

1 Summary

The modification of the analytical method 00952 / M001 was performed to extend the existing method 00952 for the analysis of the BYF00587 metabolite BYF00587-desmethyl in soil.

Soil samples of 20 g are extracted in a microwave extractor with 40 mL of a mixture of acetonitrile/water (4/1, v/v). Then a subsample is centrifuged to remove fine particles of the soil. Identification and quantitation of the test items is done by high performance liquid chromatography using MS/MS detection in the Multiple Reaction Monitoring mode. Possible Matrix effects of BYF00587-desmethyl are eliminated by using an internal standard solution of isotopically labelled reference item. This solution is added to the sample solutions after extraction.

The method was validated using a silt soil (Höfchen) and a sandy loam soil (Laacher Hof).

Specificity: Apparent residues in control samples were below $0.3 \times \text{LOQ}$. The recoveries were not corrected for interferences. Two MRM transitions were monitored for each matrix tested, m/z 398 \rightarrow 378 for quantitation and m/z 398 \rightarrow 358 for confirmation of BYF00587-desmethyl. In addition, this method was validated using internal standard solution of isotopically labelled reference items. Therefore, this HPLC-MS/MS method is regarded as highly specific.

Linearity: The correlation between the injected amount of substance and the detector response was linear for solvent standards ranging from 1.0 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$ (corresponding to about 2.0 to 200 $\mu\text{g/kg}$ sample equivalents). The correlation coefficient was 0.9997.

LOQ and LOD: The limit of quantitation (LOQ) for BYF00587-desmethyl is 5.0 $\mu\text{g/kg}$ in soil. The limit of determination (LOD) is 1.5 $\mu\text{g/kg}$.

Blank Values: The blank values in all control samples were below 1.5 $\mu\text{g/kg}$ ($<1/3 \times \text{LOQ}$), demonstrating that no background level of BYF00587-desmethyl was present in the test systems.

Recovery Rates (Accuracy): Mean recoveries for each fortification level and the overall mean recovery were within the 70 - 110% range for all matrices (see Table 1).

3 Compounds

3.1 Reference Item

Generally, only sufficiently characterised and certified substance was used as reference item.

BYF00587-desmethyl

Substance: BYF00587-desmethyl
Chemical Name: N-(3',4'-dichloro-5-fluorobiphenyl-2-yl)-3-difluoromethyl-1H-pyrazole-4-carboxamide

Mol-ID: 19969 Empirical Formula: C ₁₇ H ₁₀ Cl ₂ F ₃ N ₃ O Molecular Mass: 400.19 g/mol Analysis Certificate: AZ 13593 Expiry Date: 2008-07-07 Purity: 99.1%	
---	--

3.2 Internal Standard

Generally, only sufficiently characterised and certified substance was used as internal standard.

BYF00587-desmethyl IS

Substance: [chlorophenyl-¹³C₆, fluorophenyl-3-d] BCS-AA10008
Chemical Name: N-(3',4'-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1H-pyrazole-4-carboxamide

Empirical Formula: ¹³ C ₆ C ₁₁ H ₉ D Cl ₂ F ₃ N ₃ O Molecular Mass: 407.13 g/mol Analysis Certificate: KML 3656-1-4 Purity: >98 %	
---	--

4 Experimental Section

4.1 Test System

The method was validated using the two German soils Höfchen and Laacher Hof. Two different soils were used in order to assess a possible influence of different soil characteristics. The soil samples were classified according to USDA specifications. Soil characteristics of the used soils are summarised in Table 2.

Table 2: Soil Characteristics

	Soil Höfchen	Soil Laacher Hof
Description	Plot 4011; 0-30 cm soil layer	Plot 712/718; 0-30 cm soil layer
pH (in CaCl ₂ solution)	6.7	6.8
pH (in H ₂ O)	7.4	7.4
Organic Carbon [%]	0.92	1.20
Organic Matter [%] *	1.58	2.06
Cation Exchange Capacity [meq / 100 g dry soil]	12.4	9.8
max. Water Holding Capacity [g / 100 g dry soil]	39.5	37.9
Textural Description according to USDA [Fraction %]	Fraction [%]	Fraction [%]
Clay (<0.002 mm)	19.4	12.0
Silt (0.002-0.050 mm)	76.3	18.3
Sand (0.050-2.000 mm)	4.3	69.7
Soil type	Silt loam	Sandy loam

* Organic matter = Organic carbon x 1.72

4.2 Safety

The German guidelines for laboratories of the Employees' Liability Insurance Association, e.g. Bulletin M006 [4] or comparable guidelines in other countries should be observed.

The following chemicals were used, which are classified by the hazardous material regulations. The classification is based on the German guidelines [5] and has to be adapted to the respective national guidelines in case the method is used outside Germany.

BYF00587-desmethyl	very toxic * T+
Acetic acid	corrosive C
Acetonitrile	harmful Xn, highly flammable F
Methanol	toxic T, highly flammable F

*) A classification is not yet available. Due to this fact the compound has to be treated as very toxic substance.

The pertinent safety instructions must be observed when working with all compounds mentioned in this method (e.g. R- and S-phrases). It has to be made sure that the working place is properly ventilated when working with dry ice. Sample vessel and deep-freezing cabinet must guarantee pressure equalisation.

4.3 Materials

4.3.1 Apparatus and Reagents

For apparatus and reagents please see Appendix 2.

4.3.2 Stock Solutions

The stock solutions were prepared by weighing a defined amount of reference item into a volumetric flask and making up to volume with acetonitrile.

Table 3: Preparation Scheme of Reference Item Stock Solutions.

No.	Reference Item	Mass [mg]	Volume [mL]	Solvent	Final Concentration	
					Required [mg/L]	Actual * [mg/L]
1	BYF00587-desmethyl	10.10	20	**	500	501
2	BYF00587-desmethyl IS	5.12	10	**	500	502

*: Concentrations are corrected for purity.

** : Acetonitrile

4.3.3 Standard Solutions

Standard solutions (secondary standards) were prepared from the stock solutions by dilution with methanol/water (2/8 v/v).

Table 4: Preparation Scheme for Reference Standards.

No.	Reference Item:	Target Concentration [µg/L]	Prepared by Removal of [mL]	No. of Solution	Dilution to [mL]	Solvent
3	BYF00587-desmethyl (STD)	50000	2	1	20	*
4	BYF00587-desmethyl **	5000	10	3	100	*
5	BYF00587-desmethyl **	500	10	4	100	*
6	BYF00587-desmethyl IS (ISTD)	50000	2	2	20	*
7	BYF00587-desmethyl IS	5000	10	6	100	*
8	BYF00587-desmethyl IS	500	10	7	100	*
9	(STD) (ISTD)	100 5	2 1	4 8	100	*
10	(STD) (ISTD)	50 5	1 1	4 8	100	*
11	(STD) (ISTD)	20 5	4 1	5 8	100	*
12	(STD) (ISTD)	5 5	1 1	5 8	100	*
13	(STD) (ISTD)	2 5	0.4 1	5 8	100	*
14	(STD) (ISTD)	1 5	0.2 1	5 8	100	*
15	(STD) (ISTD)	0.75 5	0.15 1	5 8	100	*

*: Methanol/Water (2/8 V/V).

: **These standard solutions were used for fortification of the recoveries.

4.4 Sample Preparation

Extraction Procedure

1. Weigh 20 g of the soil sample into a 100-mL beaker containing a magnetic bar.
REMARK: For recoveries add standard solution in the appropriate volume for the corresponding fortification level.
2. Add 40 mL of a mixture of acetonitrile/water (4/1, v/v).
3. Place ten beakers with soil-solvent mixture into the microwave extractor.
4. Switch on the magnetic stirrer.
5. Extract for three minutes at 250 W.
6. Add an appropriate volume of internal standard solution, e.g. 400 µL of standard solution 8, and homogenise.
7. Transfer about 1.5 mL of the extract into a centrifuge tube. Centrifuge for 5 minutes at >12000 g to remove fine particles of soil.
8. Transfer about 1 mL into a HPLC sample vial and determine by liquid chromatography and MS/MS.

4.5 Instrumental Analysis

4.5.1 Principle of Measurement

An aliquot of the sample solution was injected into the high performance liquid chromatograph and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electro spray ionisation. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM). The pseudo molecular ions of the analytes ($[M+H]^+$, $[M-H]^-$ or any adducts) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were selected: one product ion (MRM-transition) serving for quantitation and the second for confirmation.

4.5.2 Variations in Instrument Conditions

Variations in equipment or sample characteristics and/or deterioration of system performance may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Instrument parameters and mobile phase may be adjusted to improve separation from unexpected interfering peaks.

Therefore, the given LC/MS/MS parameters listed may require adaptation.

4.5.3 Chromatography

Instrument: Agilent 1100 or equivalent
 Injector: HTC PAL, CTC Analytics or equivalent
 Column: Purospher STAR RP-18, length 55 mm, i.d. 4 mm
 Injection Volume: e.g. 10 µL or as needed for the sensitivity
 Oven temperature: e.g. 40 °C

Mobile Phase: Bin Pump A: Water/acetonitrile (9/1, v/v) containing 0.1 mL/L acetic acid. (Transfer 0.1 mL acetic acid into a 1 L volumetric flask and make up to volume with water/acetonitrile (9/1, v/v).)
 Bin Pump B: Acetonitrile containing 0.1 mL/L acetic acid. (Transfer 0.1 mL acetic acid into a 1 L volumetric flask and make up to volume with acetonitrile.)
 Iso Pump C: Water/acetonitrile (1/1, v/v) containing 0.1 mL/L acetic acid. (Transfer 0.1 mL acetic acid into a 1 L volumetric flask and make up to volume with water/acetonitrile(1/1, v/v).)

Time Table:

Time [min]	A [%]	B [%]	Flow Bin Pump [mL/min]	Into MS	Into Waste
0.0	50	50	1.0	Iso pump	Bin pump
1.7	--	--	--	Bin pump	Iso pump
3.4	50	50	1.0	--	--
3.5	10	90	1.0	--	--
4.0	--	--	1.0	Iso pump	Bin pump
5.6	50	50	1.0	--	--
8.0	50	50	1.0	--	--
8.0	Stop time				

Flow (Iso Pump): 1.0 mL/min
 Flow (into MS): splitted to optimize the spray
 Retention times: BYF00587-desmethyl approx. 2.1 min
 BYF00587-desmethyl IS approx. 2.1 min

4.5.4 Detection

The detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in positive ion mode and multiple reaction monitoring (MRM). Unit mass resolution was established and maintained in the mass resolving quadrupoles by maintaining a full width at half-maximum (FWHM) of about 0.7 amu. Optimal collisionally-activated dissociation (CAD) conditions for fragmentation of the pseudomolecular ions of the analytes and the corresponding stable isotopically labelled internal standards were applied with nitrogen as the collision gas.

Representative mass spectra are given in Appendix 5.

Detector: Triple Quadrupole Tandem Mass Spectrometer, Applied Biosystems MDS Sciex API 3000, Windows XP, Analyst 1.4.1 software versions or any equivalent HPLC-MS/MS System

Interface: Turbo IonSpray (ESI)
Gas Temperature: 300 °C or as needed for the sensitivity

Scan Type: MRM (Multiple Reaction Monitoring)

Table 5: MS/MS Parameters for the Determination of BYF00587-desmethyl.

	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)	Polarity
BYF00587-desmethyl Quantitation	398	378	300	-16	Neg
BYF00587-desmethyl Confirmatory	398	358	300	-24	Neg
BYF00587-desmethyl IS	405	385	300	-16	Neg

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

4.6 Calculation

The example calculation displayed below was used by the laboratory developing this method. Alternative calculation procedures appropriate to the reporting requirements may be used.

4.6.1 Calculation of Individual Residues and Recovery Rates

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystem quantitation software Analyst (Version 1.4) using linear regression. Further calculations were performed using the software EXCEL 2002 (Office 2002®). The linear equation is expressed as:

$$y = \text{Intercept} + \text{Slope} \times x$$

By means of the linear equation, the content of BYF00587 and BYF00587-desmethyl in dry soil or recoveries can be calculated as follows:

Area Analyte / Area Internal Standard	y	
Standard Concentration / Internal Standard Concentration	x	[(µg/L) / (µg/L)]
Sample Weight	G	[kg]
Fortified Amount	A	[µg]
Final Volume	VEND	[L]
Internal Standard Concentration	STI	[µg/L]
Residue in Dry Soil (only used for residue sample)	R	[mg/kg]
Recovery	Rec	[%]
Moisture	M	

$$R = \frac{y - \text{Intercept}}{\text{Slope}} \times \text{STI} \times \frac{\text{VEND}}{G} \times \frac{1}{1 - M}$$

$$\text{Rec} = \frac{y - \text{Intercept}}{\text{Slope}} \times \text{STI} \times \frac{\text{VEND}}{A} \times 100\%$$

Example for calculation of a BYF00587-desmethyl recovery 5.0 µg/kg in soil Höfchen:

Area Analyte / Area Internal Standard	y	10043 / 21582
Sample Weight	G	0.02 kg
Fortified Amount	A	0.1 µg
Final Volume	VEND	0.04 L
Internal Standard Concentration	STI	5 µg/L
Recovery	Rec	98 %
Intercept		-0.00318543
Slope		0.932786

$$100\% = \frac{\frac{10043}{21582} + 0.00318543}{0.932786} \times 5 \times \frac{0.04}{0.1} \times 100$$

4.6.2 Calculation of the Repeatability (Precision)

The repeatability or precision of the method is defined as the deviation of the validation results and is expressed as the relative standard deviation (RSD).

At each fortification level, the relative standard deviation was calculated as follows:

$$\text{Relative Standard Deviation [\%]} = \frac{\text{Standard Deviation}}{\text{Mean of Recoveries}} \times 100$$

Appendix 1: Method Characteristics

Table 14: Summary Parameters for the Analytical Method Used for the Quantitation of BYF00587 Residues in Soil. (DER TABLE B.1.1).

Method ID	00952 / M001		
Analyte(s)	BYF00587-desmethyl		
Extraction solvent/technique	Acetonitrile/water (4/1, v/v) Microwave		
Cleanup strategies	Extract, centrifuged for residues of BYF00587-desmethyl		
Instrument/Detector/Column	Agilent 1100LC Applied Biosystems API3000 LC/MS/MS Purospher STAR RP-18, length 55 mm, i.d. 4 mm		
Standardization method	Linear regression with 1/x weighting		
Stability of std solutions	Not been performed on this method		
Retention times		BYF00587-desmethyl	approx. 2.1 min
		BYF00587-desmethyl IS	approx. 2.1 min

Table 15: Characteristics for the Data-Gathering Analytical Method Used for the Quantitation of BYF00587-desmethyl Residues in Soil. (DER TABLE C.1.2).

Analyte	BYF00587-desmethyl
Equipment ID	Agilent 1100LC Applied Biosystems API3000 LC/MS/MS
Limit of detection (MDL)	Soil: 0.66 µg/kg for BYF00587-desmethyl
Limit of quantitation (LOQ)	Soil: 5.0 µg/kg for BYF00587-desmethyl
Accuracy/Precision	BYF00587-desmethyl: Soil 94 ± 2.7%
Reliability of the Method/ [ILV]	An ILV has not been performed on this method
Linearity	The correlation between the injected amount of substance and the detector response was linear for solvent standards ranging from 1.0 µg/L to 100 µg/L (corresponding to about 2.0 to 200 µg/kg sample equivalents). The correlation coefficient was 0.9997.
Specificity	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well defined and symmetrical. There appeared to be no carryover to the following chromatograms

Appendix 2: Apparatus and Reagents Apparatus

Apparatus

- Liquid chromatograph, Agilent 1100 column compartment G1316A, Agilent 1100 binary pump G1312A, Agilent 1100 iso pump G1313A, Agilent 1100 degasser G1379A, Agilent Technologies, Böblingen, Germany or equivalent
- Autosampler, HTC PAL, CTC Analytics, Switzerland or equivalent
- Mass spectrometer, API 3000 with ESI interface and mass spectrometric detector, Applied Biosystems, Darmstadt, Germany or equivalent
- Microwave, MLS-Ethos or equivalent
- Reversed phase chromatography column, Purospher STAR RP-18, length 55 mm, i.d. 4 mm or equivalent
- Volumetric flasks, 20-mL, 50-mL, 200-mL
- Variable dispenser 50-mL
- Glass beakers, 100-mL
- Calibrated pipettes, 0.5-mL, 1-mL, 10-mL
- Small instruments, e.g. Pasteur pipettes, autosampler vials, filter frits for reservoir

Reagents

- Acetonitrile for chromatography, LiChrosolv Merck KGaA, Darmstadt, Germany or equivalent
- Water, HPLC grade, purified with a Milli-Q-water system, Millipore Co., Eschborn, Germany or equivalent
- Acetic acid, Suprapur, Merck KGaA, Darmstadt, Germany or equivalent
- Nitrogen 5.0, 99.9990% purity, as bath, nebulizer, collision, curtain, and turbo gas, Linde AG, Höllriegelskreuth, Germany or equivalent

Appendix 3:
Analytical Procedure of Method 00952 / M001

Extraction Procedure

