburgerhof (Germany).

The method was validated at two fortification levels (0.002 and 0.02 mg/kg) for two soils. For each fortification level and matrix, five replicates were analysed. Additionally, at least two replicates of unfortified control samples were analysed. For analytes Reg. No. 5834378 and Reg. No. 5924326 two mass transitions were used for high selective MS/MS-detection. For analyte Reg. No. 87084 one mass transition was used for high selective MS/MS-detection, however two runs with two different analytical columns were performed. The method has a limit of quantification (LOQ) of 0.002 mg/kg for all analytes. The limit of detection (LOD) in soil for each analyte is 0.0004 mg/kg.

Matrix- and solvent-matched standards were analysed within this study to assess potential matrix effects.

Principle of the Method

5 g soil samples are 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 and after centrifugation another aliquot of 10 mL (extract 2) is taken and combined with extract 1 and mixed until complete homogeneity. The residues of Reg. No. 5834378 and metabolite Reg. No. 5924326 are directly analysed by LC-MS/MS. For analysis of Reg. No. 87084, 5 mL of the combined extracts 1 and 2 are transferred into a tared culture tube and the volume is reduced in a nitrogen evaporator to a volume ≤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 using ultra-pure water and analysed by LC-MS/MS.

Specificity

The analytes Reg. No. 5834378, Reg. No. 5924326 and Reg. No. 87084 were unequivocally identified and quantified as individual compounds. The method allows the specific determination of Reg. No. 5834378 and its metabolites Reg. No. 5924326 and Reg. No. 87084 in soil by using LC-MS/MS. Detection was accomplished by high selective MS/MS-detection using two mass transitions for Reg. No. 5834378 and Reg. No. 5924326 and one mass transition for Reg. No. 87084 on two different chromatographic systems.

MATERIALS AND METHODS

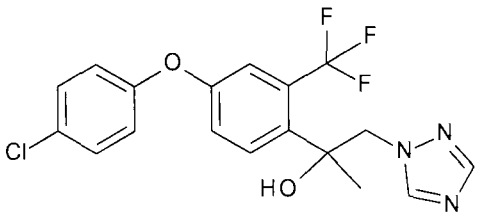
Test systems

The following test systems were considered in this study of validation:
Test System 1: Field soil LUFA 2.2 (Details are presented in Figure 70)
Test System 2: Field soil LUFA 2.3 (Details are presented in Figure 71)
Test System 3: Solvent (Acetonitrile/water 70/30, v/v)

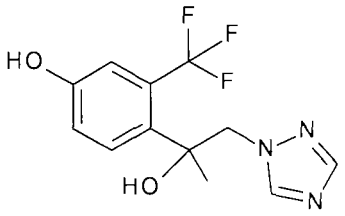
Test and Reference Items

Test Items

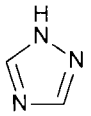
Common Name	-/-
BAS-Code	750 F
Reg. No.	5834378
IUPAC Name	2-[4-(4-chlorophenoxy)-2-(trifluoromethyl)phenyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol
CAS-No.	1417782-03-6
Molecular Formula	$C_{18}H_{15}ClF_3N_3O_2$
Molecular Weight	397.8 g/mol
Batch No.	L84-238, L85-12
Purity	99.7%, 99.4%
Expiration Date	01.04.2015, 01.01.2016



Common Name	-/-
Metabolite-Code	-/-
Reg. No.	5924326
IUPAC Name	4-[2-hydroxy-1-(1H-1,2,4-triazol-1-yl) propan-2-yl]-3-(trifluoromethyl)phenol
CAS-No.	-/-
Molecular Formula	$C_{12}H_{12}F_3N_3O_2$
Molecular Weight	287.2 g/mol
Batch No.	L84-250
Purity	99.6%
Expiration Date	01.07.2015



Common Name	Triazole
Metabolite-Code	-/-
Reg. No.	87084
IUPAC Name	1,2,4-(1H)-triazole
CAS-No.	288-88-0
Molecular Formula	$C_2H_3N_3$
Molecular Weight	69.1 g/mol
Batch No.	AC10194-134
Purity	99.0%
Expiration Date	01.04.2022



Materials and Methods

Equipment

Equipment	Size, Description	Manufacturer
Balance	XP205DR	Mettler Toledo, Giessen, Germany
Balance	LP 5200 P	Sartorius, Göttingen, Germany
Nitrogen evaporator	Turbo Vap LV	Biotage, Uppsala, Sweden
Centrifuge	Eppendorf 5810	Eppendorf, Hamburg, Germany
Lab shaker	EB Swip SM25	Edmund Bühler, Hechingen, Germany
Volumetric pipettes	various volumes	
Handystep electronic dispenser	various volumes	Brand, Wertheim, Germany
PP tubes with screw cap and conical bottom, sterile	50 mL	Sarstedt, Nümbrecht, Germany
Culture tubes	16 mL	Duran Group, Wertheim/Main, Germany
Culture tubes screw caps	GL18 with PTFE protected seal	Duran Group, Wertheim/Main, Germany
Pasteur plastic pipets	Up to 3 mL	Ratiolab GmbH, Dreieich, Germany

Reagents

Chemicals

Chemical	Grade	Manufacturer/Supplier
Acetonitrile	Gradient Grade	Merck KGaA, Darmstadt, Germany
Ultra-pure water	Ultra-pure	ELGA LabWater, Celle, Germany
Formic Acid	98 – 100%	Sigma – Aldrich, Taufkirchen, Germany

Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solvent	S1	Acetonitrile-water, 70/30, v/v Add 700 mL of acetonitrile and 300 mL of ultra-pure water into a, e.g., 1 L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A	LC1	0.1% Formic Acid in Water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1 L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% Formic Acid in Acetonitrile Add 1000 mL of acetonitrile and 1 mL of concentrated formic acid into a, e.g., 1 L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

Remark: If smaller volumes than 1 L were used, then the volumes were adjusted accordingly, but overall ratios as given above, were maintained.

Working Solutions

Stock Solutions

A 1 mg/mL stock solution was prepared by weighing an appropriate amount of each analyte into a 10 mL volumetric flask and adding the required volume of solvent. For stock solutions of Reg. No. 5834378 and Reg. No. 5924326 acetonitrile was used for dilution and for stock solutions of Reg. No. 87084 ultra-pure water was used.
In each case, a stock solution was used for preparing fortification or calibration solutions, respectively. From a stock solution that was used for preparing fortification solutions, no calibration standards were prepared and vice versa.

Fortification Solutions

Fortification solutions were prepared from 1 mg/mL stock solutions. To prepare 10 mL of 1 mg/mL stock solution of Reg. No. 5834378 and Reg. No. 5924326, 10 mg of each analyte were weighed into a 10 mL volumetric flask and diluted to the mark with acetonitrile. To prepare 10 mL of 1 mg/mL stock solution of Reg. No. 87084, 10 mg of the analyte were weighed into a 10 mL volumetric flask and diluted to mark with ultra-pure water. Homogeneous solutions were achieved by sonication or vortexing of the solutions. Dilution series were prepared by using the appropriate solvent, as shown in the tables below.

Preparation of mixed fortification solutions of Reg. No. 5834378 and Reg. No. 5924326

Take stock solution	Volume [mL]	Dilute with S1 to a final volume of [mL]	Concentration [µg/mL]
Reg. No. 5834378 Reg. No. 5924326	1 1	10	100
Take solution (µg/mL)	**Volume [mL]**	**Dilute with S1 to a final volume of [mL]**	**Concentration [µg/mL]**
100	0.5	10	5
5	1	10	0.5

Preparation of fortification solutions of Reg. No. 87084

Take stock solution	Volume [mL]	Dilute with ultra-pure water to a final volume of [mL]	Concentration [µg/mL]
Reg. No. 87084	1	10	100
Take solution [µg/mL)	**Volume [mL]**	**Dilute with ultra-pure water to a final volume of [mL)**	**Concentration [µg/mL]**
100	0.5	10	5
5	1	10	0.5

Calibration Standard Solutions

Calibration solutions for HPLC-MS/MS analysis were prepared by using stock solutions whose preparation procedure is described in the previous section "stock solutions". Stock solutions for Reg. No. 5834378 and Reg. No. 5924326 were prepared with acetonitrile and stock solutions for Reg. No. 87084 were prepared with ultra-pure water. For further concentrations, calibration solutions of Reg. No. 5834378 and Reg. No. 5924326 were diluted with acetonitrile/water (70/30, v/v) and calibration solutions of Reg. No. 87084 were diluted with ultra-pure water. Homogeneity of the solutions was achieved by sonication or vortexing of the solutions. The solutions were prepared up as described in the tables below.

Preparation of standards solutions of Reg. No. 5834378 and Reg. No. 5924326 for calibration

Take stock solution	Volume [mL]	Dilute with S1 to a final volume of [mL] [1)]	Concentration [µg/mL]
Reg. No. 5834378 Reg. No. 5924326	**1 1**	**10**	**100**
Take solution [µg/mL]	**Volume [mL]**	**Dilute with S1 to a final volume of [mL] [1)]**	**Concentration [µg/mL]**
100	**1**	**10**	**10**
10	**1**	**10**	**1**
1	**1**	**10**	**0.1**
0.1	**1**	**10**	**0.01**
Take solution [ng/mL]	**Volume [mL]**	**Dilute with S1 [1)] to a final volume of [mL]**	**Concentration [ng/mL]**
10	3.0	10	3
10	1.25	10	1.25
10	0.5	10	0.5
10	0.25	10	0.25
1.25	1.0	10	0.125
0.5	1.0	10	0.05
0.25	1.0	10	0.025

[1)] The use of matrix-matched standards of Reg. No. 5834378 and Reg. No. 5924326 was needed for the evaluation of the influence of matrix effects on the analysis of Reg. No. 5834378 and Reg.No. 5924326 (see section 3.4), extract stability of Reg. No. 5834378 (see section 3.2), validation of Reg. No. 5834378 (see section 3.3) and instrument recovery samples (Quality control samples). Matrix-matched standard solutions were prepared using a matrix solution (extracts of a control sample taken through the entire analytical procedure). Two different procedures were carried out to prepare matrix-matched standard solutions of Reg. No. 5834378 and Reg. No. 5924326, one with concentrated matrix and the other one by direct dilution of calibration standards with matrix (for details see below section 2.3.2.3 "Matrix-matched Solutions"). The matrix load in each matrix-matched standard solution was at least 90 %.

Preparation of standards solutions of Reg. No. 87084 for calibration

Take stock solution	Volume [mL]	Dilute with water to a final volume of [mL] [1)]	Concentration [µg/mL]
Reg. No. 87084	**1**	**10**	**100**
Take solution [µg/mL]	**Volume (mL)**	**Dilute with S1 to a final volume of (mL) [1)]**	**Concentration [µg/mL]**
100	**1**	**10**	**10**
10	**1**	**10**	**1**
1	**1**	**10**	**0.1**
Take solution [ng/mL]	**Volume [mL]**	**Dilute with water [1)] to a final volume of [mL]**	**Concentration [ng/mL]**
100	1.5	10	15
100	1.0	10	10
100	0.625	10	6.25
100	0.25	10	2.5
10	1.25	10	1.25
6.25	1.0	10	0.625
2.5	1.0	10	0.25
1.25	1.0	10	0.125

[1)] The use of matrix-matched standards of Reg. No. 87084 was needed for the evaluation of the influence of matrix effects on the analysis (see section 3.4) and instrument recovery samples (Quality control samples). Matrix-matched standard solutions were prepared using a matrix solution (extracts of a control sample taken through the entire analytical procedure). Matrix-matched standards were prepared with a concentrated control sample extract (for details see below section 2.3.2.3 "Matrix-matched Solutions"). The matrix load in each matrix-matched standard solution was at least 90 %.

Matrix-matched Solutions

Preparation of matrix-matched solutions for Analysis Package No. L007, L010, L039 to L042 (Experiment "concentrated"):
After sample preparation, 200 µL of a control sample extract were directly transferred in HPLC vials. Seven matrix-matched standards were prepared. Then, the control sample extracts were concentrated to dryness under a stream of nitrogen at 40°C using an N-evaporator and reconstituted with 200 µL of standard calibration solutions with concentrations between 0.025 ng/mL to 3.0 ng/mL for Reg. No. 5834378 and Reg. No. 5924326 and 0.125 ng/mL to 15.0 ng/mL for Reg. No. 87084.

Preparation of matrix-matched solutions for Analysis Package No. L040, L045 to L048 (Experiment "diluted"):
Matrix-matched standards were prepared by dilution of standard calibration solutions with appropriate amounts of a control sample extract starting with a concentration of 10 ng/mL to obtain matrix-matched standards covering a concentration range from 0.025 ng/mL to 3.0 ng/mL for Reg. No. 5834378 and Reg. No. 5924326.

Set-up of the Analytical Run

Reagent blanks or blanks were injected as necessary. Each measurement began with an injection of a blank (exception: measurement of matrix-matched validation L046 & L048. There are not injections of a blank), followed by an injection of a calibration standard and ended with a calibration standard. Standards were interspersed with samples. Each calibration standards was at least injected twice. At least seven calibration levels were injected.

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

<u>For Reg. No. 5834378 and Reg. No. 5924326:</u>
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.

<u>For Reg. No. 87084:</u>
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] $= \dfrac{Response - Intercept}{Slope} = C_A$

II. Residues in the Sample Matrix [mg/kg] $= \dfrac{V_{end} \times C_A}{G \times A_F \times 1000}$

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.

Summary of Method

Type of method:	LC-MS/MS
Test systems:	Field soil LUFA 2.2 (Figure 70) Field soil Li 10 (Figure 71) Solvent (acetonitrile/water, 70/30, v,v)

Analytes and selected mass transitions:

Reg. No. 5834378 (BAS 750 F)	398 → 182 398 → 133
Reg. No. 5924326	288 → 159 288 → 103
Reg. No. 87084 (1,2,4-Triazole)	70 → 43

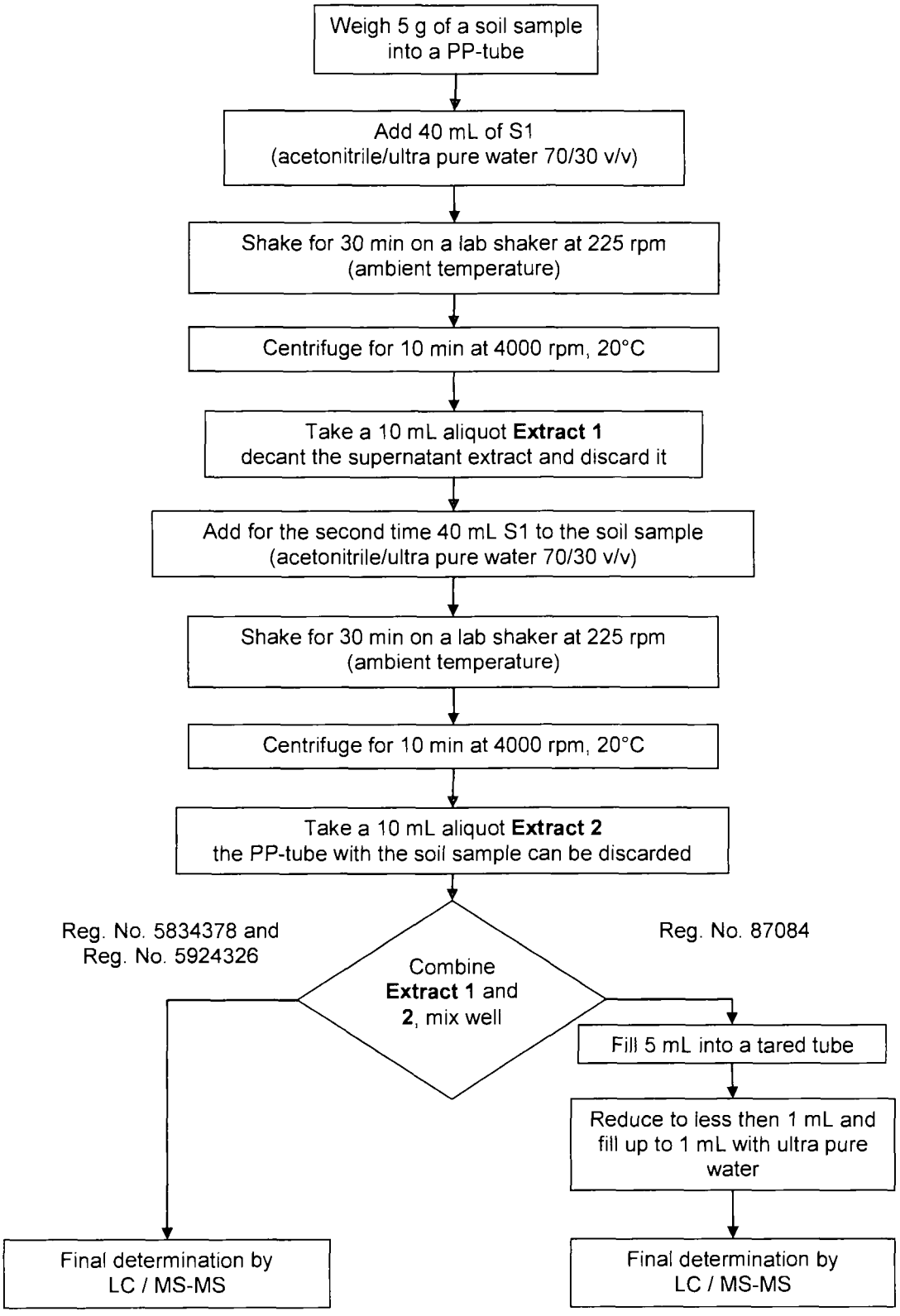
Analytical procedure:	Liquid extraction of the analytes with acetonitrile/water (70/30, v/v).
Confirmatory technique:	The quantification of Reg. No. 5834378 and Reg. No. 5924326 is based on the monitoring of two mass transitions for each analyte. Recovery data was reported for each mass transition and matrix considered. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. In the case of Reg. No. 87084 one mass transition was considered within two analytical runs using two different analytical columns, whereas the use of analytical column Hypercarb™ was used for quantification and the use of analytical column Synergi Hydro RP for confirmation. However, both analytical systems for Reg. No. 87084 are freely interchangeable.
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 which could not be neglected. No matrix effect was observed for both metabolites Reg. No. 5924326 and Reg. No. 87084.
Limit of detection (LOD):	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.
Limit of quantification (LOQ):	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.

Levels of fortification:	0.002 mg/kg and 0.02 mg/kg (equal. LoQ and 10x LoQ)
Time required:	A set of 16 samples requires about 12 hours of work (calculation of the results included)
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.
Specificity:	The method L0214/01 determines 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 of the analyte.
Repeatability:	The relative standard deviation (RSD, %) for all fortification levels was below 20%.
Reproducibility:	Reproducibility of the method was not determined within this validation study.

It could be demonstrated that method L0214/01 fulfils the requirements with regard to specificity, linearity, repeatability, limit of quantification 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.

Additional Information on the Method

Figure 18: Method Flowchart



1 Parts of the report to be changed or appended:

1.1

Present

Study Title:
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

New

Study Title:
Validation of analytical method L0214/01 for the Determination of BAS 750 F (Reg. No. 5834378) and its Metabolites Reg. No. 5924326 and 1,2,4-Triazole (Reg. No. 87084) in soil by LC-MS/MS

Reason for alteration Correction of Title

1.2

Present

2.2.1 Test Items

Common Name	-/-
Metabolite-Code	-/-
Reg. No.	5924326

Common Name	Triazole
Metabolite-Code	-/-
Reg. No.	87084

New

2.2.1 Test Items

Common Name	-/-
Metabolite-Code	M750F003
Reg. No.	5924326

Common Name	Triazole
Metabolite-Code	M750F001
Reg. No.	87084

Reason for alteration Update of Metabolite Codes

1 Parts of the report to be changed or appended:

1.1 Table on page 26

Present

Preparation of standards solutions of Reg. No. 87084 for calibration

Take stock solution	Volume [mL]	Dilute with water to a final volume of [mL] [1)]	Concentration [µg/mL]
Reg. No. 87084	1	10	100
Take solution [µg/mL]	Volume (mL)	Dilute with S1 to a final volume of (mL) [1)]	Concentration [µg/mL]
100	1	10	10
10	1	10	1
1	1	10	0.1

New

Preparation of standards solutions of Reg. No. 87084 for calibration

Take stock solution	Volume [mL]	Dilute with water to a final volume of [mL] [1)]	Concentration [µg/mL]
Reg. No. 87084	1	10	100
Take solution [µg/mL]	Volume (mL)	Dilute with water to a final volume of (mL) [1)]	Concentration [µg/mL]
100	1	10	10
10	1	10	1
1	1	10	0.1

Reason for alteration Correction of the solvents (S1 -> water), used for preparation of the final volume.

1.2 Table 3, page 35

Present

Table 3: Storage Stability of Standard Solutions of Reg. No. 87084 in Acetonitrile/Water (70/30)

New

Table 3: Storage Stability of Standard Solutions of Reg. No. 87084 in Water

Reason for alteration Correction of the solvents (Acetonitrile/Water -> Water),

1.3 Table 30, page 72

Present

Table 30: Response Factor of Reg. No. 87084 based on Standard Solutions in Acetonitrile/Water (70/30, v/v) and Matrix-matched Standards in LUFA 2.2 Soil Extracts, Hypercarb (mass transition 70→43)

New

Table 30: Response Factor of Reg. No. 87084 based on Standard Solutions in Water and Matrix-matched Standards in LUFA 2.2 Soil Extracts, Hypercarb (mass transition 70→43)

Reason for alteration Correction of the solvents (Acetonitrile/Water -> Water),

1.4 Table 31, page 73

Present

Table 31: Response Factor of Reg. No. 87084 based on Standard Solutions in Acetonitrile/Water (70/30, v/v) and Matrix-matched Standards in LUFA 2.2 Soil Extracts, Synergi (mass transition 70→43)

New

Table 31: Response Factor of Reg. No. 87084 based on Standard Solutions in Water and Matrix-matched Standards in LUFA 2.2 Soil Extracts, Synergi (mass transition 70→43)

Reason for alteration Correction of the solvents (Acetonitrile/Water -> Water),

1.5 Table 44, page 86

Present

Table 44: Response Factor of Reg. No. 87084 based on Standard Solutions in Acetonitrile/Water (70/30, v/v) and Matrix-matched Standards in LUFA 2.3 Soil Extracts, Hypercarb, Experiment "diluted" (mass transition 70→43)

New

Table 44: Response Factor of Reg. No. 87084 based on Standard Solutions in Water and Matrix-matched Standards in LUFA 2.3 Soil Extracts, Hypercarb, Experiment "diluted" (mass transition 70→43)

Reason for alteration Correction of the solvents (Acetonitrile/Water -> Water),

1.6 Table 45, page 87

Present

Table 45: Response Factor of Reg. No. 87084 based on Standard Solutions in Acetonitrile/Water (70/30, v/v) and Matrix-matched Standards in LUFA 2.3 Soil Extracts, Synergi, Experiment "diluted" (mass transition 70→43)

New

Table 45: Response Factor of Reg. No. 87084 based on Standard Solutions in Water and Matrix-matched Standards in LUFA 2.3 Soil Extracts, Synergi, Experiment "diluted" (mass transition 70→43)

Reason for alteration Correction of the solvents (Acetonitrile/Water -> Water),

1.7

Reason for alteration of author D.Lueer is no longer an active study director within the test facility

---

Head of Quality Assurance for Dr. Ulrich Schepers

Date 18.07.16 **Signature** Deniz Akyol

for **Study Director** Dominic Lueer

Date 18.07.16 **Signature** Martin Obermann

Management

Date 18/07/16 **Signature** Dr. Lars Wittkowski