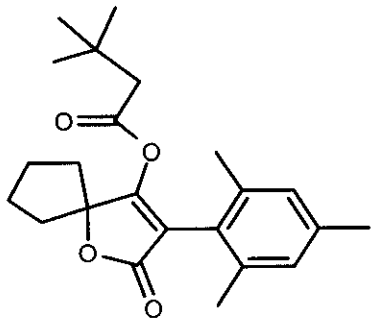
Analytical Method for the Determination of BSN2060 and Metabolite Residues in Soil

1.0 SUMMARY

A method was developed to determine trace amounts of BSN2060 and four metabolites, including BSN2060-enol, BSN2060-4-carboxy, BSN2060-enol photoisomer and the BSN2060-cyclobutyl photoisomer, in soil. After accelerated solvent extraction (ASE), three of the corresponding isotopically labeled internal standards were added, and the samples were analyzed by HPLC/electrospray/MS/MS (LC/ESI/MS/MS). The deuterated internal standards for BSN2060 and BSN2060-enol were used as reference compounds for the BSN2060-cyclobutyl photoisomer and BSN2060-enol photoisomer, respectively.

2.0 EXPERIMENTAL

2.1 Test Substances

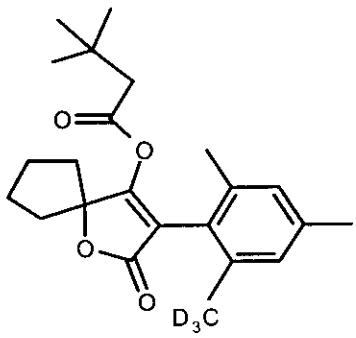
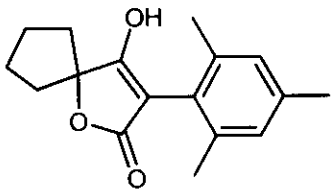
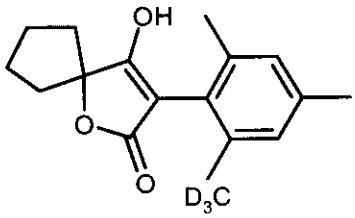
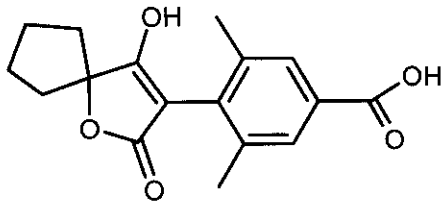


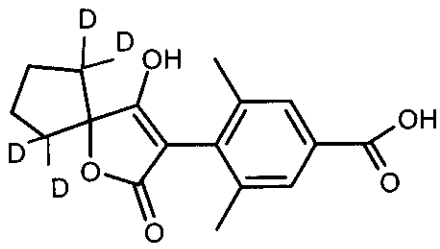
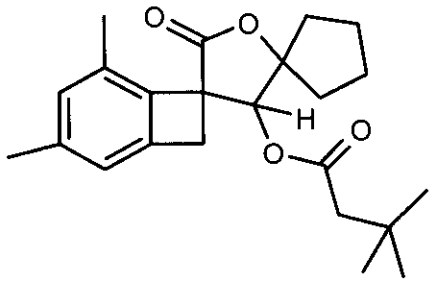
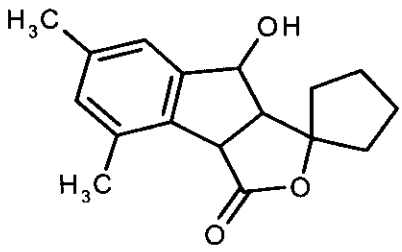
Report Name: BSN2060, K-856

Chemical Name: Butanoic acid, 3,3-dimethyl-2-oxo-3(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non- 3-en-4-yl ester

Purity/Expiration: 97.5%/5-30-05

mol wt = 370.5 g/mol

	*Report Name:* BSN2060-d_3, K-926 *Chemical Name:* Butanoic acid, 3,3-dimethyl-2-oxo-3(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non- 3-en-4-yl ester-d_3 *Purity/Expiration:* 99.0%/9-11-05 *mol wt* = 373.5 g/mol
	*Report Name:* BSN2060-enol, K-860, *Chemical Name:* 1-Oxaspiro[4.4]non-3-en-2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl) *Purity/Expiration:* 98.8%/2-08-06 *mol wt* = 272.4 g/mol
	*Report Name:* BSN2060-enol-d_3, K-925, *Chemical Name:* 1-Oxaspiro[4.4]non-3-en-2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl)-d_3 *Purity/Expiration*: 99.3%/9-11-05 *mol wt* = 275.4 g/mol
	*Report Name:* BSN2060-4-carboxy, K-912 *Chemical Name:* Benzoic acid, 4-(4-hydroxy-2-oxo-1-oxaspiro{4.4}non-3-en-3-yl)3-5-spirononyl *Purity/Expiration:* 100%/5-26-05 *mol wt* = 302.3 g/mol

	*Report Name:* BSN2060-4-carboxy-d_4, K-920 *Chemical Name:* Benzoic acid, 4-(4-hydroxy-2-oxo-1-oxaspiro{4.4}non-3-en-3-yl)3-5-spirononyl-6,6,9,9-d_4 *Purity/Expiration:* 99.7%/9-11-05 *mol wt* = 306.4 g/mol
	*Report Name:* BSN2060-cyclobutyl photoisomer, K-957 *Chemical Name:* 3,3-Dimethylbutanoate, 3,5-dimethyl-5'-oxospiro [bicyclo[4.2.0]octa-1,3,5-triene-7-4'(5',*H*) -furan-2'(3', H),1''-cyclopentan]-3'-yl *Purity/Expiration:* 99.8%/2-26-06 *mol wt* = 370.5 g/mol
	*Report Name:* BSN2060-enol photoisomer, K-966 *Chemical Name:* 8',8'a-Dihydro-8'-hydroxy-4',6'-dimethylspiro [cyclopentane-1,1'-[1*H*]indeno[1,2-c]furan- 3'(3'a*H*)-one *Purity/Expiration:* 97.5%/2-26-06 *mol wt* = 272.4 g/mol

2.2 Chemicals

Methanol (Fisher Optima, A-454-4, UN1230)
Water (Millipore)
Formic Acid (J. T. Baker, 88%)
Acetonitrile (Fisher Optima, A996-4, UN1648)

2.3 Equipment

ASE Extractor (Dionex)
Thirty three milliliter extraction tubes (Dionex)
Triple Quadrupole Mass Spectrometer (TSQ, ThermoFinnigan or equivalent)
HPLC (P-4000, ThermoFinnigan)
Autosampler (A-3000, ThermoFinnigan)
HPLC Column - Eclipse (150 x 4.6, 3.5 μ, MacMod))
Various general laboratory glassware, equipment and utensils
Autosampler vials and caps (2-mL, Baxter #C4800-135 or equivalent)

2.4 Procedures

2.4.1 Standard Stock Solutions

A stock solution of each standard, K-856 (native BSN2060), K-860 (native BSN2060-enol), K-912 (native BSN2060-4-carboxy), K-925 (d_3 - labeled BSN2060-enol),K-926 (d_3 - labeled BSN2060), K-920 (d_4- labeled BSN2060-4-carboxy), K-966 (native BSN2060-enol photoisomer) and K-957 (native BSN2060-cyclobutyl photoisomer) was prepared at a nominal concentration of 100 μg/mL (each) as follows:

Using a spatula, micro balance and glass weigh boat, measure a nominal 10 mg of each standard into respective 100-mL volumetric flasks. Add ACN/water (80:20) containing 0.05% formic acid (Solvent A) to the mark on the neck of the flask. Label each flask with the standard name and actual weighed amount as well as the date. Sonicate each solution for approximately 5 minutes.

2.4.2 Primary Standard Solutions

Using the stock solutions from 2.4.1, prepare the following standards:
Note: Since the stock solutions above were not exact measurements, the volumes used to prepare Mix 1, 2 and 3 must be adjusted to result in an exact concentration for these solutions.

Mix 1 (native spike solution): Add 100 μg (approx. 1 mL) of each native standard to a 100-mL volumetric flask. Dilute to 100 mL with solvent A. The final concentration is 1μg/mL or 1 ppm.

Mix 2: Add 1000 μg (approx. 10 mL) of each native standard to a 100-mL volumetric flask. Dilute to 100 mL with Solvent A. The final concentration is 10 μg mL or 10 ppm.

Mix 3 (internal standard spike solution): Add 200 μg (approx. 2 mL) of each

labeled standard (K-925, K-926, K-920) to a 100-mL volumetric flask. Dilute to the mark with Solvent A. The final concentration is 2 μg/mL.

2.4.3 Secondary Standard Solutions - Linearity and Calibration Curves

Mix 4: (0.4 μg/mL, 1000 ppb), Add 4 mL Mix 2 and 2 mL Mix 3 to a 100-mL volumetric flask. Dilute to the mark with Solvent A.

Mix 5: (0.2 μg/mL, 500 ppb), Add 2 mL Mix 2 and 2 mL Mix 3 to a 100-mL volumetric flask. Dilute to 100 mL with solvent A.

Mix 6: (0.04 μg/mL, 100 ppb), add 4 mL Mix 1 and 2 mL Mix 3 to a 100-mL volumetric flask. Dilute to 100 mL with solvent A.

Mix 7: (0.02 μg/mL, 50 ppb), add 2 mL Mix 1 and 2 mL Mix 3 to a 100-mL volumetric flask. Dilute to the mark with Solvent A.

Mix 8: (0.004 μg/mL, 10 ppb), add 0.4 mL Mix 1 and 2 mL Mix 3 to a 100-mL volumetric flask. Dilute to 100 mL with Solvent A.

Mix 9: (0.002 μg/mL, 5 ppb), add 0.2 mL Mix 1 and 2 mL to a 100-mL volumetric flask. Dilute to the mark with Solvent A.

Note: ppb values are in sample equivalents based on a 20-g soil sample extracted with a final volume of 50 mL.

2.4.4 Accelerated Solvent Extraction (ASE)

Step 1: Mix 20 g of soil and 4 g of Hydromatrix™ together in a 100-mL beaker.

Step 2: Add the mixture from Step 1 to a 33-mL stainless steel Dionex™ extraction tube.

Step 3: Place the tube (tubes) on a Dionex™ ASE and process with the following conditions:

Preheat: 0 min	Flush Volume: 60%	Pressure: 1500 psi
Heat : 5 min	Purge: 5 min	Temp.: 80°C
Static: 5 min	Cycles: 1 min	

Note: ACN and water are mixed by the ASE extractor at a ratio of 7:3.

Step 4: Remove the glass collection vessels from the ASE and add 1 mL of Mix 3 (IS solution).

Step 5: Dilute to 50 mL with ACN:water (8:2) containing 0.05% formic acid. Invert tube twice to mix.

Step 6: Aliquot approximately 1.5 mL into an autosampler vial and cap.

2.4.5 Instrumental Analysis

Fifty microliters of the above extract (2.4.4, Step 6) is analyzed by Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). Parameters for the analysis are as follows:

HPLC: ThermoFinnigan P-4000 quaternary pump with a ThermoFinnigan degasser and A 3000 autosampler attached.

Column: Eclipse (Zorbax), 150 x 4.6 mm, 3.5 μ particle size.

Loop Size: 200 μL

Injection Volume: 50 μL

Temperature: 30° C

Flow : 0.8 mL/min,

Split Ratio: 4:1, 200 μL to MS

Gradient: Gradient Table - Water (Millipore, containing 0.1% formic acid)

Time (min)	Flow (mL/min)	Water %	Methanol %
Initial	0.8	40	60
1.00	0.8	40	60
6.00	0.8	20	80
11.00	0.8	20	80

Time (min)	Flow (mL/min)	Water %	Methanol %
15.00	0.8	5	95
20.00	0.8	5	95
21.00	0.8	40	60
25.00	0.8	40	60

Retention times:

Compound	Retention Time min
BSN2060 (K-856)	15.2
BSN2060-cyclobutyl photoisomer (K-957)	14.6
BSN2060-enol (K-860)	8.3
BSN2060-enol photoisomer (K-966)	8.1
BSN2060-4-carboxy (K-912)	6.9

MS System: ThermoFinnigan TSQ triple quadrupole

Source: Atmospheric Pressure API II in electrospray (ESI) mode

Capillary Temp., °C: 325

Sheath Gas/Psi: Nitrogen/95

Auxillary Flow: 10 mL/min

Ion Transitions: Mode represents the ionization state of the instrument (table on next page.)

Compound	Precursor Ion (amu/Mode)	Product Ion (amu)	Scan Time (s)	Collision Energy (mv)
BSN2060	371/+	273	0.3	-17
BSN2060-cyclobutyl photoisomer	371/+	209	0.3	-22
BSN2060IS	374/+	276	0.3	-17
BSN2060-enol	273/+	255	0.3	-18
BSN2060-enol photoisomer	255/+	209	0.3	-17
BSN2060-enolIS	276/+	258	0.3	-18
BSN2060-4-carboxy	301/-	195	0.4	32
BSN2060-4-carboxyIS	305/-	198	0.4	32

Note: The BSN2060 internal standard was used as the reference standard for the BSN2060-cyclobutyl photoisomer. The BSN2060-enol internal standard was used as the reference standard for the BSN2060-enol photoisomer.

2.4.6 Quantitation

Residue values are determined by producing a calibration curve from the above prepared standards. At 10, 50, 100 and 1000 ppb (sample equivalents). The curve plots concentration in sample equivalents on the abscissa against the response ratio of the native divided by the internal standard (NA/IS) on the ordinate. Unknown values then are determined by the equation of the line solving for x:

$y = mx + b$ where:

y = response ratio

m = slope of the line

x = native concentration (sample equivalents)

b = y intercept

The calibration line was forced through zero resulting in the following simplification:

$$x = y/m$$

All chromatograms, calculations and summary information was generated using Xcalibur ® version 1.2 software provided by ThermoFinnigan (San Jose, CA.).

3.0 LINEARITY CURVE

Linearity in solvent and soil matrix were performed to determine the range of quantitaiton.

Control soil extracts (in duplicate) were prepared see (Section 2.4.4) and fortified with BSN native and internal standards as follows:

Linear response in matrix will be determined by extracting 20-g control soil samples (see 2.4.4, [Steps 1-3]), and preparing duplicate samples as follows:

0.4 μg/mL (1000 ppb sample equivalents): Add 2 mL Mix 2 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A (section 2.4.1).

0.2 μg/mL (500 ppb sample equivalents): Add 1 mL Mix 2 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A.

0.04 μg/mL (100 ppb sample equivalents): Add 2 mL Mix 1 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A.

0.02 μg/mL (50 ppb sample equivalents): Add 1 mL Mix 1 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A.

0.004 μg/mL (10 ppb sample equivalents): Add 0.2 mL Mix 1 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A.

0.002 μg/mL (5 ppb sample equivalents): Add 0.1 mL Mix 1 and 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A.

0 μg/mL (control): Add 1 mL Mix 3, and dilute to approximately 50 mL with Solvent A .

The samples are mixed, and analyzed as describe in Section 2.4.4 (Steps 5-6), and 2.4.5.

4.0 RECOVERY EXPERIMENTS

Recovery tests and experiments are generally performed according to a particular protocol. In general, control soil samples (20 g) will be fortified with 0.2 mL (0.2 μg) and 1 mL (1 μg) of standard Mix 1 representing 10 and 50 ppb sample equivalents, respectively. The samples are extracted using the ASE as described in Section 2.4.4. Following extraction, 1 mL (2 μg) of the internal standard solution (Mix 3) was added to each sample eluant and the solutions diluted to 50 mL with ACN.

5.0 DETERMINATION OF DETECTION LIMITS

The Limit of Detection (LOD) was determined according to a modified procedure listed in the Federal Register, 40 CFR Part 136, Appendix B.[5] Briefly, seven recoveries at 10 ppb are extracted and the standard deviation (SD) of the residue values determined. The LOD is calculated according to the following formula:

$$LOD = t_{0.99} \times SD$$

Where: LOD = Limit of Detection

$t_{0.99}$ = students t value for n-1 determinations (t = 3.3 for seven replicates)

SD = Standard Deviation

The LOQ is defined at 3.3 X LOD. However, the recovery tests were performed at 10 ppb as required by regulatory guidelines.