2. BACKGROUND

BYF14182 is a fungicide currently being developed by Bayer CropScience with potential uses in several crops.

The purpose of this study was to demonstrate that the "Modification M001 to the analytical method 01035 for the determination of residues of BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP in soil and sediment by HPLC-MS/MS"[3], can be performed with acceptable recoveries at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience AG, Development-Residues, Operator and Consumer Safety, at their laboratory in Monheim, Germany and reported by Thomas Freitag.

This study was performed in accordance with US EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue Analytical Method, August 1996 [2], and US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996 [1].

The method extraction was performed as written. The LC/MS/MS parameter settings were in general as described in analytical method 01035 (modification M001) but optimized for the instrument being used.

The method was successfully validated for BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP in both soil and sediment.

3. EXPERIMENTAL DETAILS

Study initiation date: September 24, 2008
Experimental Completion Date: October 7, 2008

The following personnel were involved in the conduct of this study.

Sarah Stoughton (Study Director, Principal Analytical Investigator)
R&D Specialist I
Residue and Environmental Chemistry
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3.1 Test Substances

The test substances for this study were BYF14182 and its metabolites, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP. See Appendix 3 for complete nomenclature and chemical structures.

3.2 Analytical Reference Substances

The test substances also served as the analytical reference substances. See Appendix 3 for complete nomenclature, chemical structures and reference information for the reference substances. The test and reference substances were stored in a freezer until used to prepare fortification and calibration solutions. All stock solutions were stored in a freezer set at an average temperature of -19°C when not in use. All fortification and calibration solutions were stored in a refrigerator set at an average temperature of 8°C when not in use.

3.3 Test System

The test systems were sub-samples of bulk untreated soil and sediment samples. The soil samples were obtained from Bayer CropScience Study Numbers MEELP055 [4], Terrestrial Field Dissipation of BYF14182 in California Soil, 2007 (Sample ID MEELP055-B1-014-1UT1) and MEELP053 [5], Terrestrial Field Dissipation of BYF14182 in Ontario, Canada Soil, 2007 (Sample ID MEELP053-B1-014-1UT1). The sediment sample was obtained from Bayer CropScience Study Number RAELP039 [6], Aquatic Field Dissipation of BYF14182 FS240 in a Cropped Arkansas Rice Field, 2008 (Sample Number RAELP039-A10053-NUT1). Characterization data for each of these samples is presented in Appendix 4.

The soil and sediment samples were stored at room temperature prior to fortification.

3.4 Method Summary

Each analytical set included one reagent blank, two unfortified control samples, five samples fortified at the LOQ (5.0ng/g or ppb) and five samples fortified at 10x LOQ (50.0ng/g).

BYF14182 and its metabolites were extracted from soil and sediment using microwave extraction. An isotopic internal standard was added to the extract, the sample mixed and an aliquot analyzed by LC/MS/MS for BYF14182, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP.

3.5 Instrumentation

- Sciex API 4000 LC/MS/MS System (Applied Biosystems)
- Shimadzu LC-10AD$_{VP}$ HPLC Pumps (2) with a high pressure mixer and Shimadzu SCL-10A$_{VP}$ Pump Controller
- CTC PAL Autosampler
- Eppendorf CH-30 Column Oven

3.6 LC/MS/MS Conditions

Note that the gradient parameters and LC/MS/MS settings listed below were for the API 4000 MS/MS system which was used in this study. These conditions differ from the original method which used an API 3200 MS/MS, but were found to be optimum for the LC/MS/MS instrument used in this study.

Column:YMC–Pack ProC18 33 x 4.0 mm i.d. 3µm
(Part Number AS12S030304QT)

Column oven temperature: 60ºC

Injection volume: 10 µL
Mobile phase: A: Water/acetonitrile (9:1 v/v) with 0.1mL/L acetic acid
B: Acetonitile containing 0.1 mL/L acetic acid
Flow rate (column): 1.00 mL/min

Retention times: BYF14182: approx. 1.8 minutes
BYF14182-3-hydroxy-butyl: approx. 0.9 minutes
BYF14182-pyrazolyl-AAP: approx. 1.0 minutes

HPLC Gradient Parameters

Time [min]	% A	% B
0.00	60	40
0.01	60	40
0.20	60	40
2.20	25	75
2.30	10	90
3.00	10	90
3.10	60	40
4.00	System Controller	Stop

MS/MS Conditions

	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)
BYF14182 Quantitation MRM	318.2	141.0	75	50	14	43	45
BYF14182 Confirmatory MRM	318.2	234.0	75	75	14	25	45
BYF14182 ^{13}C Internal Standard	324.2	240.0	75	55	14	25	45
BYF14182-3-hydroxy-butyl Quantitation MRM	334.2	141.0	75	85	7	35	10
BYF14182-3-hydroxy-butyl Confirmatory MRM	334.2	146	75	80	6	30	10
BYF14182-3-hydroxy-butyl ^{13}C Internal Standard	340.2	152.0	75	85	7	35	10
BYF14182-pyrazolyl-AAP Quantitation MRM	276.2	140.9	75	80	12	20	35
BYF14182-pyrazolyl-AAP Confirmatory MRM	276.2	116.1	75	80	12	50	35
BYF14182-pyrazolyl-AAP ^{13}C Internal Standard	280.2	114.9	75	55	11	20	30

The ^{13}C isotope transitions are used for both the quantitation and confirmatory analyses

CAD (Collision Gas)	12
CUR (Curtain Gas) [psi]	35
GS1 (Ion Source Gas 1) [psi]	40
GS2 (Ion Source Gas 2) [psi]	15
Source Temperature [°C]	750
Ihe (interface heater)	ON
Ion Transfer Voltage (IS) [V]	5500
Resolution of Q1 and Q3	Q1 Unit, Q3 Low
Scan type	MRM
Polarity	Positive
Ion Source	Turbo Spray

Detector Parameters	
Channel Electron Multiplier (CEM)	2300 V
Deflector (DF)	-50 V

3.7 Calculations

An example calculation for BYF14182 from sample RAELP007-ON-LOQ1-004, which was analyzed during the method validation study, is shown below. This sample was fortified with 5ng/g of BYF14182, BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP. The chromatogram used in this example is presented in Appendix 2 (Chromatogram 5).

The standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

The example shown below is for the calculation of BYF14182 residues. BYF14182-3-hydroxy-butyl and BYF14182-pyrazolyl-AAP residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in ng/g was determined. The ng/g of BYF14182 in the soil was calculated using the following equation,

$$\text{BYF14182 found (ng/g)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume}(V_1)}{\text{Initial sample wt. (W)}}$$

W	V_1	Native Peak Area	IS Peak Area	**Y**	**M**	**B**
20g	40mL	74902.3	1683038.4	0.0445	0.0186	2.81×10^{-8}

The slope and intercept were obtained from the calibration curve generated by Analyst, and is presented in Appendix 1 (Figure 1). The calibration points were weighted 1/x to provide better fit near the limit of detection

From the above equations:

$$\text{Dilution Factor (D)} = \frac{40}{20} = 2$$

$$\text{BYF14182 found} = \frac{(0.0445 - (2.81 \times 10^{-8})) \times 2}{0.0186} = 4.79 \text{ng/g}$$

Therefore sample RAELP007-ON-LOQ1-004 contains 4.79ng/g BYF14182.

The % recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{R}{T} \times 100$$

Where: R = ng/g of target analyte found in fortified sample
T = theoretical ng/g in fortified sample

Therefore, for sample RAELP007-ON-LOQ1-004,

R = 4.79ng/g
T = 5.0 ng/g

$$\text{\% Recovery} = \frac{(4.79)}{5.0} \times 100 = 96\%$$

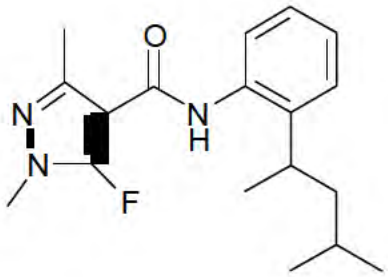
Remark: Example calculations shown above were performed using the LC/MS/MS software *Analyst (version 1.4.1)*. The example calculation was performed using the area values reported by the instrument. The instrument software carries additional figures not shown in the intermediate results. Therefore, instrument software calculated values will differ slightly from the results derived using a calculator.

Appendix 3. Identity and Purity of the Test and Reference Materials Used

BYF14182

Code Name:	BYF14182
CAS Name:	N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Molecular Formula:	$C_{18}H_{24}FN_3O$
Molecular Weight:	317 g/mol
ID No.:	K-1683
Expiration Date:	10/5/08; recertified, new expiration date: 9/17/11
Purity:	99.5 %
Storage Conditions:	Frozen
Source:	Bayer CropScience, Kansas City, MO

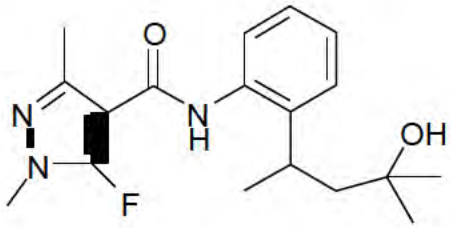
Structural Formula:



BYF14182-3-hydroxy-butyl

Code Name:	BYF14182-3-hydroxy-butyl
Molecular Formula:	$C_{18}H_{24}FN_3O_2$
Molecular Weight:	333 g/mol
ID No.:	K-1773
Expiration Date:	6/20/10
Purity:	99.2 %
Storage Conditions:	Frozen
Source:	Bayer CropScience, Frankfurt, Germany

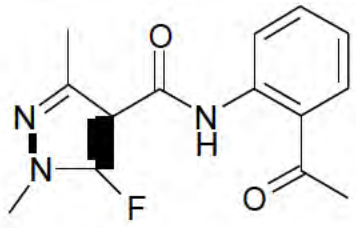
Structural Formula:



BYF14182-pyrazolyl-AAP

Code Name:	BYF14182-pyrazolyl-AAP
Molecular Formula:	$C_{14}\ H_{14}\ F\ N_3\ O_2$
Molecular Weight:	275 g/mol
ID No.:	K-1757
Expiration Date:	12/8/10
Purity:	98.6%
Storage Conditions:	Frozen
Source:	Bayer CropScience, Kansas City, MO

Structural Formula:



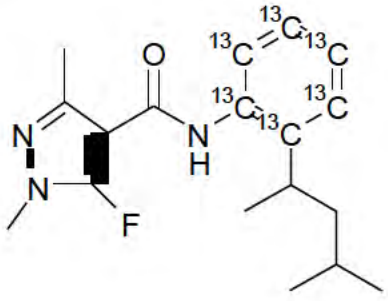
Internal Standards

BYF14182 Internal Standard

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

Code Name:	[phenyl-$^{13}C_6$] BYF 14182
Molecular Formula:	$^{13}C_6C_{12}\ H_{24}\ F\ N_3\ O$
Molecular Weight:	323 g/mol
ID No.:	K-1686
Expiration Date:	3/28/17
Purity:	99.8 %
Storage Conditions:	Frozen
Source:	Bayer CropScience, Wuppertal, Germany

Structural Formula:

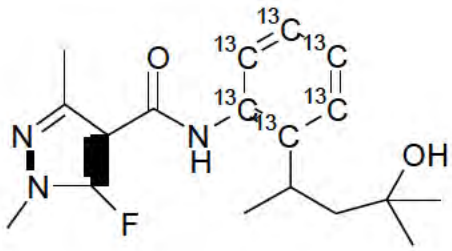


BYF14182-3-hydroxy-butyl Internal Standard

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

Code Name:	[phenyl-$^{13}C_6$] BCS-AA10006
Molecular Formula:	$^{13}C_6C_{12}H_{24}FN_3O_2$
Molecular Weight:	339 g/mol
ID No.:	K-1752
Expiration Date:	2/1/18
Purity:	99.8 %
Storage Conditions:	Frozen
Source:	Bayer CropScience, Wuppertal, Germany

Structural Formula:



BYF14182-pyrazolyl-AAP Internal Standard

Code Name:	[3-methyl-^{13}C,pyrazolyl-$^{13}C_3$]BCS-AF73126
Molecular Formula:	$^{13}C_4C_{10}H_{14}FN_3O_2$
Molecular Weight:	279 g/mol
ID No.:	K-1771
Expiration Date:	6/4/18
Purity:	100 %
Storage Conditions:	Frozen
Source:	Bayer CropScience, Wuppertal, Germany

Structural Formula: