1 Summary

The modification M001 to the analytical method 01035 was developed for the determination of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP in soil and sediment. Soil or sediment samples of 20 g are extracted in a microwave extractor with 40 mL of a mixture of acetonitrile/water (4+1, v+v). 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 BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP are eliminated by using an internal standard solution of isotopically labelled reference items. This solution is added to the sample solutions after extraction. 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.
The method was validated using two different soils and sediment.

Specificity: Apparent residues in control samples were below 0.3 × LOQ. The recoveries were not corrected for interferences. Two MRM transitions were monitored for each analyte and each matrix tested, m/z 318 → 141 for quantitation and m/z 318 → 234 for confirmation of BYF14182 , m/z 334 → 141 for quantitation and m/z 334 → 146 for confirmation of BYF14182-3-hydroxy-butyl and m/z 276 → 141 for quantitation and m/z 276 → 116 for confirmation of BYF14182-pyrazolyl-AAP .

Therefore, the HPLC-MS/MS method is highly specific but an additional confirmatory method is necessary.

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 50 µg/L. The correlation coefficients ranged from 0.9990 to 0.9997.

LOQ and LOD: The limit of quantitation (LOQ) for each single analyte is 5 µg/kg in soil. The limit of determination (LOD) for each single analyte is 1 µg/kg.

Blank Values: The blank values in all control samples were below 1 µg/kg (<1/3 x LOQ), demonstrating that no background level of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP were 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 sediment (see Table 1).

3 Compounds

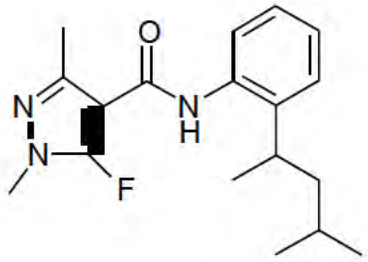
3.1 Reference Items

Generally, only sufficiently characterised and certified substances were used as reference items.

BYF14182

Active Substance:	BYF14182
Chemical Name:	N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Empirical Formula:	$C_{18}H_{24}FN_3O$
Molecular Mass:	317.41 g/mol
Analysis Certificate:	AZ 13704
Expiry Date:	October 2008
Purity:	99.5 %
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

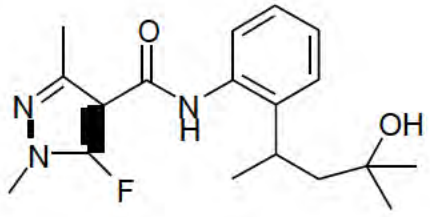
Structural Formula:



BYF14182-3-hydroxy-butyl

Active Substance:	BYF14182-3-hydroxy-butyl
Chemical Name:	5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide
Empirical Formula:	$C_{18}H_{24}FN_3O_2$
Molecular Mass:	333.41 g/mol
Analysis Certificate:	AZ 13567
Expiry Date:	July 2008
Purity:	97.4 %
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

Structural Formula:



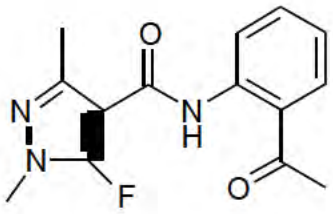
3.1 Reference Items (contd)

BYF14182-pyrazolyl-AAP

Active Substance: BYF14182-pyrazolyl-AAP
Chemical Name: N- (2-acetylphenyl)-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide

Empirical Formula: $C_{14}\,H_{14}\,F\,N_3\,O_2$
Molecular Mass: 275.28 g/mol
Analysis Certificate: AZ 14665
Expiry Date: 2010-12-08
Purity: 98.6%

Structural Formula:



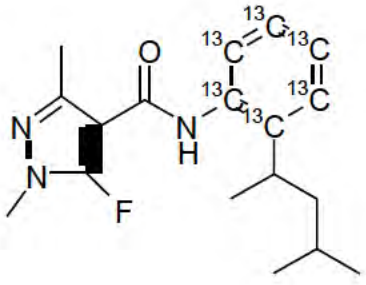
3.2 Internal Standards

BYF14182 IS

[phenyl-$^{13}C_6$] BYF14182

Active Substance: [phenyl-$^{13}C_6$] BYF 14182
Chemical Name: N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Empirical Formula: $^{13}C_6C_{12}\,H_{24}\,F\,N_3\,O$
Molecular Mass: 323.34 g/mol
Batch-Number: KML 3450-1-6
Purity: >98 %
Origin: Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

Structural Formula:



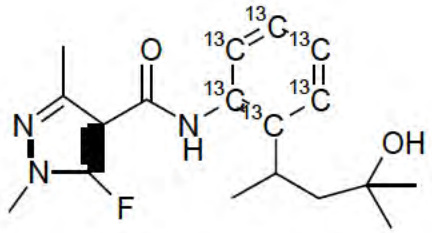
3.2 Internal Standards (contd)

BYF14182-3-hydroxy-butyl IS

[phenyl-$^{13}C_6$] BCS-AA10006

Active Substance:	[phenyl-$^{13}C_6$] BCS-AA10006
Chemical Name:	5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide
Empirical Formula:	$^{13}C_6C_{12}H_{24}FN_3O_2$
Molecular Mass:	339.34 g/mol
Batch-Number:	KML 3694-2-30
Purity:	>99 %
Origin:	Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

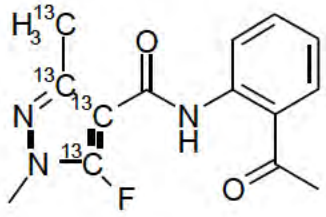
Structural Formula:



BYF14182-pyrazolyl-AAP IS

Standard Name:	[3-methyl-^{13}C, pyrazolyl-$^{13}C_3$] BCS-AF73126
Empirical Formula:	$^{13}C_4C_{10}H_{14}FN_3O_2$
Molecular Mass:	279.24 g/mol
Analysis Certificate:	KML 3937-1-15
Purity:	>99%

Structural Formula:



4 Experimental Section

4.1 Test System

The method was validated using sediment and 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 DIN and/or USDA specifications. Soil characteristics of the used soils are summarised in Table 2.

Table 2: Sediment and Soil Characteristics

	Sediment	Soil Höfchen	Soil Laacher Hof
Description	From Halver-Osenberg	Plot 4011; 0-30 cm soil layer	Plot 712/718; 0-30 cm soil layer
pH (in $CaCl_2$ solution)	5.9	6.7	6.8
pH (in H_2O)	6.6	7.4	7.4
Organic Carbon [%]	2.7	0.92	1.20
Organic Matter [%] *	4.70	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 [%]	Fraction [%]
Clay (<0.002 mm)	16.7	19.4	12.0
Silt (0.002-0.050 mm)	64.7	76.3	18.3
Sand (0.050-2.000 mm)	18.6	4.3	69.7
Soil type	Silt loam	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.

Acetic acid	Corrosive
Acetonitrile	Harmful, highly flammable

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 items into a volumetric flask and making up to volume with Acetonitrile.

Table 3: Preparation Scheme of Reference Item Stock Solutions.

Reference Item No.		Mass [mg]	Volume [mL]	Solvent	Final Concentration	
					Required [mg/L]	Actual [mg/L]
1	BYF14182 [STMBYF]	10.69	10	ACN	Not used	1064 *
2	BYF14182 IS [STMBYC]	4.98	10	ACN	Not used	488
3	BYF14182-3-hydroxy-butyl [STMBY3]	10.85	10	ACN	Not used	1057 *
4	BYF14182-3-hydroxy-butyl IS [STMBFC]	5.01	10	ACN	Not used	496
5	BYF14182-pyrazolyl-AAP [STMBYP]	11.28	10	ACN	Not used	1112 *
6	BYF14182-pyrazolyl-AAP IS [STMBRC]	5.60	10	ACN	Not used	554

* Concentrations are corrected for purity.

4.3.3 Standard Solutions

Standard solutions (secondary standards) were prepared from the stock solutions by dilution with acetonitrile/water/acetic acid (500/500/1 v/v/v).

Table 4: Preparation Scheme for Reference Standards.

No.	Reference Items:	Target Concentration [µg/L]	Prepared by Removal of [mL]	No. of Solution	Dilution to [mL]	Solvent
1 LSGBYP	BYF14182-pyrazolyl-AAP	111221	2	STMBYP	20	*
2 LSGBYP	BYF14182-pyrazolyl-AAP	11122	2	1 LSGPYP	20	*
3 LSGBYP	BYF14182-pyrazolyl-AAP	1112	2	1 LSGPYP	20	*
4 LSGBYP	BYF14182-pyrazolyl-AAP	556	5	1 LSGPYP	100	*
5 LSGBYP	BYF14182-pyrazolyl-AAP	111	1	1 LSGPYP	100	*

*** Acetonitrile/water/acetic acid (500/500/1 v/v/v).**

4.3.4 Calibration Standard Solutions

Standard mixture solutions were prepared by dilution with acetonitrile/water/acetic acid mixture (500/500/1, v/v/v). These standards were used to run the linearity investigations and for quantitation of residues.

Table 5: Preparation Scheme for Calibration Standards.

No.	Reference Item:	Target Concentration [µg/L]	Prepared by Removal of [mL]	No. of Solution	Dilution to [mL]
1 MIXBYF*	BYF14182 BYF14182-3-hydroxy-butyl BYF14182-pyrazolyl-AAP	9998 10040 10010	0.94 0.95 0.90	STMBYF STMBY3 STMBYP	100
2 MIXBYF*	BYF14182 BYF14182-3-hydroxy-butyl BYF14182-pyrazolyl-AAP	100 100 100	1.0	1 MIXBYF	100
3 MIXBYF	[phenyl-13C6] BYF 14182 [phenyl-13C6] BCS-AA10006 [3-methyl-13C] BCS-AF73126	4880 4960 4990	1.0 1.0 0.9	STMBYC STMBFC STMBRC	100
4 MIXBYF**	(All STD) (All ISTD)	0.5 20	0.5 0.4	2 MIXBYF 3 MIXBYF	100
5 MIXBYF	(All STD) (All ISTD)	1.0 20	1.0 0.4	2 MIXBYF 3 MIXBYF	100
6 MIXBYF	(All STD) (All ISTD)	2.5 20	2.5 0.4	2 MIXBYF 3 MIXBYF	100
7 MIXBYF	(All STD) (All ISTD)	5.0 20	5.0 0.4	2 MIXBYF 3 MIXBYF	100
8 MIXBYF	(All STD) (All ISTD)	25 20	0.25 0.4	1 MIXBYF 3 MIXBYF	100
9 MIXBYF	(All STD) (All ISTD)	50 20	0.5 0.4	1 MIXBYF 3 MIXBYF	100

*** This standard mix solutions were used for fortification of the recoveries.**
** **This standard mix solutions were used for the LOD estimation.**

All STD: Mix of BYF14182 , BYF14182-3-hydroxy-butyl, BYF14182-pyrazolyl-AAP , with the same concentration of each compound.

All ISTD: Mix of BYF14182 IS, BYF14182-3-hydroxy-butyl IS, BYF14182-pyrazolyl-AAP IS with the same concentration of each compound.

4.4 Sample Preparation

Extraction Procedure

1. Weigh 20 g of the soil or sediment sample into a 100-mL beaker containing a magnetic bar.

   REMARK: For recoveries add standard solution in the appropriate height 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 or sediment-solvent mixture into the microwave extractor.
4. Switch on the magnetic stirrer.
5. Extract for three minutes at 250 W.
6. Add appropriate internal standard solution, 160 µl of solution 3 MIXBYF 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 approximate 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: YMC: Pro C18, 120A, 3µm,33 x 4.0mm i.d.
Injection Volume: e.g. 25 µL or as needed for the sensitivity
Oven temperature: e.g. 60°C

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

Time Table:

Time [min]	A [%, v/v]	B [%, v/v]	Flow Bin Pump [mL/min]	Into MS	Into Waste
0.0	60	40	1.0	Iso pump	Bin pump
0.2	60	40	1.0		
0.8	--	--	--	Bin pump	Iso pump
2.2	25	75	1.0	--	--
2.3	10	90	2.0	--	--
2.4	--	--	--	Iso pump	Bin pump
3.0	10	90	2.0	--	--
3.1	60	40	1.0	--	--
3.5	Stop time				

Flow (Iso Pump): 1.0 mL/min
Flow (into MS): approx. 0.15 mL/min
Retention times:

BYF14182-3-hydroxy-butyl	approx. 0.9 min
BYF14182-3-hydroxy-butylIS	approx. 0.9 min
BYF14182-pyrazolyl-AAP	approx. 1.0 min
BYF14182-pyrazolyl-AAP IS	approx. 1.0 min
BYF14182	approx. 1.8 min
BYF14182 IS	approx. 1.8 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.

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: 350°C or as needed for the sensitivity
Scan Type: MRM (Multiple Reaction Monitoring)

Table 6: MS/MS Parameters for the Determination of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP.

	Pre-cursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)	Polarity
BYF14182 Quantitation	318	141	75	39	pos
BYF14182 Confirmatory	318	234	75	39	pos
BYF14182 IS	324	240	75	21	pos
BYF14182-3-hydroxy-butyl Quantitation	334	141	75	39	pos
BYF14182-3-hydroxy-butyl Confirmatory	334	146	75	39	pos
BYF14182-3-hydroxy-butyl IS	340	152	75	21	pos
BYF14182-pyrazolyl-AAP Quantitation	276	141	75	21	pos
BYF14182-pyrazolyl-AAP Confirmatory	276	116	75	30	pos
BYF14182-pyrazolyl-AAP IS	280	145	75	21	pos

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. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

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:

$$y = Intercept + Slope \times x$$

By means of the linear equation, the content of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP 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	[mg]
Final Volume	VEND	[L]
Internal Standard Concentration	STI	[µg/L]
Residue in Dry Soil (only for residue samples)	R	[mg/kg]
Recovery	Rec	[%]
Moisture	M	

$$R = \frac{y - Intercept}{Slope} \times STI \times \frac{VEND}{G} \times \frac{1}{1-M} \qquad Rec = \frac{y - Intercept}{Slope} \times STI \times \frac{VEND}{A} \times 100\%$$

Example for a calculation for an BYF14182 recovery 5.0 µg/kg in soil Höfchen:

Area Analyte / Area Internal Standard	y	44856/ 1485957
Sample Weight	G	0.02 kg
Fortified Amount	A	0.0001 mg
Final Volume	VEND	[L]
Internal Standard Concentration	STI	20 µg/L
Recovery	Rec	99 %
Intercept		-0.000146233
Slope		0.245715

$$100\% = \frac{\frac{44856}{1485957} + 0.000146233}{0.245715} \times 20 \times \frac{0.04}{0.02} \times 100$$

Appendix 1: Method Characteristics

Table 20: Summary Parameters for the Analytical Method Used for the Quantitation of [Chemical] Residues in [Matrices]. (DER TABLE B.1.1).

Method ID	01035/M001	
Analyte(s)	BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP	
Extraction solvent/technique	water/acetonitrile (1+4, v+v) Microwave	
Cleanup strategies	Extract, centrifuged for residues of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP	
Instrument/Detector/Column	Agilent 1100LC Applied Biosystems API3000 LC/MS/MS YMC: Pro C18, 120A, 3µm,33 x 4.0mm i.d.	
Standardization method	Linear regression with 1/x weighting	
Retention times	BYF14182-3-hydroxy-butyl BYF14182-3-hydroxy-butylIS BYF14182-pyrazolyl-AAP BYF14182-pyrazolyl-AAP IS BYF14182 BYF14182 IS	approx: 0.9min approx: 0.9min approx: 1.0min approx: 1.0min approx: 1.8min approx: 1.8min

Appendix 1:
Method Characteristics (contd)

Table 21: Characteristics for the Data-Gathering Analytical Method Used for the Quantitation of [Chemical] Residues in [Matrices]. (DER TABLE C.1.2).

Analyte	BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP
Equipment ID	Agilent 1100LC Applied Biosystems API4000 LC/MS/MS
Limit of detection (LOD)	0.38 to 0.62 µg/kg for BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP
Limit of quantitation (LOQ)	5.0 µg/kg for BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP
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 50 µg/L. The correlation coefficients ranged from 0.9990 to 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

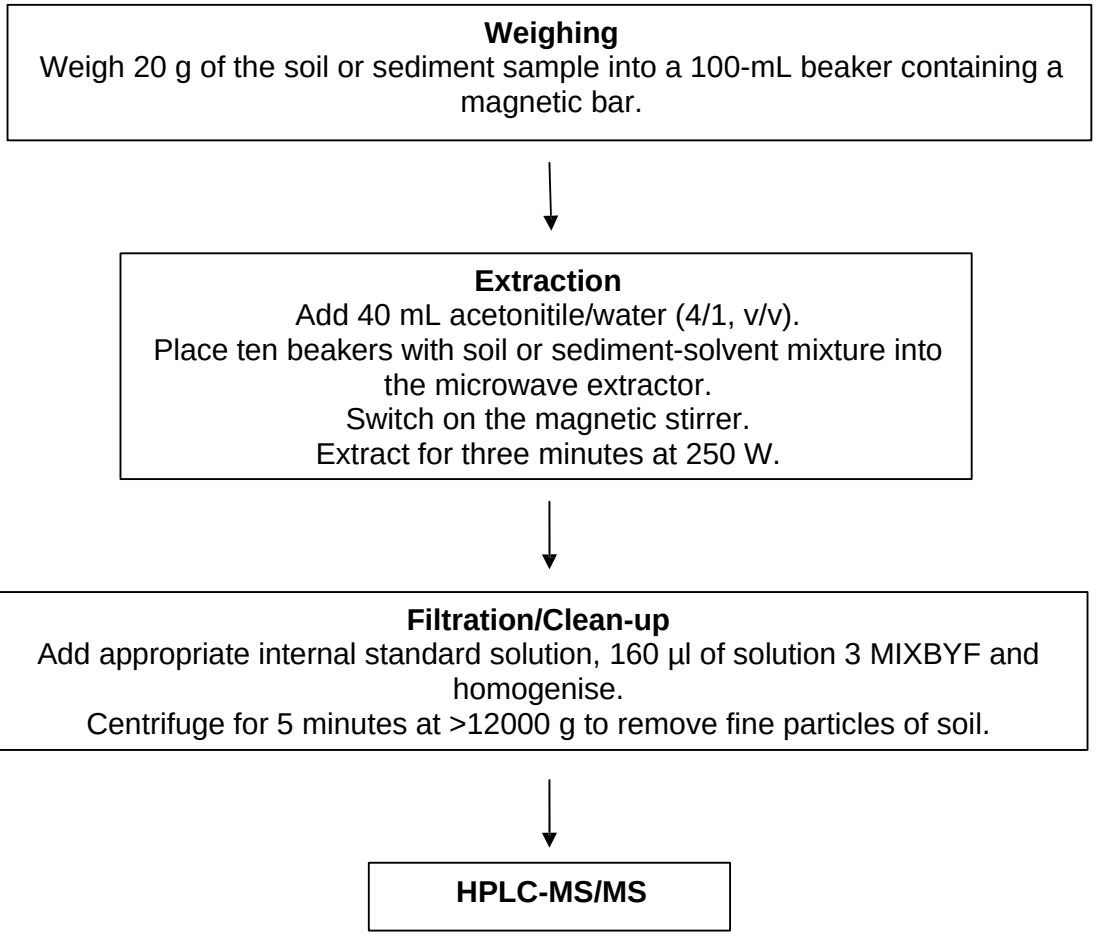
- Liquid chromatograph, Agilent 1100 column compartment G1316A, Agilent 1100 binary pump G1312A, Agilent 1100 iso pump G1310A, Agilent 1100 degasser G1379A, Agilent Technologies, Böblingen, Germany or equivalent
- Autosampler, HTC PAL, CTC Analytics, Switzerland or equivalent
- Mass spectrometer, API 3000 with ESI interface and mass spectrometric detector, Applied Biosystems, Darmstadt, Germany or equivalent
- Microwave, MLS-Ethos or equivalent
- Reversed phase chromatography column,
  YMC: Pro C18, 120A, 3µm,33 x 4.0mm i.d. 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 01035/M001

Extraction Procedure



Appendix 4:
Detailed Summary of Chromatographic and Mass Spectrometric Conditions

```
Comment:                        BYF14182 IS  incl.BYF14182-pyrazolyl-AAP
Synchronization Mode:           LC Sync
Auto-Equilibration:             Off
Acquisition Duration:           3min30sec
Number Of Scans:                250
Periods In File:                1
Acquisition Module:             Acquisition Method
Software version  Analyst 1.4.1
Agilent 1100 LC Pump Method Properties
Pump Model: Agilent 1100 LC Binary Pump
Minimum Pressure (psi):         0.0
Maximum Pressure (psi):         5801.0
Dead Volume (µl):               40.0
Maximum Flow Ramp (ml/min²):    100.0
Maximum Pressure Ramp (psi/sec):    290.0

Step Table:
@Step Total Time(min)   Flow Rate(µl/min) A (%) B (%)
0     0.00              1000              60.0  40.0
1     0.20              1000              60.0  40.0
2     2.20              1000              25.0  75.0
3     2.30              2000              10.0  90.0
4     3.00              2000              10.0  90.0
5     3.10              1000              60.0  40.0
6     3.50              1000              60.0  40.0

Left Compressibility:               50.0
Right Compressibility:              115.0
Left Dead Volume (µl):              40.0
Right Dead Volume (µl):             40.0
Left Stroke Volume (µl):            -1.0
Right Stroke Volume (µl):           -1.0
Left Solvent:                       A1
Right Solvent:                      B1


CTC PAL Autosampler Method Properties
Loop Volume1 (µl):      100
Loop Volume2 (µl):      100
Injection Volume (µl):  25.000
```

Appendix 4:
Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

Method Description:

Syringe: 100ul

01	Analyst LC-Inj	
	Air Volume (µl)	0
	Pre Clean with Solvent 1 ()	0
	Pre Clean with Solvent 2 ()	0
	Pre Clean with Sample ()	0
	Filling Speed (µl/s)	10
	Filling Strokes ()	2
	Inject to	LC Vlv1
	Injection Speed (µl/s)	100
	Pre Inject Delay (ms)	500
	Post Inject Delay (ms)	500
	Post Clean with Solvent 1 ()	1
	Post Clean with Solvent 2 ()	1
	Valve Clean with Solvent 1 ()	1
	Valve Clean with Solvent 2 ()	1
	Replicate Count ()	1
	Analysis Time (s) ()	0

Agilent 1100 Column Oven Properties

Left Temperature (°C):	60.00
Right Temperature (°C):	60.00
Temperature Tolerance +/- (°C):	1.00
Start Acquisition Tolerance +/- (°C):	0.50

Time Table (Not Used)
Column Switching Valve Installed
Position for first sample in the batch: Left (1->6)
Use same position for all samples in the batch

Valco Valve Method Properties
Valco Valve Diverter

	Total Time (min)	Position
1	0.8	Bin Pump
2	2.4	Iso Pump

Appendix 4:
Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

MS Method Properties:
Period 1:

Scans in Period:	250
Relative Start Time:	0.00 msec
Experiments in Period:	1

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Thres.:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0000 msec
MCA:	No
Step Size:	0.00 amu

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
318.20	141.00	75.00	DP	40.00	40.00
			CE	39.00	39.00
			CXP	55.00	55.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
318.20	234.00	75.00	DP	40.00	40.00
			CE	39.00	39.00
			CXP	55.00	55.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
334.20	141.00	75.00	DP	40.00	40.00
			CE	39.00	39.00
			CXP	55.00	55.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
334.20	146.00	75.00	DP	40.00	40.00
			CE	39.00	39.00
			CXP	55.00	55.00

Appendix 4:
Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
324.20	240.00	75.00	DP	35.00	35.00
			CE	21.00	21.00
			CXP	8.00	8.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
340.20	152.00	75.00	DP	35.00	35.00
			CE	21.00	21.00
			CXP	8.00	8.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
276.20	140.90	75.00	DP	35.00	35.00
			CE	21.00	21.00
			CXP	8.00	8.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
276.20	116.10	75.00	DP	20.00	20.00
			CE	30.00	30.00
			CXP	15.00	15.00

@Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
280.20	144.90	75.00	DP	35.00	35.00
			CE	21.00	21.00
			CXP	8.00	8.00

Parameter Table(Period 1 Experiment 1):

NEB:	12.00
CUR:	12.00
IS:	5000.00
TEM:	350.00
CAD:	4.00
FP	200.00
EP	10.00

Appendix 4:
Detailed Summary of Chromatographic and Mass Spectrometric Conditions (contd)

Agilent 1100 LC Pump Method Properties

Pump Model:	Agilent 1100 LC Isocratic Pump
Minimum Pressure (psi):	0.0
Maximum Pressure (psi):	5801.0
Compressibility:	100.0
Dead Volume (µl):	40.0
Stroke Volume (µl):	-1.0
Maximum Flow Ramp (ml/min²):	100.0
Maximum Pressure Ramp (psi/sec):	290.0

Step Table:

@Step	Total Time(min)	Flow Rate(µl/min)
0	0.00	1000
1	3.50	1000

Primary Flow Rate (ul/min): 200.0
Flow Sensor Calibration Table Index: 0