SUMMARY

An independent validation of the analytical method for the determination of residues of BSN 2060 and four relevant soil metabolites, provided by Bayer, was conducted in accordance with OPPTS 850.7100. The method validation was conducted by fortifying samples of homogenized soil with BSN 2060, enol, 4-carboxy, cyclobutyl photoisomer, and enol photoisomer. Homogenized soil samples were extracted with a mixture of acetonitrile/water (7:3) using a Dionex Accelerated Solvent Extractor (ASE). The residues were then analyzed by LC-MS/MS.

1.0 PURPOSE

The objective of this study was to conduct a GLP independent laboratory validation (ILV) of Bayer's Method "Determination of BSN 2060 and Four Metabolites in Soil by LC-MS/MS", as described in draft Bayer Report 110478, to meet regulatory requirements as described in OPPTS *Ecological Effects Test Guidelines* and the OPP *Pesticide Assessment Guidelines*. The method was developed for analysis of BSN 2060 and relevant metabolites to meet U.S. EPA environmental fate regulatory requirements. This study was conducted in accordance with U.S. Environmental Protection Agency (EPA) Federal Insecticide, Fungicide and Rodenticide Act; Final Rule (40 CFR Part 160), and Good Laboratory Practice Standards.

2.0 METHOD SUMMARY

The validation procedure followed Bayer's Method "Determination of BSN 2060 and Four Metabolites in Soil by LC-MS/MS", as described in draft Bayer Report 110478. BSN 2060, enol, 4-carboxy, cyclo photoisomer, and enol photoisomer were extracted from fortified soil by extraction with a mixture of acetonitrile and water (7:3) using ASE with subsequent dilution and filtration. The extracted residues were then determined by high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) for the five analytes. A flow chart for the method can be found in Figure 1.

Sample concentrations were calculated by comparison of the response factor between the native analyte and a deuterated analog (internal standard) to an average response factor obtained from six standard concentrations. Micromass MassLynx software was used to determine

chromatographic peak areas and calculate concentrations. MicroSoft Excel 2000 was used for statistical calculations including averages and standard deviations.

3.0 MATERIALS AND METHODS

3.1 Test Substances/Reference Substance

The test substances for this study were BSN 2060, enol, 4-carboxy, cyclobutyl photoisomer, and enol photoisomer. The reference substances for this study were BSN 2060-d_3, enol-d_4, and 4-carboxy-d_4. The test and reference substances were received at room temperature from Bayer Agriculture Division, Stilwell Kansas. The test/reference substances were stored at approximately ≤ -20 °C. Documentation of the synthesis as well as chemical and physical characterizations of the test/reference substances is maintained by the Sponsor. The chemical names, molecular weights, lot numbers, purity, and chemical structures of the test substances are presented below:

Common Name:	BSN 2060
Chemical Name:	3-(2,4,6-trimethylphenyl)-4-(3,3-dimethylbutyl-carbonyloxy)-5-spirocyclopentyl3-dihydrofuranon-2
CAS Name:	Butanoic acid, 3,3-dimethyl-, 2-oxo-3-(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non-3-en-4-yl ester
CAS Number:	283594-90-1
Standard Ref. No. (lot No.):	K-856
Purity:	97.5%
Expiration Date:	5/30/05
Empirical Formula:	$C_{23}H_{30}O_4$
Molecular Weight:	370.49 g/mol

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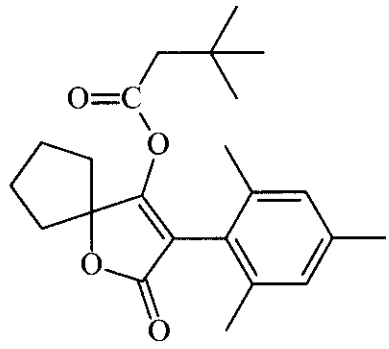
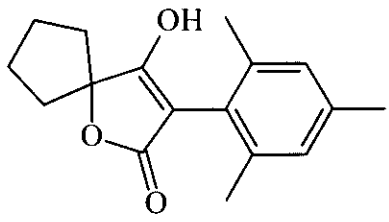
Structure:



Common Name:	Enol
Chemical Name:	1-Oxaspiro[4.4]non-3-en-2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl)
CAS Number:	Not Assigned
Standard Ref. No. (lot No.):	K-860
Purity:	98.8%
Expiration Date:	02/08/06
Empirical Formula:	$C_{17}H_{20}O_3$
Molecular Weight:	272.34 g/mol

Structure:



Common Name:	4-Carboxy
Chemical Name:	3-(4-acetyl-2,6-dimethylphenyl)-4-hydroxy-oxaspiro[4.4]non-3-en-2-one
CAS Number:	Not Assigned

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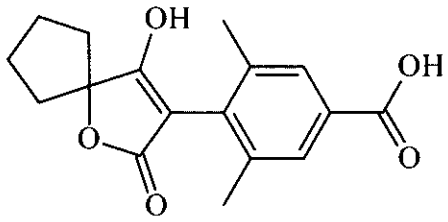
Standard Ref. No. (lot No.):	K-912
Purity:	100.0%
Expiration Date:	05/26/05
Empirical Formula:	$C_{17}H_{18}O_5$
Molecular Weight:	302.32 g/mol
Structure:	

Common Name:	Cyclobutyl Photoisomer
Chemical Name:	3,5-Dimethyl-5'-oxaspiro[bicyclo[4.2.0]octa-1,3,5-triene-7,4'(5'H)-furan-2'(3'H),1"-cyclopentan]-3'-yl 3,3-dimethylbutanoate
CAS Number:	Not Assigned
Standard Ref. No. (lot No.):	K-957
Purity:	99.8%
Expiration Date:	02/26/06
Empirical Formula:	$C_{23}H_{30}O_4$
Molecular Weight:	370.49 g/mol

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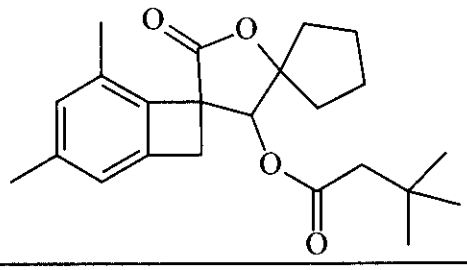
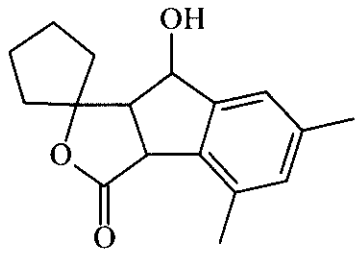
200168

Structure:



Common Name:	Enol Photoisomer
Chemical Name:	8',8'a-Dihydro-8'-hydroxy-4',6'-dimethylspiro[cyclopentane-1,1'-[1H]indeno [1,2-c]furan]-3'(3'aH)-one
CAS Number:	Not Assigned
Standard Ref. No. (lot No.):	K-966
Purity:	97.5%
Expiration Date:	02/26/06
Empirical Formula:	$C_{17}H_{20}O_3$
Molecular Weight:	272.35 g/mol

Structure:



Common Name:	BSN 2060-d_3
Chemical Name:	Butanoic acid, 3,3-dimethyl-, 2-oxo-3-(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non-3-en-4-yl ester-methyl-d_3
CAS Number:	Not Assigned

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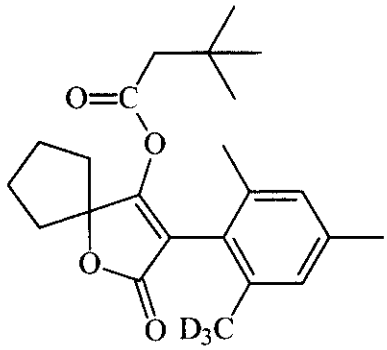
Standard Ref. No. (lot No.):	K-926
Purity:	99.0%
Expiration Date:	09/11/05
Empirical Formula:	$C_{23}H_{27}D_3O_4$
Molecular Weight:	373.50 g/mol
Structure:	

Common Name:	Enol-d_3
Chemical Name:	1-Oxaspiro[4.4]non-3-en-2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl)-methyl-d_3
CAS Number:	Not Assigned
Standard Ref. No. (lot No.):	K-925
Purity:	99.3%
Expiration Date:	09/11/05
Empirical Formula:	$C_{17}H_{17}D_3O_3$
Molecular Weight:	275.37 g/mol

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Agriculture Division

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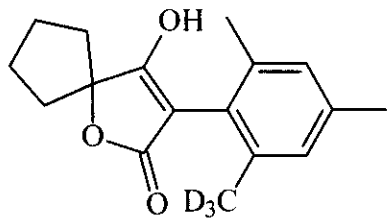
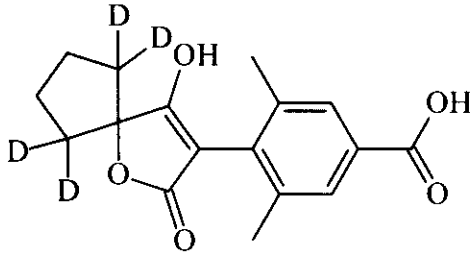
Structure:



Common Name:	4-Carboxy-d_4
Chemical Name:	Benzoic acid, 4-(4-hydroxy-2-oxo-1-oxaspiro[4.4]non-3-en-3-yl)-3,5-dimethyl-spirononyl-6,6,9,9-d_4
CAS Number:	Not Assigned
Standard Ref. No. (lot No.):	K-920
Purity:	99.7%
Expiration Date:	11/16/02
Empirical Formula:	$C_{17}H_{14}D_4O_5$
Molecular Weight:	306.40 g/mol

Structure:



3.2 Test Systems

The test system was comprised of homogenized control soil. The test system chosen was classified by the sponsor as a Fresno, California soil. The soil sample was selected because it represents a "difficult" matrix. The homogenized control soil was received frozen from Bayer Agriculture Division, Stilwell Kansas on October 10, 2001. The homogenized test system was stored at approximately 1-9 °C.

3.3 Analytical Method

The linearity assessment and recovery data for the study was generated using the method described in Bayer Report No. 110478, "Determination of BSN 2060 and Four Metabolites by LC-MS/MS." In brief, 20 g of soil and 4 g of Hydromatrix™ were mixed together and extracted with acetonitrile and water (7:3) using an Accelerated Solvent Extractor at 80 °C and 1500 psi for approximately 10 minutes. After extraction, 1 mL of internal standard solution was added and the extract was brought to 50 mL with acetonitrile and water (8:2) containing 0.05% formic acid. The extracts were analyzed by LC-MS/MS using a 4.6 x 150 mm, 3.5 µm, Eclipse XDB-C8 column (Zorbax), electrospray ionization, and MS/MS detection in the Multiple Reaction Monitoring mode. A method flow chart is presented in Figure 1.

The sample method trial was conducted as one set. The set consisted of a reagent blank, two unspiked matrix control samples, five matrix control samples fortified at LOQ (10 ppb), and five matrix control samples fortified at 10 X LOQ (100 ppb). There were a total number of thirteen samples for the entire method validation. The test substance was administered to the test system using a variable volume positive displacement pipette.

Prior to the method validation trial, instrumental analysis parameters were optimized, linearity was assessed, and the control matrix was evaluated for the presence of interferences. To assess linearity, seven calibration solutions of the analytes were prepared at nominal sample equivalent concentrations of 0, 5, 10, 50, 100, 500, and 1000 ppb of each standard (0.0, 0.002, 0.004, 0.02, 0.04, 0.2, and 0.4 µg/mL). Standards were prepared in solvent and in control soil extract. Each standard solution contained 100 ppb (0.04 µg/mL) of each isotopically labeled reference standard. The standards were analyzed in a random order and each was injected twice. Duplicate control matrix samples were prepared as described by the method.

A few minor equipment substitutions and quantitation methods were made in order to accommodate equipment and practices frequently utilized at our facility (discussed below).

3.4 Modifications/Observations for the Method Described in Bayer Report 110478

The following is a list of observations and modifications that were noted while validating the Bayer Method 110478.

Section 3.4.3. To accommodate the instrumentation used (discussed below) the calibration curve was generated differently than specified in the method. The residue values were determined from

a calibration curve produced from a 1/y-weighted linear regression of data obtained for six calibration standards (5 – 1000 ppb). All chromatograms, calculations, and summary information were generated using MassLynx software proved by Micromass.

Section 4.3.3. The LC-MS/MS conditions and equipment differ from the original method. A Micromass Quattro LC was used instead of a Finnigan Triple Quadropole Mass Spectrometer. Modifications to the instrumental analysis conditions were made to accommodate the different LC-MS/MS instrument used. The injection volume was decreased to 15 µL because the 4-carboxy peak fronted badly when using a 50-µL injection as given in the method. The analysis conditions are detailed in Section 3.5 below.

3.5 LC-MS/MS System

The following conditions, based on Section 3.4 of Bayer Method 110478, were used for LC-MS/MS analysis of prepared extracts.

Equipment and Conditions

HPLC:	Hewlett Packard 1090
Column:	Zorbax, Eclipse XBD-C8, 3.5 µm, 150 x 4.6 mm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	Methanol
Flow Rate:	0.8 mL/min. Post column split to approximately 160 µL/min to mass spectrometer.

Gradient:

Time (minutes)	% Mobile Phase A	% Mobile Phase B
0	40	60
1	40	60
6	20	80
11	20	80
14	5	95
15	5	95
16	40	60
20	40	60

Injection Volume: 15 µL

Run Time: Approximately 20 min

Retention Times

4-Carboxy	~ 4.7 min
Enol Photoisomer	~ 6.8 min
Enol	~ 7.1 min
Cyclobutyl Photoisomer	~12.5 min
BSN 2060	~13.3 min

Mass Spectrometer: Micromass, Quattro LC with Z-Spray® interface

Ionization: electrospray, negative ion mode (ESP-) for 4-carboxy and positive ion mode (ESP+) for all other analytes.

Ion transitions Monitored:

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage(v)	Collision Energy (eV)
4-carboxy	301	195	45	27
4-carboxy – d_4	305	198	45	27
Enol photoisomer	255	209	18	16
Enol	273	255	25	14
Enol – d_3	276	258	25	14
Cyclobutyl photoisomer	371	209	18	23
BSN 2060	371	273	23	15
BSN 2060 – d_3	374	276	23	15

Source Temp.: 80 °C

Drying Gas:	Nitrogen at ~460 L/h
Collision Gas:	Argon at ~2.1 x 10^{-3} mb (gas cell pressure)
Detector Voltage:	700 V

3.6 Data Calculations/Statistical Methods

3.6.1 Linearity Assessment

The linearity assessments of analyte response were determined by using standard curves obtained from the peak area ratio between the native analyte and its deuterated analog, (internal standard) at seven standard concentrations. Standards with concentrations of 0, 5, 10, 50, 100, 500, and 1000 ppb sample equivalents were prepared in solvent and control extract. Each standard was injected twice.

The relative response of each standard was calculated using the following equation.

$$\text{Relative Response} = \frac{\text{(Peak Area STD)}}{\text{(Peak Area IS)}}$$

The calibration curve was plotted as a function of the individual relative response vs. concentration. A 1/y-weighted linear least squares regression was calculated from the response vs. concentration data. The correlation coefficient (r) was determined for each calibration curve.

3.6.2 Method Validation Trial

The concentrations of BSN 2060 and its metabolites present in the fortified samples were determined by using a standard curve obtained from the peak area ratio between the native analyte and its deuterated internal standard, at six sample-equivalent concentrations, with three injections per concentration. The standard injections were interspersed between the fortified sample extracts in the sample analysis set.

The calibration curve for each test substance was constructed by plotting the 1/y-weighted peak area ratio of each standard versus the concentration of the standard injected. The y-intercept and slope of the standard curve were used to calculate the concentration of each test substance in the samples as follows:

$$\text{Peak Area Ratio} = \frac{\text{Area of Native Analyte}}{\text{Area of Deuterated Internal Standard}}$$

$$\text{Residue Level (ppm)} = \frac{\text{(Sample Peak Area Ratio - Intercept)}}{\text{Slope}}$$

The method recovery was calculated by dividing the determined residue level by the fortification concentration and multiplying by 100. The following formula was used:

$$\text{Method Recovery}(\%) = \frac{\text{Residue Level (found)}}{\text{Fortification Level}} \times 100$$

For example, the concentration of enol in soil sample BSV1- 4, fortified at approximately 10 ppb, was calculated as follows:

$$\text{Peak Area Ratio} = \frac{1810.405}{19464.768} = 0.09301$$

$$\text{Enol Residue Level (ppm)} = \frac{(0.09301 - 0.0148818)}{0.00928786} = 8.41 \text{ ppm}$$

and

$$\text{Method Recovery}(\%) = \frac{8.41 \text{ ppm}}{10.0 \text{ ppm}} \times 100 = 84.1\%$$

Figure 1. Flowchart for the Method Described in Bayer Report No. 110478