2 Objective

The objective of this study was to demonstrate that method FN-002-S05-02 ("BYI08330: Analytical method for the determination of BYI08330 and its metabolites BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide in soil and sediment by LC/MS/MS") can be performed with acceptable recoveries for determination of the compounds BYI08330, BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience LP, Stilwell, USA, and validated in that laboratory with results reported as Method FN-002-S05-02, by D.J. Netzband & J.M. Wade, in report dated January 25, 2006. Soil Höfchen (Germany) and soil Laacher Hof (Germany) were chosen as representative matrices for validation within the present study.

This study was performed in accordance with EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 of March 17, 2004, Commission Directive 96/46/EC amending Council Directive 91/414/EEC of July 16, 1996, and BBA Guideline on Residue Analytical Methods for Post-Registration Control Purposes of July 21, 1998 US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996 and US EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue Analytical Method, EPA 712-C-96-174, August 1996.

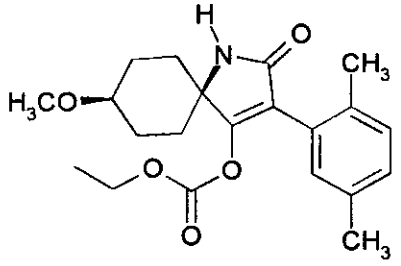
3 Materials

3.1 Test and Reference Items

The structures for BYI08330 and its metabolites BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide are presented below:

BYI08330

Structural formula:



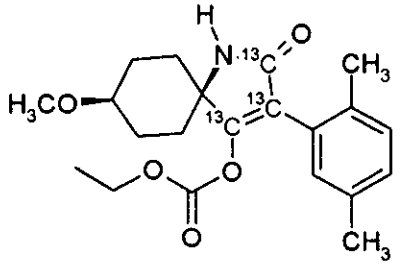
Chemical code:	BYI08330, AE 1302943
Chemical name (CAS):	cis-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate
CAS No.:	203313-25-1
Empirical formula:	$C_{21}H_{27}NO_5$
Molecular weight:	373.45 g/mol

Reference standard:

Certificate of analysis:	AZ 12545
Purity:	99.2 %
Expiry date:	May 2008
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

$^{13}C_3$-BYI08330
(used as internal standard for the active ingredient BYI08330):

Structural formula:



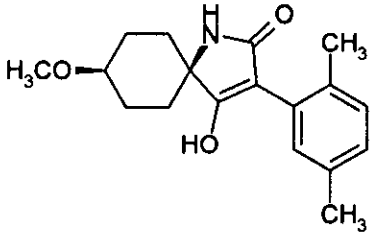
Code name:	[azaspirodecenyl-*2,3,4-*$^{13}C_3$] cis BYI08330
Chemical name (CAS):	3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl-*2,3,4-*$^{13}C_3$ ethyl carbonate
Empirical formula:	$C_{18}\ ^{13}C_3\ H_{27}\ N\ O_5$
Molecular weight:	376.41 g/mol

Reference standard:

Batch no.:	KML3386-1-2
Purity:	95.1%
Origin:	Bayer CropScience AG, R-PT Isotope Chemistry D-42096 Wuppertal, Germany

BYI08330-enol

Structural formula:



Chemical code:	AE 1302944
Chemical name (CAS):	cis-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one
CAS No.:	203312-38-3

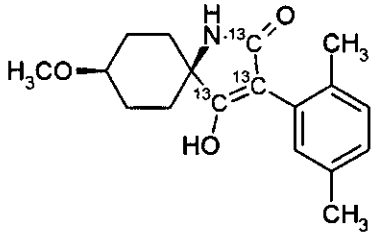
Empirical formula: $C_{18}H_{23}NO_3$
Molecular weight: 301.39 g/mol

Reference standard:

Certificate of analysis: AZ 12333
Purity: 99.4 %
Expiry date: February 2008
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

$^{13}C_3$-BYI08330-enol
(used as internal standard for the test item BYI08330-enol):

Structural formula:



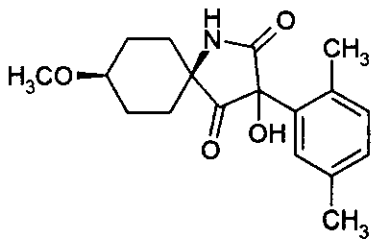
Code name: [azaspirodecenyl-2,3,4-$^{13}C_3$] FHN13777
Chemical name (CAS): cis-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one-2,3,4-13C3
Empirical formula: $^{13}C_3\,C_{15}H_{23}NO_3$
Molecular weight: 304.35 g/mol

Reference standard:

Batch no.: KML 3384-1-1
Purity: 96.3%
Origin: Bayer CropScience AG, R-PT Isotope Chemistry D-42096 Wuppertal, Germany

BYI08330-keto-hydroxy

Structural formula:



Chemical code: AE 1422479

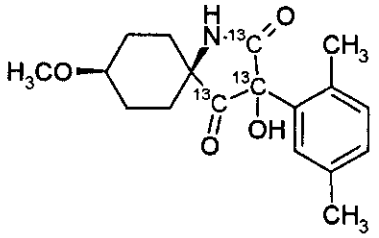
Chemical name (CAS): cis-3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione
Empirical formula: $C_{18}H_{23}NO_4$
Molecular weight: 317.38 g/mol

Reference standard:

Certificate of analysis: AZ 11970
Purity: 92 %
Expiry date: September 2006
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

$^{13}C_3$-BYI08330-keto-hydroxy
(used as internal standard for the test item BYI08330-keto-hydroxy):

Structural formula:



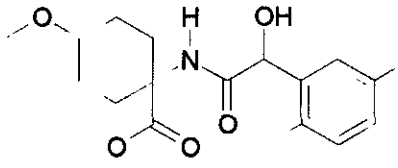
Code name: [azaspirodecane-2,3,4-$^{13}C_3$] FHN14066
Chemical name (CAS): 3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione-2,3,4-$^{13}C_3$
Empirical formula: $^{13}C_3\,C_{15}H_{23}NO_4$
Molecular weight: 320.35 g/mol

Reference standard:

Batch no.: KML 3387-1-8
Purity: 99.4%
Origin: Bayer CropScience AG, R-PT Isotope Chemistry D-42096 Wuppertal, Germany

BYI08330-MA-amide

Structural formula:



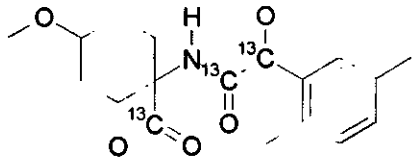
Chemical code: AE 1786350

Chemical name (CAS): cis-1-[[(2,5-Dimethylphenyl)hydroxyacetyl]amino]-4-methoxycyclohexanecarboxylic acid
Empirical formula: $C_{18}H_{25}NO_5$
Molecular weight: 335.40 g/mol

Reference standard:
Certificate of analysis: AZ 11525
Purity: 96.1 %
Expiry date: April 2006
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

$^{13}C_3$ -BYI08330-MA-amide
(used as internal standard for the test item BYI08330-MA-amide):

Structural formula:



Code name: [acetyl-$^{13}C_2$, carboxylic acid-^{13}C] FHN14065
Chemical name (CAS): 1-[[(2,5-Dimethylphenyl)hydroxyacetyl-$^{13}C_2$]amino]-4-methoxycyclohexanecarboxylic-^{13}C acid
Empirical formula: $^{13}C_3\,C_{15}H_{25}NO_5$
Molecular weight: 338.37 g/mol

Reference standard:
Batch no.: KML 3389-1-4
Purity: 88.1%
Origin: Bayer CropScience AG, R-PT Isotope Chemistry D-42096 Wuppertal, Germany

3.2 Test System

The method was validated using 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 1.
Complete soil parameterisation is reported in Table 9 and Table 10.

Table 1: Soil Types

Soil	Texture of Soil	Organic Matter [%]
Höfchen	silt loam (USDA)	1.58
Laacher Hof	sandy loam (USDA)	2.06

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:	FN-002-S05-02
Title of the method:	BYI08330: Analytical Method for the Determination of BYI08330 and its Metabolites BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide in Soil and Sediment by LC/MS/MS
Author of the method:	Derek J. Netzband Jami M. Wade Bayer CropScience LP 17745 S Metcalf Avenue Stilwell, Kansas 66085
Reference:	Method FN-002-S05-02
Limit of quantitation:	0.005 mg/kg

The following sample sets were analysed:

Table 2: Level and Number of Recoveries per Fortification Level

Soil	Control sample	Level 0.005 mg/kg	Level 0.05 mg/kg
Höfchen	2	5	5
Laacher Hof	2	5	5

Additionally, two solvent blanks with internal standards added were analysed.

4.1.1 Outline of the Method

Residues of BYI08330 and its metabolites BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide are extracted from soil using an acidic extraction solution in the presence of cysteine hydrochloride and utilizing microwave extraction. The extraction solvent consists of a mixture of water (containing 8 g/L cysteine hydrochloride), acetonitrile, ethyl acetate and formic acid. An aliquot of the final extract is analyzed by LC/MS/MS. Quantification of residues is based on the use of isotopically labeled internal standards and comparison of peak areas with those of known standards.
The final quantitative detection of BYI08330 and its metabolites BYI08330-enol, BYI08330-keto-hydroxy and BYI08330-MA-amide is accomplished by LC/MS/MS.

4.1.2 Instruments

Microwave Extractor:	MLS-Ethos MWS Vertriebs GmbH 88299 Leutkirch, Germany
Balances:	PC 4400, PM 4800 and AT 261 Mettler Instruments GmbH 35387 Giessen, Germany
Ultrasonic Bath:	Transsonic 890/H Heinrich Faust 51145 Cologne, Germany
Liquid Chromatograph:	HP 1100 Column Compartment G1316A HP 1100 Binary Pump G1312A HP 1100 Isocratic Pump G1310A HP 1100 Degasser G1322A Agilent 40880 Ratingen, Germany
Autosampler:	HTC PAL System CTC Analytics AG 4222 Zwingen, Switzerland
Mass Spectrometer:	API 4000 with turbo-ionspray interface mass selective detector (MS/MS) Perkin Elmer Sciex Instruments 64331 Weiterstadt, Germany
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):	Luna 3u C8(2) 100A, size 50 mm x 2 mm Part No. 00B4248-B0 Phenomenex 63741 Aschaffenburg, Germany
Magnetic stirring bar:	plain (large, e.g. 40 x 8 mm [length x i.d.])
Syringe filter:	Acrodisc® CR 13 mm syringe filter with 0.45 µm PTFE membrane, parts no. 514-4008 VWR International GmbH 64301 Darmstadt, Germany

Acetonitrile:	for HPLC, super gradient grade Riedel de Haen, No. 34998 30926 Seelze, Germany
Ethyl acetate:	for residue analysis Promochem 46485 Wesel, Germany
Formic acid:	p.a. (100%) Merck, No.1.00264.0100 64271 Darmstadt, Germany
L-Cysteine hydroclorid:	for biochemistry Merck, No.1.02839.1000 64271 Darmstadt, Germany
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 Chromatographic Conditions and Mass Spectrometric Parameters

Liquid chromatographic conditions were identical to those described in Appendix 1 of the original method report FN-002-S05-02.
MS/MS parameter settings were optimized for the instrument being used and therefore not identical with those reported in method FN-002-S05-02.

4.1.5 Calculation

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 (vers. 1.4) using linear regression. Further calculations were performed using the software EXCEL 2002 (Office 2002®).
Matrix effects for BYI08330 and its metabolites are eliminated by using an internal standard solution of the isotopically labelled reference items. Generally, the concentration of the internal standards should be in the range of the concentration of the analytes in the sample solutions.

The linear equation is expressed as:

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

y = Area, x = Concentration

If an internal standard is used:

$$y = \frac{Area_{Standard}}{Area_{Internal\ Standard}} = Int.\ Ratio \quad and \quad x = \frac{Conc_{Standard}}{Conc_{IS}} = Conc_{ratio}$$

Int. Ratio: *intensity ratio*
$Conc_{Standard}$: *concentration of standard solution [µg/L]*
$Conc_{IS}$: *concentration of internal standard solution [µg/L]*
$Conc_{ratio}$: *concentration ratio*

Since the concentrations of the isotopically labelled internal standards were the same in all sample and standard solutions that were injected into the HPLC instrument, their concentrations can be neglected for calculations. However, the unit (µg/L or ng/mL) of the internal standard concentration has always to be considered in the calculation while the value is set to 1 (see example calculation). In cases were the concentration is taken into account in the formula for $Conc_{ratio}$, it has to appear in the formula for $Conc_{Soil}$, too. By means of the linear equation, the compounds concentration in soil can be calculated as follows:

$$Dilution_{Factor} = \frac{Volume_{Extraction}}{Weight}$$

$$Conc_{Analyte} = \frac{Int.Ratio - Intercept}{Slope}, \quad Int.\ Ratio = \frac{Area_{Analyte}}{Area_{Internal\ Standard}}$$

$$Conc_{Soil} = Conc_{Analyte} \times Dilution_{Factor} \times Conc_{IS}$$

$Volume_{Extraction}$: *volume of the extraction solvent [L]*
Weight: *weight of the soil sample [kg]*
Intercept: *intercept of the linear regression curve*
Slope: *slope of the linear regression curve*
$Area_{Analyte}$: *area of the analyte in the sample solution*
$Conc_{Soil}$: *concentration of the analyte in soil (sediment) [µg/kg]*

The recovery is calculated according to the following equation:

$$Recovery = \frac{Conc_{Soil\ (Sediment)} \times 100\%}{Conc_{Soil\ (Sediment)\ Spiked}}$$

$Conc_{Soil\ (Sediment)\ Spiked}$: *concentration of the reference item spiked [µg/kg]*