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Final Report

Study Title

Validation of Analytical Method L0353/01 for the Determination of BAS 595 F (Triticonazole) and its Metabolites M595F001, M595F002 and M595F014 in Soil by LC-MS/MS

Test Guideline(s)

SANCO/825/00 rev. 8.1
SANCO/3029/99 rev. 4
OCSPP 850.6100 Environmental Chemistry Methods and Associated ILV

Abbreviations

AI	Active ingredient
CalL	Calibration sample
ConL	Untreated control sample
ESI	Electrospray ionisation
ForL	Fortified sample
LC	Liquid chromatography
LOQ	Limit of quantification
LOD	Limit of detection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
n.d.	Not detected
QcsL	Quality control sample
RSD	Relative standard deviation
SsoL	Substance solution

1 INTRODUCTION

1.1 Scope of the Study

The purpose of this study was to validate the existing analytical method, Aventis method no. 0051, BASF analytical method no. L0353/01, for the determination of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in soil by LC-MS/MS according to the cited guidelines. The principle of the given method stayed unchanged, however, it was downscaled by a factor of 10 to reduce the weigh in, as well as the amount of solvents and other consumables. To cover actual requirements, metabolite M595F014 was additionally validated to the already covered analytes.

Therefore, a residue analytical method for the determination of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in soil with a limit of quantification (LOQ) of 0.002 mg/kg was required.

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

1.2 Principle of the Method

A 5 g soil sample was extracted by solid-liquid extraction using sonication and shaking with 0.1M ammonium hydroxide and acetone. After each extraction, the suspension was centrifuged and decanted over cotton wool into a graduated cylinder. Then, an aliquot of the combined extracts was evaporated until the aqueous phase remained and subsequent redissolved in water. A sample clean-up by solid-phase extraction using a C_{18}-SPE column was followed. The residues were eluted with acetonitrile/methanol (95/5, v/v) from the C_{18}-SPE column. Afterwards, the liquid phase was evaporated to nearly dryness using a nitrogen evaporator and the remaining liquid phase was redissolved in water and acetonitrile. Final determination of the residues was conducted by LC-MS/MS.

The limit of quantification (LOQ) is 0.002 mg/kg for BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in soil. The limit of detection (LOD) is 0.0004 mg/kg (20% of LOQ).

1.3 Specificity

The method is highly specific for analysis of the four test items (mass transitions from the positively charged molecule ions to two typical fragment ions in MS/MS mode). The retention times of the test items in extracts matched the retention times in calibration solutions. No peak interferences occurred at the retention times of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014. Since detection by MS/MS with two characteristic mass transitions is regarded to be highly specific, no further confirmatory method is required.

2 MATERIALS AND METHODS

2.1 Test systems

The following test systems were considered in this study of validation:

Test System 1: Field soil LUFA 2.4 (certificate of analysis see Figure A 90)

Test System 2: Field soil LUFA 2.2 (certificate of analysis see Figure A 91)

Test System 3: Acetonitrile for stability testing in stock solutions (Section 3.1)

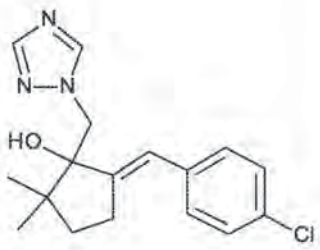
Test System 4: Water/acetonitrile (80/20, v/v) for stability testing in fortification and calibration solutions (section 3.1) and final soil extracts (Section 3.2).

Test System 5: 0.1M Ammonium hydroxide and acetone for stability testing in raw soil extracts (section 3.2).

2.2 Test and Reference Items

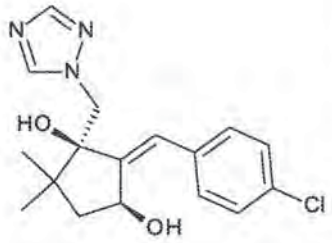
2.2.1 BAS 595 F (Triticonazole)

BAS-Code	BAS 595 F
Common Name	Triticonazole
Reg.No.	4378513
IUPAC Name	(RS)-(5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
CAS-No.	138182-18-0
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8 g/mol
Batch No.	L85-136
Purity	99.4%
Expiration Date	June 01, 2017



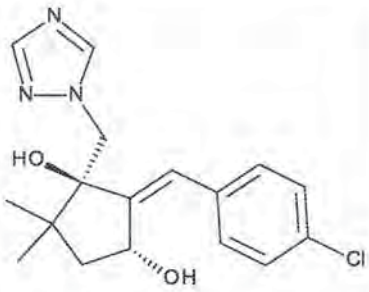
2.2.2 M595F001

Metabolite-Code	M595F001
Reg.No.	5079285
IUPAC Name	(1RS,2E,3SR)-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol
CAS-No.	-/-
Molecular Formula	$C_{17}H_{20}ClN_3O_2$
Molecular Weight	333.8 g/mol
Batch No.	L67-148
Purity	99.3%
Expiration Date	July 01, 2020



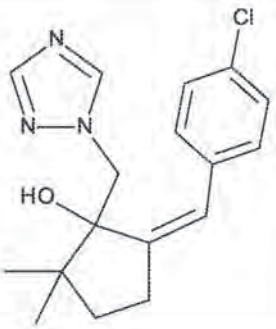
2.2.3 M595F002

Metabolite-Code	M595F002
Reg.No.	5059144
IUPAC Name	(1RS,2E,3RS)-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol
CAS-No.	-/-
Molecular Formula	$C_{17}H_{20}ClN_3O_2$
Molecular Weight	333.8 g/mol
Batch No.	L85-198
Purity	72.5%
Expiration Date	July 01, 2018



2.2.4 M595F014

Metabolite-Code	M595F014
Reg.No.	5079359
IUPAC Name	(1RS)-(5Z)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
CAS-No.	-/-
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8 g/mol
Batch No.	BESS0578
Purity	99.9%
Expiration Date	April 01, 2024



2.3 Materials and Methods

2.3.1 Equipment

Equipment	Size, Description	Manufacturer
Balance	PM4800 Delta Range	Mettler (Germany)
Balance	Mettler Toledo XP205 Delta Range	Mettler (Germany)
Turbo Vap LV / N-Evap	Evaporator	Biotage (Sweden)
Ultrasonic bath	Transsonic 460/H	Elma
Shaker	SM25 SM-30	Edmund Bühler (Germany)
Centrifuge	5810R	Eppendorf (Germany)
Bakerbox	VacMaster	Biotage (Sweden)
Rotary evaporator	Laborota 4003	Heidolph
Pipettes Handy Step	Microman, various volumes DispenserTips, various volumes	Gilson (Germany) Brand (Germany)

2.3.2 Reagents

2.3.2.1 Chemicals

Chemical	Grade
Acetonitrile	Analytical grade
Methanol	Analytical grade
Purified Water	Analytical grade
Acetone	Analytical grade
Ammonium hydroxide (concentrated)	Analytical grade
Formic acid	Analytical grade
Bond Elut C_{18} (200 mg, 3 mL ; Agilent Technologies)	--

2.3.2.2 Solutions and Solvent Mixtures

Description	Code	Composition
Solvent for Stock Solution	S1	Acetonitrile
Solvent for Standard and Fortification Solution	S2	Water / Acetonitrile (80/20, v/v)
Solvent for Extraction	S3	0.1M Ammonium hydroxide
Solvent for Extraction	S4	Acetone
Solvent for Extraction	S5	Acetonitrile / Methanol (95/5, v/v)
HPLC Mobile Phase A	LCSS1	Water / Formic acid (1000/2, v/v)
HPLC Mobile Phase B	LCSS2	Methanol / Formic acid (1000/2, v/v)

2.3.2.3 Working Solutions

Stock Solutions
Individual stock solutions with a concentration of 1 mg/mL were prepared for each of the four analytes by weighing an appropriate amount of each analyte into a flask and adding the required volume of acetonitrile, e.g. 4.97 mg AI + 4.97 mL acetonitrile.

Fortification Solutions
Fortification solutions were prepared by adding 1 mL of each individual prepared stock solution (see above) of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in a flask and diluting with water/acetonitrile (80/20, v/v, S2) to a mixed fortification solution with a concentration of 100 µg/mL. Further dilution series were prepared by using water/acetonitrile (80/20, v/v, S2). Homogeneous solutions were achieved by vortexing of the solutions. The solutions were prepared as exemplarily described in the table below.

Preparation of mixed fortification solutions (exemplified for analysis packages L003 and L010)

Take solution [mg/mL]	Volume [mL]	Solution Identifier	Dilute to a volume of S2 [mL]	Concentration [µg/mL]	Solution Identifier
1 (BAS 595 F) 1 (M595F001) 1 (M595F002) 1 (M595F014)	1 1 1 1	SsoL163489 SsoL163491 SsoL163492 SsoL163490	10	100 (mixed)	SsoL163493
Take mixed solution [ng/mL]	**Volume [mL]**	**Solution Identifier**	**Dilute to a volume of S2 [mL]**	**Concentration mixed [ng/mL]**	**Solution Identifier**
100 000	1	SsoL163493	10	10 000	SsoL163494
10 000	1	SsoL163494	10	1 000 [1)]	SsoL163495
1 000	1	SsoL163495	10	100 [1)]	SsoL163496

[1)] Used as fortification solution.

Calibration Standard Solutions

Mixed calibration solutions for LC-MS/MS analysis were prepared by adding 1 mL of each individual prepared stock solution (see above) of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in a flask and diluting with water/acetonitrile (80/20, v/v, S2) to a mixed calibration solution with a concentration of 100 µg/mL. Further dilution series with the mix of the four analytes were prepared with water/acetonitrile (80/20, v/v, S2). Homogeneity of the solutions was achieved by vortexing of the solutions. The solutions were prepared as exemplarily described in the table below.

Preparation of mixed calibration standard solutions (exemplified for analysis package L003)

Take stock solution [mg/mL]	Volume [mL]	Solution Identifier	Dilute to a volume of S2 [mL]	Concentration [µg/mL]	Solution Identifier
1 (BAS 595 F) 1 (M595F001) 1 (M595F002) 1 (M595F014)	1 1 1 1	SsoL163497 SsoL163499 SsoL163500 SsoL163498	10	100 (mixed)	SsoL163501
Take mixed solution [ng/mL]	**Volume [mL]**	**Solution Identifier**	**Dilute to a volume with S2 [mL]**	**Concentration mixed [ng/mL]**	**Solution Identifier**
100 000	1.0	SsoL163501	10	10 000	SsoL163502
10 000	1.0	SsoL163502	10	1 000	SsoL163503
10 000	0.2	SsoL163502	10	200	SsoL163504
1 000	1.0	SsoL163503	10	100	SsoL163505
1 000	0.5	SsoL163503	10	50	SsoL163506
200	1.0	SsoL163504	10	20 [1)]	SsoL163507
100	1.0	SsoL163505	10	10 [1)]	SsoL163508
50	1.0	SsoL163506	10	5.0 [1)]	SsoL163509
20	1.0	SsoL163507	10	2.0 [1)]	SsoL163510
10	1.0	SsoL163508	10	1.0 [1)]	SsoL163511
5.0	1.0	SsoL163509	10	0.5 [1)]	SsoL163512
2.0	1.0	SsoL163510	10	0.2 [1)]	SsoL163513

[1)] Used as calibration level.

Matrix-matched Solutions
Matrix-matched standards of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 were prepared to investigate the influence of the matrix load after sample preparation on the analysis (for details see section 3.4). For this purpose, matrix-matched standard solutions were prepared by dilution of standard calibration solutions with appropriate amounts of control samples (soil LUFA 2.4 or soil LUFA 2.2), which were taken through the entire preparation procedure as described in section 2.4.1.2. The matrix-load in the prepared matrix-matched standards was at least 90%. The preparation and dilution series are exemplarily shown for soil LUFA 2.4 matrix-matched standards in the table below.

Preparation of matrix-matched standard solutions (exemplified for analysis package L002)

Take mixed solution [ng/mL]	Volume [mL]	Solution Identifier	Dilute to a volume with ConL0003 [1] [mL]	Concentration mixed [ng/mL]	Solution Identifier
200	0.1	SsoL163504	1.0	20 [2]	SsoL163528
100	0.1	SsoL163505	1.0	10 [2]	SsoL163527
50	0.1	SsoL163506	1.0	5.0 [2]	SsoL163526
20	0.1	SsoL163507	1.0	2.0 [2]	SsoL163525
10	0.1	SsoL163508	1.0	1.0 [2]	SsoL163524
5.0	0.1	SsoL163509	1.0	0.5 [2]	SsoL163523
2.0	0.1	SsoL163510	1.0	0.2 [2]	SsoL163522

[1] To achieve enough matrix extract, ConL0003 was pooled from 2 control samples (ConL0001 and ConL0002), which were taken through the entire preparation procedure as described in section 2.4.1.2.
[2] Used as matrix-matched standard.

2.3.3 Set-up of the Analytical Run

Each analytical set began with a calibration standard and ended with the injection of a calibration standard. In general, calibration standards were interspersed with samples (e.g. fortified, control and quality control samples and blanks). Each calibration standard was measured at least twice. Seven calibration levels with concentrations ranging between 0.2 ng/mL and 20 ng/mL were injected.

2.4 Instrumental Analysis

2.4.1 Analytical Procedure

2.4.1.1 Weighing and Fortification

For the preparation of control samples, 5 g of the soil sample was transferred into a flask.
For the preparation of fortified samples, 5 g of the soil sample was transferred into a flask and spiked with the respective fortification solution. The principle of fortification is described in the table below:

Sample Type	Sample Volume [g]	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	5	--	--	0.0
Fortification (LOQ)	5	100	0.1	0.002 [1]
Fortification (10xLOQ)	5	1000	0.1	0.02

[1] Limit of quantification.

2.4.1.2 Extraction of Sample Material

Remark: The analytical procedure is based on the existing analytical method, Aventis Method No. 0051, BASF method L0353/01 [Ref. 1]. The principle of the given method stayed unchanged, but was downscaled by a factor of 10 to reduce the weigh in and the amount of solvents and other consumables.

A 5 g soil aliquot was weighed and 2.5 mL 0.1M ammonium hydroxide was added. The soil samples were manually shaken and then allowed to stand for 10 min. Subsequently, 12.5 mL acetone was added. After shaking for 1 h at 300 rpm on a mechanical shaker, the soil sample was treated for 10 min by ultra-sonication, following by subsequent centrifugation for 10 min at 3000 rpm.
The supernatant was decanted over cotton wool placed in a funnel into a 50 mL graduated cylinder. The soil remnant was additionally shaken manually with 12.5 mL acetone and centrifuged for 10 min at 3000 rpm. The supernatant was decanted over cotton wool placed in a funnel and combined in the same 50 mL graduated cylinder and the funnel was rinsed with about 2 mL acetone.
The volume of the graduated cylinder was adjusted to 30 mL with acetone. An aliquot of 15 mL was transferred into a 50 mL tapered flask and the solution was evaporated at 40°C using a rotary evaporator until the aqueous phase of about 1 – 1.5 mL remained.
The aqueous phase was transferred into a centrifuge tube, the tapered flasks was rinsed with around 0.4 mL water. The aqueous phases were combined in the centrifuge tube and the volume was adjusted to 2 mL with water.

A C_{18}-SPE column was pre-conditioned with 1.5 mL acetonitrile, followed by 1.5 mL water. Then, the aqueous sample was applied onto the C_{18}-SPE column and allowed to elute. The C_{18}-SPE column was washed with 1.5 mL water. Then, total vacuum was applied for 1 min for drying the C_{18}-SPE column.
The residues were eluted with 1.5 mL acetonitrile/methanol (95/5, v/v) into a centrifuge tube. Then, the liquid phase was evaporated to nearly dryness at 40°C using a nitrogen evaporator until about 0.45 mL remained. The remaining liquid phase was transferred into a 5 mL culture tube, the centrifuge tube was rinsed with 4 mL water and combined with the remaining liquid phase in the culture tube. The volume was adjusted with acetonitrile to 5 mL (V_{end}).

2.4.1.3 Preparation for Measurement

For residues at LOQ and 10x LOQ level, an aliquot of the combined solutions (V_{end} = final volume = 5 mL) was transferred into a LC vial and analysed by LC-MS/MS.

For the investigation of stability of stock and fortification solutions (refer to section 3.1), dilutions with water/acetonitrile (80/20, v/v) were made as described in the following tables:

Dilution series for investigation of stability of stock solutions

Take solution [ng/mL]	Volume [mL]	Dilute to a volume with S2 [mL]	Concentration [ng/mL]
1 000 000 (stock)	0.1	10	10 000
10 000	0.1	10	100
100	0.1	10	1

Dilution series for investigation of stability of fortification solutions

Take solution [ng/mL]	Volume [mL]	Dilute to a volume with S2 [mL]	Concentration [ng/mL]
1 000 (fortification)	0.1	10	10
10	0.1	1	1

2.4.2 Instrumentation and Conditions

The chromatographic system and conditions used for the analysis of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 are shown in the table below.

	Parameter		
Chromatographic System	Waters Acquity LC system		
Analytical Column	Phenomenex Luna Phenyl Hexyl, 100 x 4.6 mm, 5 µm particle size		
Guard Column[3)]	Phenomenex SecurityGuard C18, 4 x 3.0 mm		
Column Temperature	25°C		
Injection Volume[2)]	35 µL Partial loop with needle overfill; load ahead; loop offline - disabled		
Injection Procedure	Strong wash with acetonitrile (300 µL), weak wash with water / acetonitrile (500 µL)		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/2, v/v Methanol / formic acid, 1000/2, v/v		
Flow Rate	1.0 mL/min		
Isocratic Elution	Time (min)	Phase A [%]	Phase B [%]
	0.0 10.0	30 30	70 70
Divert Valve	yes		
Time to waste	1.2 min to 8.0 min to MS; remaining elution time to waste		
Detection System	AB Sciex Triple Quad 5500 Mass Spectrometer		
Ionisation	Turbo Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
BAS 595 F (Triticonazole) (Reg.No. 4378513)	318→70 [1)] 318→125	positive	approx. 5.75 min
M595F001 (Reg.No. 5079285)	334→70 [1)] 334→125	positive	approx. 2.45 min
M595F002 (Reg.No. 5059144)	334→70 [1)] 334→125	positive	approx. 3.13 min
M595F014 (Reg.No. 5079359)	318→70 [1)] 318→125	positive	approx. 6.15 min

[1)] Proposed as quantification transition.

[2)] Because of the capabilities of the used instrumentation, injection volume has been reduced to 35µL.

[3)] Material of guard column has been changed from C8 to C18 material, since C8 column was not available.

2.4.3 Calibration Procedure

The calculation of the results was based on the peak area measured using a calibration curve. Seven calibration levels were injected. Calibration curves were obtained by direct injection of solvent standards covering a concentration range of 0.2 ng/mL to 20 ng/mL. The same volume (35 µL) was injected for all samples and standard solutions.

2.4.4 Influence of Matrix Effects on the Analysis

In order to assess the influence of the matrix effects on the analysis, the response of analytes BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in the presence of the matrix soil was compared to standards prepared in water/acetonitrile (80/20, v/v) (see Table A 1 to Table A 16). For preparation of matrix-matched standards, refer to chapter 2.3.2.3 (Matrix-matched Solutions).

2.4.5 Quality Control Samples

Quality control samples were prepared to check the instrument performance during the analytical series for recovery experiments. For this purpose, 100 µL of a calibration solution with a concentration of 10 ng/mL were diluted with 900 µL of a control sample extract, resulting in a concentration of 1.0 ng/mL. The matrix load in the quality control samples was at least 90%.

2.4.6 Calculation of Residues and Recoveries

For the procedural recoveries, a sample volume of 5 g will be considered in the final calculation of residues [mg/kg]. The recovery is the percentage of the fortified amount of the analyte (µg or ng), which is recovered after the entire sample work-up steps.

The residues of BAS 595 F (Triticonazole) and its metabolites M595F001, M595F002 and M595F014 in mg/kg are calculated as shown in equations **I** and **II**:

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

II. **Residues in Soil [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 as read from the calibration curve [ng/mL]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquotation factor
1000	=	Factor for unit conversion

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 [%]** $= \dfrac{Residue\ in\ fortified\ sample \times 100}{Amount\ of\ analyte\ fortified}$

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

Example of Calculation:

BAS 595 F (Triticonazole), mass transition 318→125 in soil LUFA 2.4 fortified at 0.002 mg/kg:

The following values were used in this calculation:

Worklist no.	*2016pg70040*
Peak area of fortified sample (ForL0013)	*111258*
Peak area of control sample [1] (ConL0004 and ConL0005)	*0.0*
Slope	*104000*
Intercept	*-900*
Sample Aliquot (G)	*5 g*
Final Volume (V_{end})	*5 mL*
Aliquotation Factor (A_F)	*0.5 (= 50%)*

[1] Area of two control samples in the same worklist

$$\textbf{Concentration of fortified sample [ng/mL]} = \frac{111258 - (-900)}{104000} = 1.078\ \text{ng/mL}$$

$$\textbf{Residue fortified sample [mg/kg]} = \frac{5\ mL \times 1.078 ng/mL}{5\ g \times 0.5 \times 1000} = 0.00216 \mu g/g \equiv mg/kg$$

$$\textbf{Recovery [\%]} = \frac{0.00216 mg/kg}{0.002\ mg/kg} \times 100\% = 108.0\ \%$$

$$\textbf{Recovery corrected [\%]} = \frac{(0.00216 mg/kg - 0.0\ mg/kg) \times 100\%}{0.002\ mg/kg} = 108.0\%$$

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 numerical values.