2 Objective

The objective of this study was to demonstrate that method EL-001-W08-01 ("An Analytical Method for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP in Water Using LC/MS/MS") can be performed with acceptable recoveries for determination of the compounds BYF 14182, BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience Residue and Environmental Chemistry, Stilwell, USA, and reported as Method EL-001-W08-01, by Jami M. Wade, dated May 27, 2009. River Rhine water (taken at Leverkusen-Hitdorf, Germany) and tap water Monheim, Germany were chosen as representative matrices for validation within the present study.

This study was performed in accordance with US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996.

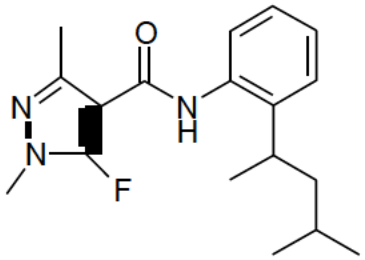
3 Materials

3.1 Test and Reference Items

Code Name: **BYF 14182** (Active Ingredient, Parent Molecule)

CAS Name: N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide

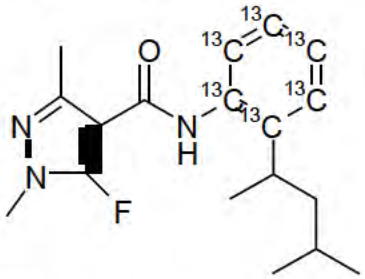
Structural formula:



Molecular Formula: $C_{18}H_{24}FN_3O$
Molecular Weight: 317 g/mol
Certificate of analysis: AZ 15302
Origin Batch No.: NLL7306-17
Purity: 99.5%
Expiry date: 2011-09-17
Appearance: light beige powder

Code Name: **BYF 14182 [phenyl-$^{13}C_6$]**
(Parent Molecule, Labeled Internal Standard)
IUPAC Name: N-[2-(1,3-dimethylbutyl)phenyl]-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide

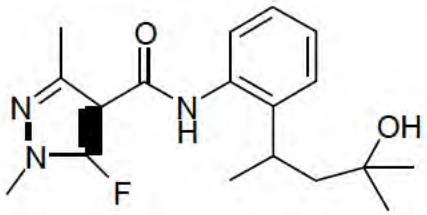
Structural formula:



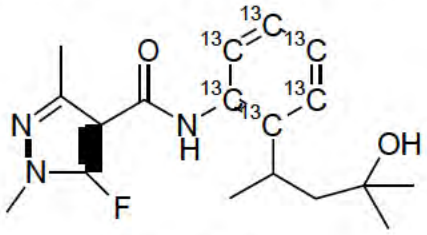
Molecular Formula: $^{13}C_6C_{12}H_{24}FN_3O$
Molecular Weight: 323 g/mol
Standard No.: KML3450-1-6
Purity: >98%
Certification date: 2004-10-21

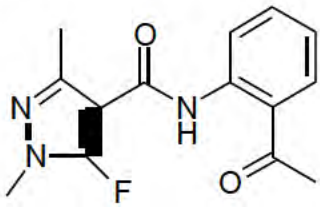
Code Name: **BYF14182-3-hydroxy-butyl** (Metabolite)
CAS Name 5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide

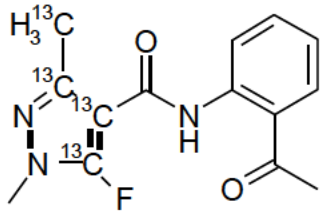
Structural formula:



Molecular Formula: $C_{18}H_{24}FN_3O_2$
Molecular Weight: 333 g/mol
Certificate of analysis: AZ 15083
Origin Batch No.: SES 10139-3-3
Purity: 99.2%
Expiry date: 2010-06-20
Appearance: light beige powder

Code Name:	**BYF14182-3-hydroxy-butyl-$^{13}C_6$**
	BCS-AA10006-[phenyl-$^{13}C_6$]
	(Metabolite, Isotopic Internal Standard)
CAS Name	5-fluoro-N-[2-(3-hydroxy-1,3-dimethylbutyl)phenyl]-1,3-dimethyl-1H-pyrazole-4-carboxamide
Structural formula:	
Molecular Formula:	$^{13}C_6C_{12}H_{24}FN_3O_2$
Molecular Weight:	339 g/mol
Batch No.:	KML 3694-2-30
Purity:	>99 %
Certification date:	2006-09-19

Code Name:	**BYF14182-pyrazolyl-AAP** (Metabolite)
CAS Name:	N-(2-acetylphenyl)-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Structural formula:	
Molecular Formula:	$C_{14}H_{14}FN_3O_2$
Certificate of analysis:	AZ 14665
Molecular Weight:	275 g/mol
Origin Batch No.:	KTS10231-2-3
Purity:	98.6%
Expiry date:	2010-12-08
Appearance:	beige powder

Code Name:	**BYF14182-pyrazolyl-AAP-$^{13}C_4$** [3-methyl-^{13}C,pyrazolyl-$^{13}C_3$] BCS-AF73126 (Metabolite, Isotopic Internal Standard)
CAS Name:	N-(2-acetylphenyl)-5-fluoro-1,3-dimethyl-1H-pyrazole-4-carboxamide
Structural formula:	
Molecular Formula:	$^{13}C_4C_{10}\ H_{14}\ F\ N_3\ O_2$
Molecular Weight:	279 g/mol
Batch No.:	KML 3937-1-15
Purity:	> 99%
Expiry date:	2010-05-05

More details about the reference items are documented in the raw data. For handling of the reference items and for preparation of standard solutions see SOP 1001 and SOP 1014.

3.2 Test System

The method was validated using river Rhine and tap water Monheim. Two different water types were used in order to assess a possible influence of different water characteristics. The water samples were analysed for TOC, DOC, conductivity, water hardness, dry residue after filtration and pH by Currenta , 51368 Leverkusen, Germany. Water types are summarised in Table 1. Complete water parameters are reported in Table 13 and Table 14.

Table 1: Water Types

Water Type	Source of Water
Surface Water	River Rhine Water
Tap Water	Tap Water Monheim

4 Experimental

4.1 Analytical Method

The recovery data for the study were generated using the following method, which gives full details of preparing the analytical sample extracts and the conditions for high performance liquid chromatography (HPLC):

Number of the method:	EL-001-W08-01
Title of the method:	An Analytical Method for the Determination of Residues of BYF 14182 And Its Metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP in Water Using LC/MS/MS
Author of the method:	Jami M. Wade Bayer CropScience Residue and Environmental Chemistry 17745 South Metcalf Avenue Stilwell, Kansas 66085
Reference:	Method EL-001-W08-01
Limit of quantitation:	0.1 µg/L

The following sample sets were analysed:

Table 2: Level and Number of Recoveries per Fortification Level

Water	Control sample	Level 0.1 µg/L	Level 1.0 µg/L
River Rhine	2	5	5
Tap Water Monheim	2	5	5

4.1.1 Outline of the Method

Appropriate volumes of fortification and internal standard solutions were added to the water samples. After mixing the analysis was performed by LC-MS/MS.

4.1.2 Instruments

Liquid Chromatograph:	1200 Column Compartment G1316B 1200 Binary Pump G1312B Bin Pump SL 1200 Isocratic Pump G1310A 1200 Degasser G1322A Agilent Technologies, 40880 Ratingen, Germany
Autosampler:	HTC PAL System CTC Analytics AG 4222 Zwingen, Switzerland

Mass Spectrometer:	Applied Biosystems API 4000 with turbo ionspray interface mass selective detector (MS/MS) Note: Some mass spectrometric conditions are instrument specific. The spectrometric conditions were optimised by a competent operator prior to analysis.

4.1.3 Reagents and Equipment

Column (HPLC):	YMC Pro C18 , length 33mm x 4.0mm, Particle size 3µm, pore size 1200A Series No.: 90306 14839
Acetonitrile:	for HPLC, optigrade LGC Promochem GmbH, UN 1648 46485 Wesel, Germany
Acetic acid (100%):	Suprapur Merck KGaA, No. 1.00066.1000 64271 Darmstadt, Germany
Deionized water:	purified in a Milli-Q unit Milli-Pore GmbH 65731 Eschborn, Germany

Volumetric flasks, pipettes and other equipment commonly used in the laboratory.

4.1.4 Liquid Chromatographic Conditions

Liquid chromatographic conditions were almost identical to those described in the original method report EL-001-W08-01. Only the HPLC gradient had to be corrected since there were two typographical errors in the original description.

Table 3: Liquid Chromatographic Conditions

Column: YMC Pro C18, length 33mm x 4mm, pore size 120A
Particle size: 3 µm
Oven temperature: 60 °C
Injection volume: 80 µL
Run time: 4 minutes
Mobile phase: A: deionized water / acetonitrile (9/1 v/v) (+ 0.01% acetic acid)
B: acetonitrile (+ 0.01% acetic acid)
Retention times: approx. 0.85 min for BYF 14182-3-hydroxy-butyl
approx. 0.93 min for BYF 14182-pyrazolyl-AAP
approx. 1.45 min for BYF 14182

Table 4: HPLC Gradient

Time [min]	Flow [µL/min]	A [%]	B [%]
0.00	1000	80	20
0.10	1000	40	60
1.00	1000	40	60
2.20	1000	25	75
2.30	1000	10	90
3.00	1000	10	90
3.01	1000	80	20
4.00	1000	80	20

Table 5: Valco Valve Method Properties

Time [min]	
0.0	switch eluent stream into waste
0.5	switch eluent stream into interface
3.8	switch eluent stream into waste

These switching times were also adjusted to the used equipment.

4.1.5 Mass Spectrometric Parameters

MS/MS parameter settings were in general as described in method EL-001-W08-01 but optimized for the instrument being used. The dwell time was reduced to 40 msec for all analytes in the second period.

4.1.6 Calculation

For calculation of the concentrations, six-point calibration curves were used. These curves were calculated using linear regression automatically after each sequence run with the Applied Biosystems quantitation software Analyst (vers. 1.5). Further calculations were performed using the software MS-EXCEL 2003. The results given are rounded values. Thus, rounding "errors" may occur if recalculations are made using the listed figures.

4.1.6.1 Calculation of Analyte Concentrations

NOTE: Evaluation is performed according to the linearity standard procedure.

1. Calculate the response factors (peak area of detected analyte / peak area of the internal standard) of all standard injections and calculate the resulting linearity of the analyte.
2. Determine the response factor (peak area of detected analyte / peak area of the internal standard) for the sample. This value will be used as x.
3. Calculate the residue level in µg/L as follows:

$$R = (x - b) / a$$

R: Determined concentration of analyte in µg/L
x: Response factor of the sample
b: Interception from linear regression
a: Slope from linear regression

4.1.6.2 Calculation of Recoveries

1. Calculate the concentration in the recovery sample according to 4.1.6.1.
2. Calculate the percent recovery as follows:

$$\text{Recovery} = \frac{\text{Concentration} \times 100}{\text{Fortification Level}}$$

Recovery:	Recovered concentration of analyte in % found in the fortified sample
Concentration:	Concentration in the fortified sample in µg/L determined according to 4.1.6.1
Fortification level:	Fortified concentration of analyte in µg/L

4.1.7 Deviations from the Method

Within the analytical procedure for determination of BYF 14182 and its metabolites BYF 14182-3-hydroxy-butyl and BYF 14182-pyrazolyl-AAP only minor details have been changed.
The HPLC gradient had to be corrected since there were two typographical errors in the original description. The dwell time was reduced to 40 msec for all analytes in the second period.