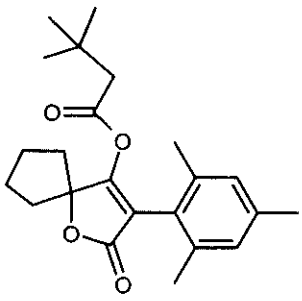
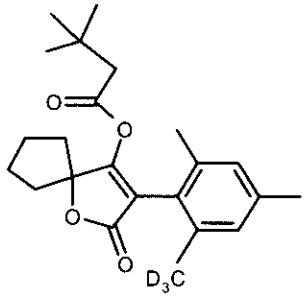
**Analytical Method for the Determination of BSN2060 and BSN206-enol in Water**

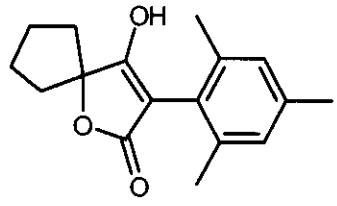
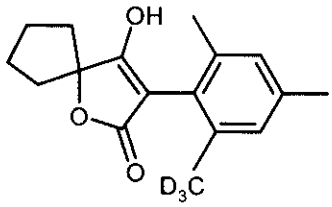
## 1.0 SUMMARY

An LC/MS/MS method to determine BSN2060 and BSN2060-enol in water was developed. The analytes from a 25-mL water sample were extracted with a reverse phase C18 cartridge. The target compounds were eluted from the cartridge with acetonitrile containing 0.1% formic acid, concentrated and analyzed by LC/MS/MS. Recovery experiments were performed from 0.025 µg/L to 25 µg/L for both BSN2060 and BSN2060-enol

## 2.0 EXPERIMENTAL

### 2.1 Test Substances

| | |
|---|---|
|  | *Report Name:* BSN2060, K-856<br><br>*Chemical Name:* Butanoic acid, 3,3-dimethyl - 2-oxo-3(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non- 3-en-4-yl ester<br><br>*Purity/Expiration:* 97.5%/5-30-05<br><br>*mol wt* = 370.5 g/mol |
|  | *Report Name:* BSN2060-$d_3$, K-926<br><br>*Chemical Name:* Butanoic acid, 3,3-dimethyl -2-oxo-3(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non- 3-en-4-yl ester-$d_3$<br><br>*Purity/Expiration:* 99.0 %/9-11-05<br><br>*mol wt* = 373.5 g/mol |

| | |
|---|---|
|  | *Report Name:* BSN2060-enol, K-860<br><br>*Chemical Name:* 1-Oxaspiro[4.4]non-3-en -2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl)<br><br>*Purity/Expiration:* 98.8%/2-08-06<br><br>*mol wt* = 272.4 g/mol |
|  | *Report Name:* BSN2060-enol-$d_3$, K-925<br><br>*Chemical Name:* 1-Oxaspiro[4.4]non-3-en -2-one, 4-hydroxy-3-(2,4,6-trimethylphenyl)-$d_3$<br><br>*Purity/Expiration:* 99.3%/9-11-05<br><br>*mol wt* = 275.4 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

Triple Quadrupole Mass Spectrometer (TSQ, ThermoFinnigan or equivalent)
HPLC (P-4000, ThermoFinnigan)
Autosampler (A-3000, ThermoFinnigan)
HPLC Column - Zorbax Eclipse XDB-C8 (150 x 4.6mm, 3.5$\mu$, MacMod))
Autosampler vials (1.8 mL, ThermoFinnigan, Part No. A4957-010)
SPE cartridges (Varian, Mega Bond Elut C-18, Part No. 1225-6023)
Turbo Vap LV evaporator (Zymark Corporation) or equivalent
Vacuum Manifold
Various laboratory glassware and utensils

## 3.0 PROCEDURES

### 3.1 Standard Solution Preparation

**Stock Standard Solutions**

3.1.1 Using an analytical balance, weigh 0.01 g of BSN2060 analytical standard (K-856, 99.5% purity), dilute to approximately 100 mL with acetonitrile containing 0.1% formic acid resulting in a concentration of 0.1 mg mL.

3.1.2 Using an analytical balance, weigh 0.01 g of BSN2060-$d_3$ analytical standard (K-926, 99% purity), dilute to approximately 100 mL with acetonitrile containing 0.1% formic acid resulting in a concentration of 0.1 mg mL.

3.1.3 Using an analytical balance, weigh 0.01 g of BSN2060-enol analytical standard (K-860, 99.6% purity), dilute to approximately 100 mL with 9:1 acetonitrile/water containing 0.1% formic acid resulting in a concentration of 0.1 mg mL.

3.1.4 Using an analytical balance, weigh 0.01 g of BSN2060-enol-$d_3$ analytical standard (K-925, 99.3% purity), dilute to approximately 100 mL with 9:1 acetonitrile/water containing 0.1% formic acid resulting in a concentration of 0.1 mg mL.

### 3.2 Internal Standard Solution

Transfer 500 µL (using a syringe) of each solution from sections 3.1.2 and 3.1.4 into a 100-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a final concentration of 0.5 µg/mL.

### 3.3 Primary Standard Solutions - Sample Fortification

3.3.1 Pipette 1.0 mL each of the stock standard solutions from sections 3.1.1 and 3.1.3 into a 100-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a final concentration of 1.0 µg/mL.

3.3.2 Pipette 10 mL of the 1.0-µg/mL solution from section 3.3.1 into a 100-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a final concentration of 0.1 µg/mL.

3.3.3 Pipette 10 mL of the 0.1-µg/mL solution from section 3.3.2 into a 100-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a final concentration of 0.01 µg/mL.

### 3.4 Secondary Standard Solutions - Calibration

Prepare the following solutions to be used for establishing a 5-point calibration curve during sample analysis. The internal standard concentration for all calibration standard solutions is 125 $\mu$g/L, equivalent to 5 ppb in a water sample.

3.4.1 Transfer 625 $\mu$L of the 0.01-$\mu$g/mL standard solution from section 3.3.3 and 2.5 mL of the 0.5 $\mu$g/mL internal standard solution from section 3.2 into a 10-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a 0.625-µg/L calibration solution, equivalent to a 0.025 ppb water sample

3.4.2 Transfer 1.25 mL of the 0.01-$\mu$g/mL standard solution from section 3.3.3 and 2.5 mL of the 0.5 $\mu$g/mL internal standard solution from section 3.2 into a 10-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a 1.25-µg/L calibration solution, equivalent to a 0.05 ppb water sample.

3.4.3 Transfer 2.5 mL of the 0.1-$\mu$g/mL standard solution from section 3.3.2 and 2.5 mL of the 0.5 $\mu$g/mL internal standard solution from section 3.2 into a 10-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a 25-$\mu$g/L calibration solution, equivalent to a 1 ppb water sample.

3.4.4 Transfer 2.5 mL of the 1-$\mu$g/mL standard solution from section 3.3.1 and 2.5 mL of the 0.5 $\mu$g/mL internal standard solution from section 3.2 into a 10-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a 250-$\mu$g/L calibration solution, equivalent to a 10 ppb water sample.

3.4.5 Transfer 6.25 mL of the 1-$\mu$g/mL standard solution from section 3.3.1 and 2.5 mL of the 0.5 $\mu$g/mL internal standard solution from section 3.2 into a 10-mL volumetric flask and dilute to volume with 50:50 acetonitrile/water containing 0.1% formic acid resulting in a 625-$\mu$g/L calibration solution, equivalent to a 25 ppb water sample.

### 3.5 Sample Preparation - Extraction

3.5.1 Measure 25 mL of test solution into a test tube. Add 250 $\mu$L of 0.5-$\mu$g/mL internal standard solution from section 3.2 and mix.

3.5.2 Condition a Mega Bond Elut C18 SPE cartridge at a moderate flow rate (5 mL/min) with one column volume of ACN containing 0.1% formic acid (approx. 20 mL) and two column volumes of water (approx. 40 mL).

3.5.3 Pass the test solution through the cartridge slowly (<2 mL/min). Do not allow the column to go dry.

3.5.4 Wash the cartridge with one column volume of water (approx. 20 mL) at a moderate flow rate (5 mL/min) and allow the cartridge to dry for approx. 15 seconds.

3.5.5 Slowly elute the analytes from the cartridge into a test tube with approx. 40 mL of ACN containing 0.1% formic acid.

3.5.6 Concentrate the sample to approx. 0.5 mL using a Turbovap® set at 40 °C. Dilute the sample to a final volume of 1.0 mL using ACN containing 0.1% formic acid.

3.5.7 Transfer the sample solution into an autosampler vial.

### 3.6 Spiking Procedure for Concurrent Recovery Samples

3.6.1 Transfer an appropriate aliquot of spiking solution (section 3.3) as described in Table 3.6.1 into a test tube containing 25 mL of control water. Add 500 µL of 5% formic acid solution and 125 µL of formulation blank (0.00037 µg/mL).

Typical validation levels are presented in the Table below. Aliquots (25 mL) of control water (blended hard water) were fortified with a mixed standard of a BSN2060 and BSN2060-enol solution (in acetonitrile/water 1:1 containing 0.1% formic acid) as specified, resulting in spikes at 0.025 ppb (two),0.050 ppb (two), 0.10 ppb (two), 1.0 ppb (two) and one each at 10 and 25 ppb.

**Table. 3.6.1**

| Fortification Level (µg/L) [1] | Fortification Solution Used[2] (µg/mL) | Volume of Fortification Solution (µL) |
|---|---|---|
| 0.025 | 0.01 | 62.5 |
| 0.050 | 0.01 | 125 |
| 0.10 | 0.01 | 250 |
| 1.0 | 0.1 | 250 |
| 10 | 1.0 | 250 |
| 25 | 1.0 | 625 |

[1] Sample volume = 25 mL.
[2] Solutions described in section 3.3

**3.6.2** Prepare the concurrent recovery sample along with the sample set as described above in Section 3.5.

### 3.7 LC/MS/MS Analysis

A Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan, San Jose, CA.) connected to a Thermo Separation Products (Thermoquest, San Jose, CA.) P4000 gradient HPLC system and an AS 3000 autosampler was used. The system was equipped with an API interface and operated in the ESI mode in the selected reaction monitoring (SRM) scan mode. A DEC Alpha workstation loaded with UNIX 4.0b, ICIS 8.3 and ICL 8.3.2 software was used to operate the system.

Instrument conditions were as follows:

| | |
|---|---|
| MS Conditions | |
| Capillary Temperature: | 300 °C |
| Sheath gas pressure: | 100 psi ($N_2$) |
| Aux. gas flow: | 20 mL/min ($N_2$) |
| Ion mode (Polarity): | Positive ion |
| SRM Transition: | Enol: *m/z* 272.8 → *m/z* 186.8 |
| | Enol-$d_3$: *m/z* 275.8 → *m/z* 189.8 |
| | BSN2060: *m/z* 370.8 → *m/z* 272.8 |
| | BSN2060 $d_3$: *m/z* 373.8 → *m/z* 275.8 |
| Collision energy: | -18 eV (BSn2060-enol); -17 eV (BSN2060) |
| HPLC Conditions | |
| Flow rate: | 0.8 mL/min |
| Inj. volume: | 50 $\mu$L |
| Conditions: | Gradient: 80% water (containing 0.1% formic acid) to 95% methanol (containing 0.1% formic acid) in 6 min; hold 6 min |
| Column: | Zorbax Eclipse XDB-C8, 150 x 4.6 mm, 3.5$\mu$ |
| Retention time (approx.): | 8.4 min (BSN2060-enol), 9.5 min (BSN2060) |
| Total analysis time: | 18 min |

### 3.8 Quantitation

Quantitation was performed using a 5 point weighted (1/X) calibration curve. The calibration points used were 0.025, 0.05, 1.0, 10.0 and 25.0 ppb. Triplicate injections of each standard were made during the analysis run of each standard level. The equation of the line was determined instrumentally using Xcalibur™, version 1.2, software. The residue values were calculated by comparing the area response ratio, i.e., the response of the native to the response of the internal standard (Native/Internal Standard) to concentration. The equation of the line in general form is given as follows:

$Y = b + mx$ where:

Y = response ratio
b = y intercept