

## 1. BACKGROUND INFORMATION

The objective of this study was to validate a method of analysis for the determination of BYI08330 and its metabolites BYI08330–enol, BYI08330–ketohydroxy and BYI08330-MA-amide in soil and sediment by LC/MS/MS.

The original protocol covered the analysis of BYI08330 and its metabolites BYI08330–enol, BYI08330–ketohydroxy and BYI08330-MA-amide in soil. The validation of sediment was added to the study by protocol amendment on July 29, 2005.

On completion of this study the analytical method FN-002-S05-02: "BYI08330: Analytical Method For The Determination of BYI08330 And Its Metabolites BYI08330–enol, BYI08330–ketohydroxy And BYI08330-MA-amide In Soil And Sediment by LC/MS/MS" was issued.

An ILV was performed on analytical method FN-002-S05-02 (See Section 8.7). The analytical method was updated to include the data generated during the ILV, and the updated method was assigned an analytical method number of FN-002-S05-03.

The study was performed in accordance with United States Environmental Protection Agency (EPA) Pesticide Assessment Guidelines and Good Laboratory Practices (and Ecological Effects Test Guidelines OPPTS 850.7100<sup>1</sup> and Residue Chemistry Test Guidelines, OPPTS 860.1340<sup>2</sup>). This validation fulfils the requirement that properly validated methods of analysis be utilized for the generation of pesticide residue data and for tolerance enforcement.

Nomenclature for BYI08330 and its metabolites are presented in Section 3.

## 2. EXPERIMENTAL DESIGN

This study was conducted following an approved protocol. All amendments to the protocol were signed and dated by the Study Director and the Sponsor's Representative. Any deviations from the protocol were documented and brought to the Study Director's attention when they were noted and maintained with the raw data.

This study was initiated on July 22, 2005. The experimental phase of the study began on July 27, 2005 and concluded on December 1, 2005. The following personnel were involved in the conduct of this study:

Derek J. Netzband  
Senior Scientist  
Environmental Chemistry

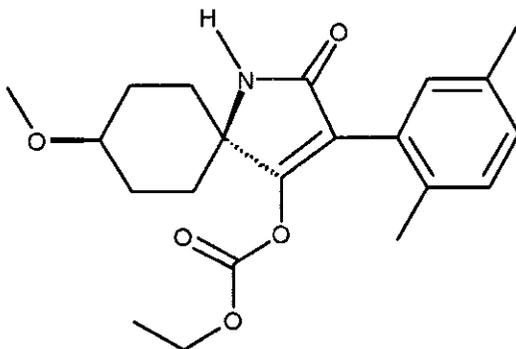
Jami M. Wade  
R&D Specialist  
Environmental Chemistry

Robert Bognar  
Technician  
Environmental Chemistry

### 3. TEST AND REFERENCE SUBSTANCES

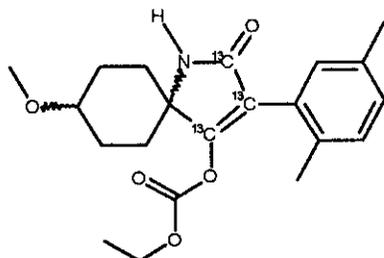
The following compounds were used as test and reference substances, and were supplied by Bayer CropScience. Neat standards and stock standard solutions were stored in a freezer at an average temperature of  $-28^{\circ}\text{C}$ . Standard solutions were stored in a refrigerator at an average temperature of  $8^{\circ}\text{C}$ .

Code Name: BYI08330 (Spirotetramat or AE 1302943)  
(Active Ingredient, Parent Molecule)



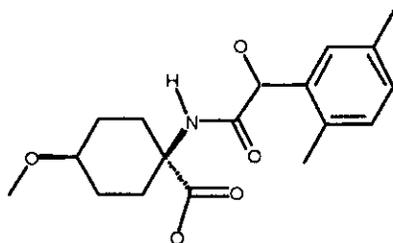
CAS name: *cis*-3-(2,5-dimethyl-phenyl)-8-methoxy-2-oxo-1-azaspiro-[4.5]dec-3-en-4-yl ethyl carbonate  
 IUPAC Name: (5*s*,8*s*)-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate  
 CAS Number [203313-25-1]  
 Molecular Formula:  $\text{C}_{21}\text{H}_{27}\text{NO}_5$   
 Molecular Weight: 373.44  
 ID No.: K-1338  
 Reference No.: 1105200304  
 Purity: 99.2%  
 Expiration Date: 05/02/08  
 Storage Conditions: Frozen  
 Source: Bayer CropScience, Stilwell, Kansas

Code Name:  $^{13}\text{C}_3$ -BYI08330 (BYI08330-azaspirodecenyl-2,3,4- $^{13}\text{C}_3$ )  
(Parent Molecule, Isotopic Internal Standard)



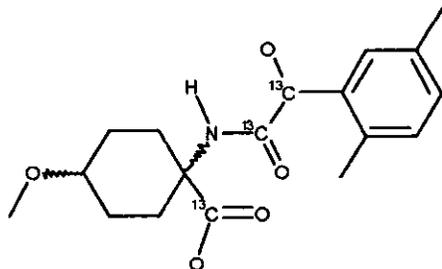
CAS Name: *cis*-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl-2,3,4- $^{13}\text{C}_3$  ethyl carbonate  
Molecular Formula:  $\text{C}_{21}\text{H}_{27}\text{NO}_5$   
Molecular Weight: 376.46  
ID No.: K-1381  
E.R. Reference No.: KML 3386-1-2  
Reference No.: 0622200501  
Purity: 99.6%  
Expiration Date: 06/21/15  
Storage Conditions: Frozen  
Source: Bayer CropScience, Wuppertal, Germany

Code Name: BYI08330-MA-amide (FHN14065 or AE 1786350)  
(Soil Metabolite)



CAS Name: 2 *cis*-1-[[[(2,5-Dimethylphenyl)hydroxyacetyl]amino]-4-methoxycyclohexanecarboxylic acid  
Molecular Formula:  $\text{C}_{18}\text{H}_{25}\text{NO}_5$   
Molecular Weight: 335.39  
ID No.: K-1387  
Reference No.: 0706200414  
Purity: 96.1%  
Expiration Date: 03/31/09  
Storage Conditions: Frozen  
Source: Bayer CropScience, Frankfurt, Germany

Code Name: FHN 14065-acetyl-13C (<sup>13</sup>C<sub>3</sub>BAY BY108330 Mandelic Acid Amide)  
(Soil Metabolite, Isotopic Internal Standard)



CAS Name: 1-[[[(2,5-Dimethylphenyl)hydroxyacetyl-<sup>13</sup>C<sub>2</sub>]amino]-4-methoxycyclohexanecarboxylic-<sup>13</sup>C acid

Molecular Formula: C<sub>18</sub> H<sub>25</sub> N O<sub>5</sub>

Molecular Weight: 338.36

ID No.: K-1384

E.R. Reference No.: KML-1-4

Reference No.: 0728200501

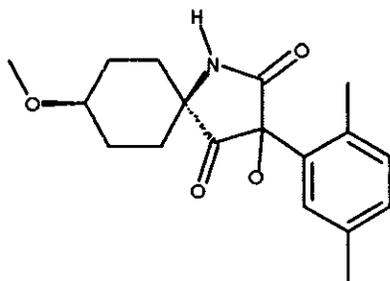
Purity: 98.8%

Expiration Date: 07/25/15

Storage Conditions: Frozen

Source: Bayer CropScience, Wuppertal, Germany

Code Name: BY108330-ketohydroxy (FHN14066 or AE 1422479)  
(Soil Metabolite)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione

Molecular Formula: C<sub>18</sub> H<sub>23</sub> N O<sub>4</sub>

Molecular Weight: 317.3795

ID No.: K-1339

Reference No.: 1105200305

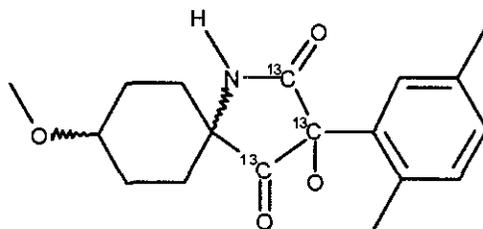
Purity: 95.3

Expiration Date: 10/19/09

Storage Conditions: Frozen

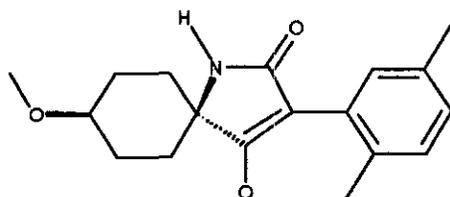
Source: Bayer CropScience, Frankfurt, Germany

Code Name:  $^{13}\text{C}_3$ -BYI 08330-keto-hydroxy, FHN 14066-azaspirodecane-2,3,4- $^{13}\text{C}_3$   
(Soil Metabolite, Isotopic Internal Standard)



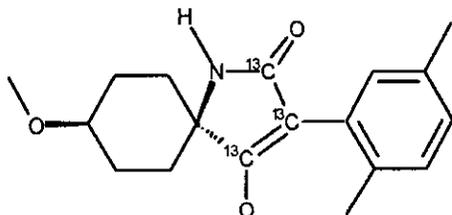
CAS Name: 3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione-2,3,4- $^{13}\text{C}_3$   
Molecular Formula:  $\text{C}_{18}\text{H}_{23}\text{NO}_4$   
Molecular Weight: 320.34  
ID No.: K-1383  
E.R. Reference No.: KML 3387-1-8  
Reference No.: 0622200503  
Purity: 100  
Expiration Date: 6/21/15  
Date of Analysis: 6/21/05  
Storage Conditions: Frozen  
Source: Bayer CropScience, Wuppertal, Germany

Code Name: BYI08330-enol (FHN13777 or AE 1302944)  
(Soil Metabolite)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one  
Molecular Formula:  $\text{C}_{18}\text{H}_{23}\text{NO}_3$   
Molecular Weight: 301.3801  
ID No.: K-1337  
Reference No.: 1105200303  
Purity: 99.4  
Expiration Date: 2/8/08  
Date of Analysis: 2/8/05  
Storage Conditions: Frozen  
Source: Bayer CropScience, Frankfurt, Germany

Code Name:  $^{13}\text{C}_3$ BAY BYI08330 cis-enol  
(Soil Metabolite, Isotopic Internal Standard)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one-2,3,4- $^{13}\text{C}_3$   
Molecular Formula:  $\text{C}_{18}\text{H}_{23}\text{NO}_3$   
Molecular Weight: 304.35  
ID No.: K-1382  
E.R. Reference No.: KML 3384-1-1  
Reference No.: 0622200502  
Purity: 98.3  
Expiration Date: 6/21/15  
Date of Analysis: 6/21/05  
Storage Conditions: Frozen  
Source: Bayer CropScience, Wuppertal, Germany

#### 4. TEST SYSTEM – SOIL AND SEDIMENT SAMPLES

The method was validated using one sediment and two soil samples. The soil samples used in this study were collected for Bayer CropScience Study Number MEFNY004<sup>3</sup>: Terrestrial Field Dissipation of BYI08330 in New York Soil, 2004, and Study Number MEFNY003<sup>4</sup>: Field Dissipation of BYI08330 in Washington Soil, 2004.

Sample ID	Original Study	Source Location
MEFNY004-SL	MEFNY004	New York
MEFNY003-SL	MEFNY003	Washington

The sediment samples used in this study were collected for Bayer CropScience Study Number EBFTY003<sup>5</sup> (Analysis of water and sediment samples taken from an investigation of the toxicity of fipronil to sediment dwelling organisms in the field).

Sample ID	Original Study	Sediment source	Source Location
05-FN-SRL-SD	EBFTY003	Sandhill Research Station Lake	Columbia, SC

## 5. STORAGE

The untreated soil samples were stored at room temperature and the sediment was stored in a refrigerator at approximately 8.4°C.

## 6. REAGENTS AND EQUIPMENT

### 6.1 Reagents and General Equipment

The reagents and equipment used in this study are listed in Sections 4 and 5 of the method of analysis presented in Appendix 3.

### 6.2 Liquid Chromatographic/Mass Spectrometer Detection System

Residues of BYI08330 and its metabolites BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide in soil and sediment were determined using an Applied Biosystems Sciex API-4000 LC/MS/MS system with Sciex TurbolonSpray Electrospray Interface; Shimadzu LC-10AD VP HPLC pumps (2) with a high pressure mixer and SCL-10A VP Pump Controller; and a Gilson 215 Series autosampler. The Applied Biosystems instrument software applications used was Analyst 1.4.

The LC conditions used for the soil and sediment validation and MS/MS operating parameters used are outlined in Appendix 1 of the analytical method which may be found in Appendix 3 of this report. Product ion spectra for each of the analytes are presented in Appendix 1 of this report.

Example Chromatograms using these LC conditions are presented in Appendix 1 of this report.

## 7. CALCULATIONS

### 7.1 Calibration Curves

At least six different standard concentrations were run with each set of samples.

Standard concentrations of BYI08330 and its metabolites ranged from 0ng/mL to 10.0ng/mL (ppb), each with 1.0ng/mL isotopic internal standard added. The calibration standards were interspersed with the samples. All calculations were performed using Applied Biosystems Analyst software (Version 1.4) or Microsoft® Excel worksheets. Linear regression coefficients were calculated for the ratio of analyte to internal standard area plotted versus the area of analyte in the calibration standards.

### 7.2 Quantification of Residues

The calculation technique, and an example calculation is presented in Appendix 2 of this report.

Appendix 2 Example Calculation For Determination Of BYI08330 and BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide Residues

BYI08330 and BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide residues were quantified using internal standard linear regression analysis. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL using Analyst Software, a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set.

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 BYI08330 residues. BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of BYI08330 in the soil was calculated using the following equation,

$$\text{BYI08330 found (ppb)} = \frac{(Y-B) \times D}{M}$$

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

An example calculation for BYI08330 from sample MEFNY003-SL-LOQ-001, which was analyzed during the method validation study, is shown below. This sample was fortified with 5ppb of BYI08330, BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide. The chromatogram used in this example is presented in Appendix 1 (Chromatogram 4), and a complete summary of results is presented at the end of this Appendix.

W	V <sub>1</sub>	Native Peak Area	IS Peak Area	Y	M	B
20	100mL	1625746.5	1520427.9	1.06927	1.01	0.0435

From the above equations:

$$\text{Dilution Factor (D)} = \frac{100}{5} = 20$$

$$\text{BYI08330 found} = \frac{(1.06927 - 0.0435) \times 5}{1.01} = 5.078 \text{ ppb}$$

Therefore sample MEFNY003-SL-LOQ-001 contains 5.078ppb BYI08330.

The % recovery was calculated using the following equation:

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

Where: R = ppb of target analyte found in fortified sample  
S = ppb of target analyte found in control sample, real or apparent  
T = theoretical ppb in fortified sample

Therefore, for sample MEFNY003-SL-LOQ-001, which was fortified with 5.0ppb BYI08330:

$$\begin{aligned} R &= 5.078 \text{ ppb} \\ S &= 0.0 \text{ ppb} \\ T &= 5.0 \text{ ppb} \end{aligned}$$

$$\% \text{ BYI08330 Recovery} = \frac{(5.078 - 0.0)}{5.0} \times 100 = 101.6\%$$

Note: The above calculations were performed using rounded numbers and may vary slightly from the results presented in the raw data.

## 1. SUMMARY

This method is suitable for the determination of the total extractable residues of BYI 08330 and its associated metabolites BYI08330-enol, BYI08330-ketohydroxy and BYI 08330-MA-amide in soil and sediment.

BYI 08330 and its associated metabolites were extracted from soil and sediment using microwave extraction.

An isotopic internal standard containing BYI 08330-<sup>13</sup>C<sub>3</sub>, BYI 08330 -enol-<sup>13</sup>C<sub>3</sub>, BYI 08330ketohydroxy-<sup>13</sup>C<sub>3</sub> and BYI08330-MA-amide-<sup>13</sup>C<sub>3</sub> was added to the sample and an aliquot of the extract analyzed for BYI 08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by LC/MS/MS.

The data generated during the method validation study<sup>1</sup> concluded that the method detection limit (MDL) was demonstrated to fall at or below the target LOQ of 5.0ng/g (ppb) for BYI 08330 and BYI 08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide, with MDL's of 0.5, 0.5, 0.9 and 2.1ppb respectively in soil and 0.5ppb, 0.6ppb, 1.0ppb and 2.7ppb in sediment.

The mean recovery and relative standard deviation (RSD) found for BYI 08330 and its metabolites based on multiple fortifications at 5 ng/g (LOQ) and 25 ng/g (5x LOQ) were all within the range of 70 to 120% and the precision values as measured by the relative standard deviation (RSD) were all less than 20%.

## 2. BACKGROUND

The insecticide BYI08330 is currently being developed by Bayer CropScience.

An analytical method was developed for the analysis of BYI08330 and its associated metabolites BYI08330-enol (AE 1302944), BYI08330-ketohydroxy (AE 1422479) and BYI08330-MA-amide (AE 1786350) in soil and sediment, and the method validated in Bayer CropScience Study Number RAFNX012<sup>1</sup>.

This method also provides LC/MS/MS conditions for detecting potential residues of two additional metabolites, ROI-6 and ROI-7. Reference standards of these two metabolites are not available.

The structures for these compounds are presented in Appendix 2. This analytical method was prepared based on the results obtained in the validation study.

Typical recovery results are presented in Appendix 3, and the data shown was obtained from the method validation study.

## 3. PRINCIPLE

Residues of BYI08330 and its metabolites BYI 08330-enol, BYI 08330-ketohydroxy 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 BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide is accomplished by LC/MS/MS.

A flow-chart outlining the procedure summarizes the method in Appendix 7.

## 4. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- Milestone Ethos E Microwave Labstation, equipped with a Model 320 Touch Screen Controller, and automatic temperature control with fiber optic sensor
- Beckman Coulter Allegra 6 Centrifuge
- Disposable pipettes
- Micropipetter, VWR brand (Calibra) and pipette tips
- Nalgene, high density polyethylene (HDPE), 125 mL clear wide mouth bottles, P/N 2189-0008 or equivalent
- Glass, 125 mL jars (Fisher, Cat. No. 02-911-731)
- Disposable magnetic stirring bars (Fisher Cat. No. 1451394)
- Graduated plastic bottles (Fisher Scientific Cat. No. 03-311-3D)

- Graduated cylinders
- Pipette bulb
- Volumetric flasks
- Volumetric pipettes
- 2 mL autosampler vials
- Rainin Microman<sup>®</sup> Classic positive-displacement pipettes (Cat. No.: M-25, M-50, and M-250)
- VWR Pyrex<sup>®</sup> Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex<sup>®</sup> Brand glass funnels
- VWR Pyrex<sup>®</sup> Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
- National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
- National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
- Javelin Direct-Connect Column Filter, 2.1 mm, (Part No.: 88200)
- Phenomenex Luna 3 $\mu$ m C8 (2) 50 x 2.0mm Column (Part No.: 00B-4248-B0)
- Applied Biosystems PE Sciex 4000 LC/MS/MS System with a Dell Optiplex PC and Analyst Software Version 1.4 or higher installed.
- Applied Biosystems Turbo Ionspray Interface.
- Shimadzu LC-10AD VP HPLC pump with Gilson 215 Liquid handler and Gilson 819 Valve Actuator.
- VICI Cheminert Valve and 2 position actuator Controller.
- Analytical Balance with accuracy of  $\pm 0.01$  grams for sample weights and  $\pm 0.0001$  grams for analytical standards.

## 5. REAGENTS

Use as a guide; equivalents or different manufactures (brands) may be substituted.

- Acetic acid GR (EM Science Cat. No. AX0073)
- Acetonitrile Omni-Solv (EM Science, Cat. No. AX0142)
- Ethyl acetate Fisher, HPLC Grade Cat. No. E196-4
- Water Omni-Solv, HPLC Grade (EM Science, Cat. No. WX0004)
- L-cysteine hydrochloride monohydrate (Fisher Cat. No. BP376-100)
- Solution of 8g/L cysteine hydrochloride in 5% ethyl acetate in water: Weigh ~ 32g of cysteine hydrochloride into a clean 4 L brown glass solvent jug. Dissolve the cysteine hydrochloride in ~ 3800 mL HPLC water. Add ~ 200 mL of ethyl acetate to this solution and mix thoroughly. (Extraction Solution A).
- Certified analytical reference standards of BYI08330, BYI 08330-enol, BYI08330-ketohydroxy and BYI 08330-MA-amide.
- Certified isotopic internal standards of BYI08330-<sup>13</sup>C<sub>3</sub>, BYI 08330-enol -<sup>13</sup>C<sub>3</sub>, BYI08330-ketohydroxy -<sup>13</sup>C<sub>3</sub> and BYI 08330-MA-amide -<sup>13</sup>C<sub>3</sub>

## 6. PREPARATION OF ANALYTICAL STANDARDS

**NOTE:** *The following procedure is an example description of how standard solutions may be prepared. Standards may be prepared as mixed solutions by dilution from individual stock solutions or prepared individually. Alternate or additional standards of appropriate weight and volume may be prepared as needed.*

*Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in amber glass bottles at or below 10°C when not in use. Solutions should be allowed to warm to room temperature prior to use.*

### 6.1 Primary Stock Standard Solutions

Prepare individual 100 µg/mL stock solutions of BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by placing 0.0100 grams of each analyte in separate 100 mL volumetric flasks. Dilute to volume with acetonitrile.

**NOTE:** Corrections for standard purities should be applied when expressing standard concentrations. For Example: If an analytical standard material has a purity of 98.0%, then 0.0102 grams (0.0100 g / 0.980) would be required to prepare a 100 µg/mL stock solution.

The stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤18°C.

### 6.2 Fortification Standard Solutions

Prepare a stock 10 µg/mL solution containing a mixture BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by taking a 10mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.

Prepare a 1.0 µg/mL solution containing a mixture of BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by taking a 10mL aliquot of the stock 10µg/mL solution and diluting to 100 mL with acetonitrile.

Prepare a 0.1µg/mL fortification solution containing a mixture of BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by taking a 10mL aliquot of the 1.0µg/mL solution and diluting to 100 mL with acetonitrile.

Prepare a 10ng/mL fortification solution containing a mixture of BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide by taking a 10mL aliquot of the 0.1µg/mL fortification solution and diluting to 100 mL with acetonitrile.

The fortification standard solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C.

### 6.3 Isotopic Internal Standard Solutions

Prepare individual 100 µg/ml stock internal standard solutions of BYI08330-<sup>13</sup>C<sub>3</sub>, BYI 08330-enol -<sup>13</sup>C<sub>3</sub>, BYI08330-ketohydroxy -<sup>13</sup>C<sub>3</sub> and BYI 08330-MA-amide-<sup>13</sup>C<sub>3</sub> by placing 0.0050 grams of each analyte in separate 50 mL volumetric flasks. Dilute to volume with acetonitrile.

Prepare a stock 10 µg/mL internal standard solution containing a mixture of BYI08330-<sup>13</sup>C<sub>3</sub>, BYI 08330-enol -<sup>13</sup>C<sub>3</sub>, BYI08330-ketohydroxy -<sup>13</sup>C<sub>3</sub> and BYI 08330-MA-amide -<sup>13</sup>C<sub>3</sub> by taking a 10mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.

Prepare a 1.0 µg/mL internal standard solution containing a mixture of BYI 08330-<sup>13</sup>C<sub>3</sub>, BYI 08330 cis-enol-<sup>13</sup>C<sub>3</sub>, BYI 08330 cis-keto-hydroxy-<sup>13</sup>C<sub>3</sub> and BYI 08330-MA-amide-<sup>13</sup>C<sub>3</sub> by taking a 10mL aliquot of the stock 10µg/mL internal standard solution and diluting to 100 mL with acetonitrile.

Prepare a 0.1µg/mL internal standard solution containing a mixture of BYI 08330-<sup>13</sup>C<sub>3</sub>, BYI 08330 cis-enol-<sup>13</sup>C<sub>3</sub>, BYI 08330 cis-keto-hydroxy-<sup>13</sup>C<sub>3</sub> and BYI 08330-MA-amide-<sup>13</sup>C<sub>3</sub> by taking a 10mL aliquot of the 1.0µg/mL internal standard solution and diluting to 100 mL with acetonitrile.

The internal standard solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C.

Further dilutions of this mixed fortification solution may be made as needed.

### 6.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0ng/mL of BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide diluted to 100mL with 25:75 acetonitrile:deionized water. Before bringing the calibration solutions to volume, add by pipet 1.0mL of the 0.1µg/mL internal standard solution prepared in acetonitrile to each of the calibration solutions. (see Section 6.3 Internal Standard Solutions)

Further calibration solutions may be prepared as needed.

Prior to the analysis of each set of samples a set of calibration solutions are prepared by adding a ~0.5mL aliquot of each of the solutions into a LC vial containing ~0.5mL of 25:75 acetonitrile:deionized water.

## 7. ANALYTICAL PROCEDURE FOR ANALYSIS OF SOIL AND SEDIMENT

A method flow chart is presented in Appendix 7, and a summary of the analytical method parameters is presented in Table 1.

Stopping points in the analytical method are designated by the following symbol: §

### 7.1 Sample Preparation

Samples of soil and sediment should be thoroughly homogenized and stored frozen until sampled for extraction.

### 7.2 Extraction

**Note:** This method uses internal standards to determine the concentrations of BY108330 and its metabolites present in soil.

If the concentration of any of these components are outside the range of the appropriate calibration curve the analysis may be repeated using either a reduced sample weight or by further diluting the sample extract. If a further dilution is made to the sample extract adjust the internal standard concentration should be adjusted so that the concentration of internal standard present in the final sample is 1ng/mL.

- 7.2.1 Weigh approximately 20 ±0.2 grams of soil or sediment into a 125 mL glass jar. §

**Note:** the glass jars used in this procedure may be replaced with 125mL high density polyethylene (HDPE) bottles.

- 7.2.2 Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile (see Section 6.2 Fortification Stock Solutions).
- 7.2.3 Add approximately 25mL of the extraction solution-A (8g/L cysteine hydrochloride and 5% ethyl acetate in water) and mix with the soil by gentle swirling. Wait approximately 5 minutes then add 0.5 mL formic acid to each sample and mix by gentle swirling.
- 7.2.4 Add approximately 25 mL of acetonitrile to each sample and a stirring bar, cap and hand shake for about 5 seconds. Add a disposable magnetic stirring bar to each sample.
- 7.2.5 Add by pipet 1.0mL of the 0.1µg/mL <sup>13</sup>C internal standard solution prepared in acetonitrile. (see Section 6.3 Isotopic Internal Standard Solutions)
- 7.2.6 Loosen the caps of each bottle and place the bottles on a microwave carousel/rotor. (A suitable carousel may be obtained from Milestone or modified from an existing carousel. The modified carousel is essentially a tray with a center pivot.) The bottles should be arranged in one or two rows around the center of the carousel.
- 7.2.7 Place the temperature probe in its sleeve in a hole punched through the cap of a 125 glass jar or HDPE bottle. This jar should contain 20 grams of untreated soil and the same volume of extraction solvents (~50mL). The probe is used to ensure control of the temperature by modifying the power input.

- 7.2.8 Microwave the samples at approximately 70°C and a maximum of 800 watts as described below.

Step Number (Nr)	Time Duration (t)	Temperature set point at end of step (T1*)	Max. Power (E)	Comments
1	10 min.	70°C	800W	ramp from ambient to 70°C
2	20 min	70°C	800W	maintain 70°C

\* Parameter T1 refers to the fiber optic probe temperature control parameter. An optional infrared probe controls a parameter T2, which is not used in this method.

Ventilation time: 1 minute  
 QP limit: 60-80% (Shut off limit in case solvent vapors in oven becoming too concentrated.)  
 Stirrer (speed setting): 72  
 Rotor control: On (Rotor rotation is on)  
 Twist control: On (Rotor rotates clockwise and then counterclockwise to keep probe cable from twisting)

- 7.2.9 Allow the bottles to cool down to room temperature then centrifuge at approximately 2000 rpm for about 10 minutes. If glass jars are used, the centrifuge speed should not exceed 2000 rpm because at higher rpm the glass jars could break.
- 7.2.10 After centrifugation, decant each sample into a graduated plastic bottle or glass jar labeled with the sample ID number.
- 7.2.11 Add 25 mL of the extraction solvent A (water containing 8g/L cysteine hydrochloride and 5% ethyl acetate) followed by 25 mL acetonitrile. Repeat the microwave extraction and centrifugation steps. (No formic acid is added at this step.)
- 7.2.12 Decant the liquid into the same bottle used in step 7.2.10. Stopper and mix the contents of the bottle/jar thoroughly. §
- 7.2.13 Filter a ~0.5mL aliquot of the solution through an Acrodisc® 0.45µm syringe filter into a LC vial containing 0.5mL of water. Cap the vial and mix to await analysis by LC/MS/MS. §

## 8. ANALYSIS

### 8.1 Sample Analysis

BYI08330, BYI 08330-enol, BYI 08330-ketohydroxy and BYI 08330-MA-amide are analyzed by LC/MS/MS using isotopic internal standards.

Inject a 80 µl aliquot of each test sample (or fortified sample matrix) from step 7.2.13 into the LC/MS/MS under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

#### 8.1.1 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting a 80 µL aliquot of each LC/MS/MS calibration solution interspersed with samples.

BYI08330 and BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide residues are quantified using internal standard linear regression analysis. Produce a separate calibration curve for each set of samples analyzed on the LC/MS/MS. A calibration curve is generated by linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL using Analyst Software, a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients a and b, respectively called slope and intercept, for each analytical set.

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 BYI08330 residues. BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of BYI08330 in the soil was calculated using the following equation,

$$\text{BYI08330 found (ppb)} = \frac{(Y-B) \times D}{M}$$

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

$$W = 20\text{g}$$

$$V_1 = 100\text{mL}$$

## 8.2 Fortification Experiments

**Note:** *Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing & validating method efficiency.*

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

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

Where:  $R$  = ppb of target analyte found in fortified sample  
 $S$  = ppb of target analyte found in control sample, real or apparent  
 $T$  = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 5ppb in soil and sediment or other appropriate level with fortification solutions. Calculate the final residue  $R$  for the control ( $S$ ) and fortified control ( $R$ ) samples.

**Note:** See Appendix 5 for typical untreated control and fortified control chromatograms

## 9. DISCUSSION

### 9.1 Method Validation

The method validation has been performed and reported in Bayer CropScience Study RAFNX012<sup>1</sup>. The results from this study are summarized in Appendix 3.

### 9.2 Independent Laboratory Validation (ILV)

An ILV has been successfully performed on this method<sup>5</sup>. The results are summarized in Table 2.

### 9.3 Time Considerations

A set of fourteen samples can be weighed and prepared for analysis in 4 to 5 hours. The samples are analyzed overnight and the data processed the following working day.

### 9.4 Analytical stopping points (IF NEEDED)

As noted in the method, the procedure may be paused if needed. These should flexibly accommodate the analyst's normal working day or schedule. It is assumed that the analysis will resume during the next working period.

## Appendix 1 Instrument Conditions

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

**NOTE:** *As the LC/MS/MS system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually affected over time. Therefore, the given LC/MS/MS parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.*

Acquisition Parameters

Instrument Used:	Perkin Elmer Sciex API 4000 LC/MS/MS System with Valco Divert Valve	
Interface:	PE Sciex Turbo Ion Spray Electrospray	
Synchronization Mode:	LC Sync	
AutoEquilibration:	Off	
Acquisition Duration:	19 min. 0 sec.	
Number of Scans:	445	
Periods in File:	1	
Acquisition Module:	Acquisition Method	
Software Version:	Analyst 1.4	
Period 1:	Period Delay:	0.00 sec.
	Scans In Period:	445
	Relative Start Time:	0.00 msec.
	Experiments in Period:	1
Period 1 Experiment 1:	Scan Type:	MRM
Duration 8.5 Minutes	Polarity:	Positive
	Scan Mode:	N/A
	Resolution Q1:	Low
	Resolution Q3:	Low
	Intensity Threshold:	0 counts
	Smart Settling:	Off
	Settling Time:	700.0000 ms
	MR Pause:	2.0000 ms
	MCA:	No
	Step Size:	0.00 amu

Appendix 1 (con't)

<u>~ Retention Time (min)</u>	<u>Analyte</u>	<u>Q1 Mass (amu)</u>	<u>Q3 Mass (amu)</u>	<u>Dwell (msec)</u>	<u>Parameter</u>	<u>Start</u>	<u>Stop</u>
6.1	BYI 08330-MA-amide	336	290	200	DP	71	71
					CE	22	22
					CXP	15	15
6.1	BYI 08330-MA-amide <sup>13</sup> C <sub>3</sub>	339	292	200	DP	71	71
					CE	22	22
					CXP	10	10
6.4	BYI 08330-enol	302	216	200	DP	86	86
					CE	30	30
					CXP	15	15
6.4	BYI 08330-enol - <sup>13</sup> C <sub>3</sub>	305	219	200	DP	86	86
					CE	30	30
					CXP	15	15
6.8	BYI08330-ketohydroxy	318	268	200	DP	26	26
					CE	29	29
					CXP	14	14
6.8	BYI08330-ketohydroxy - <sup>13</sup> C <sub>3</sub>	321	271	200	DP	26	26
					CE	29	29
					CXP	14	14
8.9	BYI08330	374	302	200	DP	31	31
					CE	23	23
					CXP	16	16
8.9	BYI08330- <sup>13</sup> C <sub>3</sub>	377	305	200	DP	31	31
					CE	23	23
					CXP	16	16

Parameter Table  
(Period 1 Experiment 1):

CAD:	4.0
CUR:	10.0
GS1:	40.0
GS2:	20.0
IS:	4200.0 volts
TEM	500° C
Ihe	on
EP	10

Appendix 1 (con't)

Autosampler Used: Gilson 215 Autosampler  
 Syringe Size: 500  $\mu$ L  
 Injection Volume: 80  $\mu$ L  
 Pre-inject Flushes: 5  
 Post inject Flushes: 5  
 Air Cushion: 50  $\mu$ L  
 Excess Volume: 20  $\mu$ L  
 Sample Speed: 5.00 mL/min.  
 Needle Level: 5%  
 Inject Delay Time: 0.00 min.  
 Needle Z-Direction Speed: Very Fast  
 Inject Time Delay: 0.0 min.  
 Loop Volume: 100  $\mu$ L  
 Needle Flush Volume: 400  $\mu$ L  
 Flush Speed: 5.00 mL/min.  
 Port Flush Volume: 200  $\mu$ L

Pump Used: Shimadzu LC-10AVP (High Pressure Mixer)  
 Minimum Pressure: 0.0 psi  
 Maximum Pressure: 5000 psi  
 Shutdown Time: 999.9 min.  
 Guard Column: Javelin-Direct Connect Column Filter (2.1 mm i.d.)

Column Temperature: Ambient  
 Column:
 

Manufacturer:	Phenomenex
Type:	Luna C8 (2)
Phase:	C8
Particle Size:	3 $\mu$ m
Diameter:	2.0 mm
Length:	50 mm

Mobile Phase A: 0.5% acetic acid in HPLC grade water  
 Mobile Phase B: acetonitrile

## Gradient Program:

Step	Total Time (min.)	Flow	A(%)	B(%)
0	0.0	200 $\mu$ L/min.	85.0	15.0
1	0.1	200 $\mu$ L/min.	85.0	15.0
2	2.9	200 $\mu$ L/min.	85.0	15.0
3	3.0	200 $\mu$ L/min.	70.0	30.0
4	5.0	200 $\mu$ L/min.	55.0	45.0
5	7.0	200 $\mu$ L/min.	55.0	45.0
6	8.1	200 $\mu$ L/min.	35.0	65.0
7	11.0	200 $\mu$ L/min.	35.0	65.0
8	11.1	200 $\mu$ L/min.	85.0	15.0
9	13.0	200 $\mu$ L/min.	85.0	15.0

Appendix 1 (con't)**Screening Samples for the presence of ROI-6 and ROI-7:**

This section provides LC/MS/MS conditions for detecting metabolites ROI-6 and ROI-7. These two compounds may be formed in small quantities as a result of direct application of BYI 8330 and BYI 8330-enol to soil. The exact structures of these two compounds are not fully known and therefore, could not be synthesized. The LC/MS/MS conditions described below were established using a purified soil extract of the two compounds. In the full scan of Q1 in the positive ion mode, these two compounds produce a parent ion of molecular 601 amu. In MS/MS mode, these parent ions produced one major daughter fragment of mass 301 amu. The mass of this fragment is the same mass as AE 1302944 (BYI 08330-enol). Therefore, it is believed that ROI-6 and ROI-7 occur in the soil as a result of some type of dimerization of BYI-8330-enol.

The compounds, ROI-6 and ROI-7, are analyzed under the same chromatographic conditions used for the other analytes, described earlier in Appendix I. Since there are no calibration reference standards, the amounts of these analytes, if found, can not be calculated. Use of the response of any of the other analytes would only give a relative estimate of the amount present. The mass spectrometry parameters are as follows:

Analyte	Mass Transitions Q1/Q3	Declustering Potential (DP)	Collision Energy (CE)	Exit Potential (CXP)
ROI-6	601/301	50	28	15
ROI-7	601/301	50	28	15

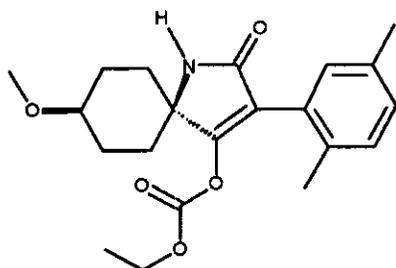
Under these conditions, the retention times for ROI-6 and ROI-7 are 9.9 and 10.9 minutes respectively.

A representative chromatogram is shown in Appendix 5, Figure 11.

Appendix 2 Structures

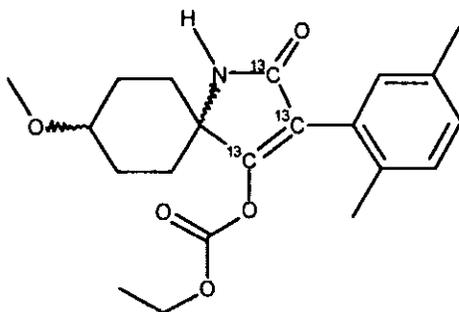
The structures for BYI 08330 and metabolites BYI 08330-enol, AE BYI 08330-ketohydroxy and BYI 08330-MA-amide are presented below:

Code Name: BYI08330 (Spirotetramat or AE 1302943)  
(Active Ingredient, Parent Molecule)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate  
CAS Number [203313-25-1]  
Molecular Formula: C<sub>21</sub> H<sub>27</sub> N O<sub>5</sub>  
Molecular Weight: 373.4

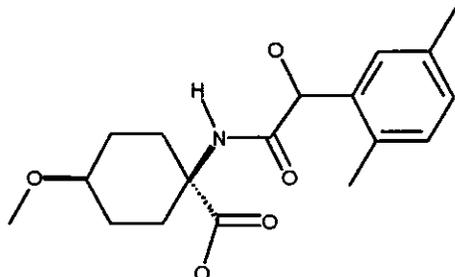
Code Name: <sup>13</sup>C<sub>3</sub>-BYI08330 (BYI 08330-azaspirodecenyl-2,3,4-<sup>13</sup>C<sub>3</sub>)  
(Parent Molecule, Isotopic Internal Standard)



CAS Name: 3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl-2,3,4-<sup>13</sup>C<sub>3</sub> ethyl carbonate  
Molecular Formula: C<sub>21</sub> H<sub>27</sub> N O<sub>5</sub>  
Molecular Weight: 376.5

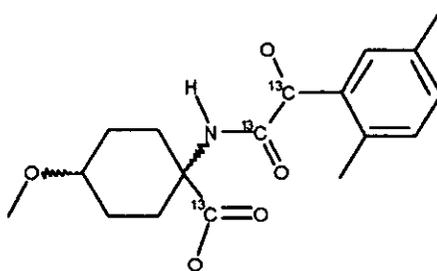
## Appendix 2 (continued)

Code Name: BYI08330-MA-amide (AE 1786350)  
(Soil Metabolite)



CAS Name: *cis*-1-[[[(2,5-Dimethylphenyl)hydroxyacetyl]amino]-4-methoxycyclohexanecarboxylic acid  
Molecular Formula: C<sub>18</sub> H<sub>25</sub> N O<sub>5</sub>  
Molecular Weight: 335.4

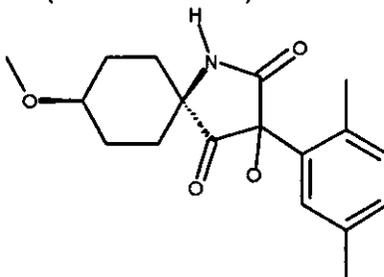
Code Name: <sup>13</sup>C<sub>3</sub>-BYI08330-MA-amide (FHN 14065-acetyl-<sup>13</sup>C)  
(Soil Metabolite, Isotopic Internal Standard)



CAS Name: 1-[[[(2,5-Dimethylphenyl)hydroxyacetyl-<sup>13</sup>C<sub>2</sub>]amino]-4-methoxycyclohexanecarboxylic-<sup>13</sup>C acid  
Molecular Formula: C<sub>18</sub> H<sub>25</sub> N O<sub>5</sub>  
Molecular Weight: 338.4

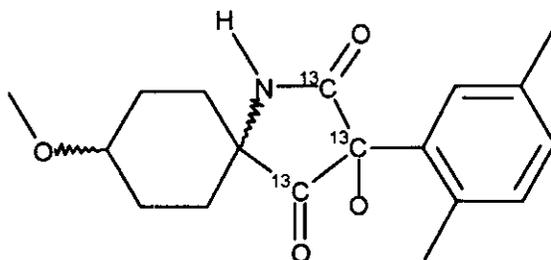
## Appendix 2 (continued)

Code Name: BYI08330-ketohydroxy (AE 1422479)  
(Soil Metabolite)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione  
Molecular Formula: C<sub>18</sub> H<sub>23</sub> N O<sub>4</sub>  
Molecular Weight: 317.4

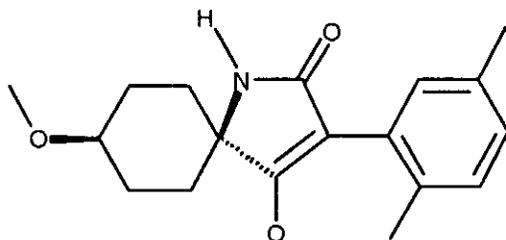
Code Name: <sup>13</sup>C<sub>3</sub>-BYI 08330 *cis*-keto-hydroxy, (FHN 14066-azaspirodecane-2,3,4-<sup>13</sup>C<sub>3</sub>)  
(Soil Metabolite, Isotopic Internal Standard)



CAS Name: 3-(2,5-Dimethylphenyl)-3-hydroxy-8-methoxy-1-azaspiro[4.5]decane-2,4-dione-2,3,4-<sup>13</sup>C<sub>3</sub>  
Molecular Formula: C<sub>18</sub> H<sub>23</sub> N O<sub>4</sub>  
Molecular Weight: 320.3

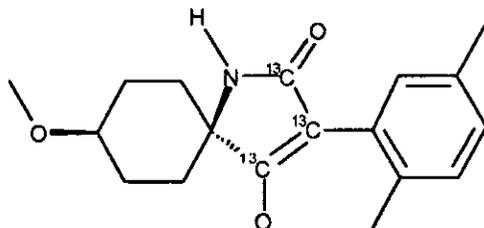
## Appendix 2 (continued)

Code Name: BYI08330-enol (AE 1302944)  
(Soil Metabolite)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one  
CAS Number: [203312-38-3]  
Molecular Formula: C<sub>18</sub> H<sub>23</sub> N O<sub>3</sub>  
Molecular Weight: 301.4

Code Name: <sup>13</sup>C<sub>3</sub>-BYI 08330 *cis*-enol  
(Soil Metabolite, Isotopic Internal Standard)



CAS Name: *cis*-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4.5]dec-3-en-2-one-2,3,4-<sup>13</sup>C<sub>3</sub>  
Molecular Formula: C<sub>18</sub> H<sub>23</sub> N O<sub>3</sub>  
Molecular Weight: 304.4

## Appendix 4 Example Calculation

An example calculation for BYI08330 from sample MEFNY003-SL-LOQ-001, which was analyzed during the method validation study is presented below. This sample was fortified with 2.0ppb BYI08330 and BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide. The chromatogram used in this example is presented in Appendix 5 (Chromatogram 8) and the calibration curve for this analysis is presented in Appendix 6.

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 BYI08330 residues. BYI08330-enol, BYI08330-ketohydroxy and BYI08330-MA-amide residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of BYI08330 in the soil was calculated using the following equation,

$$\text{BYI08330 found (ppb)} = \frac{(Y-B) \times D}{M}$$

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

W	V <sub>1</sub>	Native Peak Area	IS Peak Area	Y	M	B
20g	100mL	1625746.5	1520427.9	1.06927	1.01	0.0435

From the above equations:

$$\text{Dilution Factor (D)} = \frac{100}{20} = 5$$

$$\text{BYI08330 found} = \frac{(1.06927 - (0.0435) \times 5)}{1.01} = 5.078 \text{ ppb}$$

Therefore sample MEFNY003-SL-LOQ-001 contains 5.078ppb BYI08330.

## Appendix 4 (continued)

The % recovery was calculated using the following equation:

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

Where: R = ppb of target analyte found in fortified sample  
S = ppb of target analyte found in control sample, real or apparent  
T = theoretical ppb in fortified sample

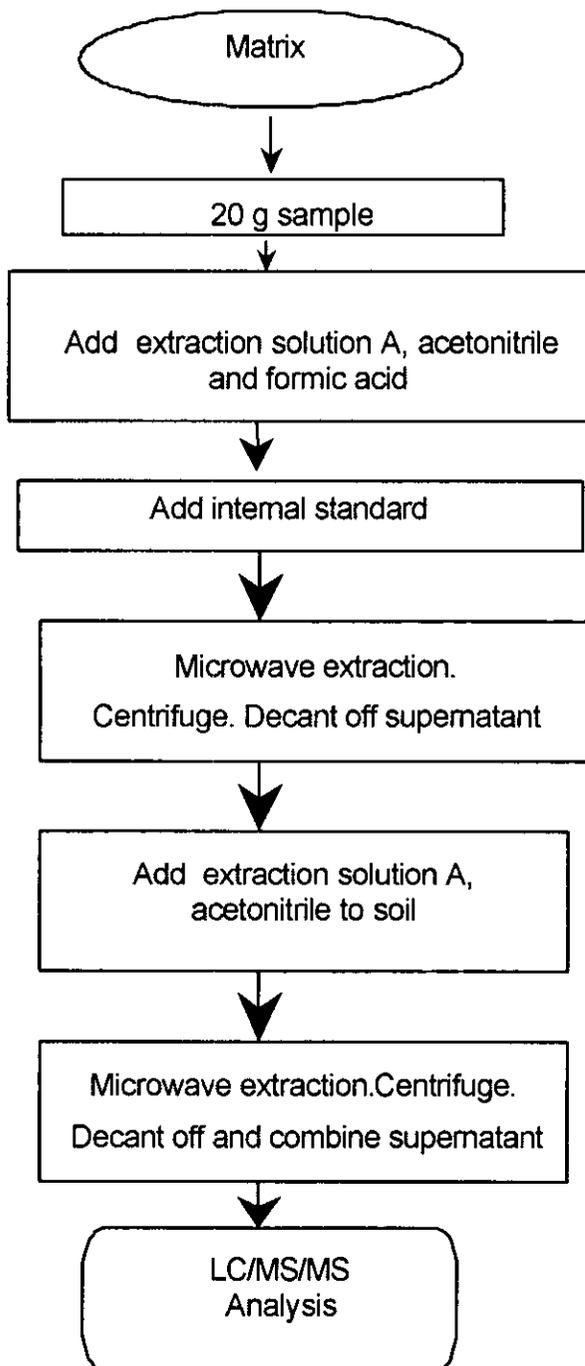
Therefore, for sample MEFNY003-SL-LOQ-001, which was fortified with 5.0ppb BYI08330:

R = 5.078 ppb  
S = 0.0 ppb  
T = 5.0 ppb

$$\% \text{ BYI08330 Recovery} = \frac{(5.078 - 0.0)}{5.0} \times 100 = 102\%$$

Note: The above calculations were performed using rounded numbers and may vary slightly from the results presented in the raw data.

## Appendix 7 Method Flow Chart



Appendix 8   Revision History

Method #	Revision	Description
FN-002-S05-01	01	Method prepared on completion of validation study <sup>1</sup> (08/10/05)
FN-002-S05-02	02	Analyte names changed from AE number to BYI08330 names (01/25/06)
FN-002-S05-03	03	Updated with ILV results, calibration curve data (08/22/06)