Cover Sheet for Analytical Method

Benzobicyclon in Soil - MRID 49506327

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1.0 INTRODUCTION

Methodology from Morse Laboratories (Meth-222, Revision #2) was validated to quantify the concentration of benzobicyclon and 1315P-070 (metabolite B) present in recovery samples prepared in three different types of soil on 17 to 19 December 2013. The detailed Sponsor-supplied method is presented in Appendix 1. This independent laboratory validation (ILV) study is required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one group, can be independently validated by a second group with no major interaction between the two groups. This method was validated by fortification of clay loam #1, silt loam and clay loam #2 soils with benzobicyclon and 1315P-070 (metabolite B) at concentrations of 0.005 ppm (LOQ) and 0.050 ppm (10X LOQ). Recovery samples were extracted once with 80:20 acetonitrile:0.5M citric acid in deionized water (v:v, 90 mL), filtered through Whatman #5 filter paper, rinsed with 80:20 acetonitrile:0.5M citric acid in deionized water (v:v, 10 mL) and brought to a final volume of 100 mL with 80:20 acetonitrile:0.5M citric acid in deionized water (v:v). Prior to analysis, recovery samples containing the test substance were further processed using solid phase extraction (SPE) and then analyzed using liquid chromatography with mass spectrometry (LC/MS/MS).

The study was initiated on 13 December 2013, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the ILV study was conducted on 17 to 19 December 2013 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data and the final report produced during this study are archived at Smithers Viscient at the above location.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled “Independent Laboratory Validation (ILV) of the Analytical Method: Determination of Benzobicyclon and 1315P-070 (Metabolite B) in Soil (Morse Meth-222, Revision #2)”, Smithers Viscient Protocol

No.: 120211/ILV/sediment (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100 for Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA, 2012).

2.2 Test Substances

The test substance, benzobicyclon, was received on 20 August 2012 from PTRL West, Hercules, California. The following information was provided:

Name:	benzobicyclon
Synonym:	3-[2-chloro-4(methylsulfonyl)benzoyl]-4-(phenylthio)bicyclo[3.2.1]oct-3-en-2-one
Lot No.:	1A0110
CAS No.:	156963-66-5
Purity:	>99.9% (Certificate of Analysis, Appendix 2)
Expiration Date:	20 May 2015

Upon receipt at Smithers Viscient, benzobicyclon (SMV No. 5812) was stored frozen in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-070, was received on 7 March 2013 from PTRL West, Hercules, California. The following information was provided:

Name:	1315P-070
Lot No.:	95Z25
CAS No.:	126656-88-0
Purity:	99.8% (Certificate of Analysis, Appendix 2)
Expiration Date:	8 April 2015

Upon receipt at Smithers Viscient, 1315P-070 (SMV No. 6090) was stored frozen in the original container. Concentrations were adjusted for the purity of the test substance.

Both test substances were used to fortify the recovery samples and prepare the calibration standards during testing. Determination of stability and characterization, verification of the test substances identity, maintenance of records on the test substances, and archival of a sample of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
2.	Citric acid, anhydrous:	BDH, reagent grade
3.	Phosphoric acid:	EMD, reagent grade
4.	Diatomaceous Earth:	EMD, reagent grade
5.	Purified reagent water:	prepared from a Millipore Milli-Q® Direct 8 system (meeting ASTM Type II requirements)
6.	Ammonium acetate:	BDH, reagent grade
7.	Methanol:	EMD, reagent grade
8.	Deionized water:	In-house, reagent grade

2.4 Equipment

1.	Instrument:	AB MDS Sciex 4000 Turbo V ESI with Shimadzu 20AD vacuum degasser, LC-20AD binary pumps, a CBM-20A communication bus, CTO-20AC column oven with a CTO-20AC column compartment, and SIL-20ACHT autosampler; Analyst 1.4.2 for data acquisition
2.	Balance:	Mettler Toledo AG 285, Mettler Toledo PJ 3000, Sartorius Moisture Analyzer MA-45
3.	Shaker table:	Orbit Shaker Table 3520
4.	Laboratory equipment:	volumetric flasks, disposable glass pipets, disposable glass vials, positive displacement pipets, Whatman#5 filter paper, Bond Elut C18 SPE cartridges, 70 mm Buchner funnels, 500 mL filter flasks, Nalgene centrifuge tubes, autosampler vials, and amber glass bottles with Teflon®-lined caps

2.5 Test Soil

The three soil types used for this ILV analysis were clay loam #1, silt loam and clay loam #2 (Appendix 3). The clay loam #1 soil was received on 31 May 2013 from Morse Laboratories, Sacramento, California and was determined to have a soil moisture content of 9.33% prior to testing. The silt loam was received on 12 June 2013 from R and D Research, Washington, Louisiana and was determined to have a soil moisture content of 22.57%. The clay loam #2 soil was received on 18 June 2013 from Research for Hire, Porterville, California and was determined to have a moisture content of 6.31%. All soil moisture analyses were conducted using a Sartorius MA-45 moisture analyzer. Soil characterization results are summarized in the table below.

Soil Type	**Soil ID**	**pH (1:1 soil:water)**	**Sand Content (% w/w)**	**Silt Content (% w/w)**	**Clay Content (% w/w)**	**Organic Matter (%)**	**Cation Exchange Capacity (meq/100 g)**
Clay loam #1[a]	Cores 1 though 6 0-6”	7.7	38	25	37	2.1	29.3
Silt Loam[b]	RB 3	6.4	14.9	64.6	20.3	2.1	9.7
Clay loam #2[a]	R06-18-13-14	7.6	43	21	36	1.3	30.8

[a] Soil characterized at Agvise Laboratories, 604 Highway 15 West, P.O. Box 510, Northwood, ND 58267

[b] Soil characterized at A&L Analytical Laboratories, Inc., 2790 Whitten Rd. Memphis, TN 38133

2.6 Preparation of Stock Solutions

A 1000 mg/L primary stock solution was prepared by placing 0.0503 g of benzobicyclon (0.0502 g as active ingredient) in a volumetric flask and bringing it to volume with 50.0 mL of acetonitrile. Two secondary stock solutions (10.0 and 100 mg/L) were prepared by placing 0.500 and 5.00 mL of the 1000 mg/L primary stock solution in separate volumetric flasks and bringing each to volume with 50.0 mL of acetonitrile.

A 1000 mg/L primary stock solution was prepared by placing 0.0503 g of 1315P-070 (0.0502 g as active ingredient) in a volumetric flask and bringing it to a volume of 50.0 mL of acetonitrile. Two secondary stock solutions (10.0 and 100 mg/L) were prepared by placing 0.500 and 5.00 mL of the 1000 mg/L primary stock solution in separate volumetric flasks and bringing each to volume with 50.0 mL of acetonitrile.

A 0.100 mg/L mixed sub-stock solution was prepared by combining 0.100 mL of the 10.0 mg/L benzobicyclon secondary stock solution with 0.100 mL of the 10.0 mg/L 1315P-070 secondary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 0.100 mg/L mixed stock solution was used fortify the low-level recovery samples and prepare the calibration standards.

A 1.00 mg/L mixed sub-stock solution was prepared by combining 0.100 mL of the 100 mg/L benzobicyclon secondary stock solution with 0.100 mL of the 100 mg/L 1315P-070 secondary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 1.00 mg/L mixed stock solution was used to fortify the high-level recovery samples and prepare the calibration standards.

A 10.0 mg/L mixed sub-stock solution was prepared by combining 0.100 mL of the 1000 mg/L benzobicyclon primary stock solution with 0.100 mL of the 1000 mg/L 1315P-070 primary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 10.0 mg/L mixed stock solution was used prepare the calibration standards.

All primary and secondary stock solutions were stored refrigerated in glass amber bottles fitted with Teflon®-lined caps. All sub-stock solutions were prepared daily and discarded after use.

2.7 Reagent Solution and Mobile Phase Preparation

A 50:50 acetonitrile:purified reagent water (v:v) liquid reagent solution was prepared by mixing 100 mL of acetonitrile with 100 mL of purified reagent water. This reagent solution was mixed well using a stir bar and stir plate.

A 0.5 M citric acid in deionized water liquid reagent solution was prepared by transferring a portion of deionized water to a 500 mL volumetric flask. A 52.23 g portion of citric acid (molecular weight = 192.124, anhydrous) was measured and transferred to the volumetric flask containing the deionized water. The solution was brought to a final volume of 500 mL with deionized water and mixed well.

An 80:20 acetonitrile:0.5 M citric acid in deionized water (v:v) liquid reagent solution was prepared by combining 800 mL of acetonitrile with 200 mL of 0.5 M citric acid in deionized water. This reagent solution was mixed well using a stir bar and stir plate.

A 50 mM phosphoric acid in deionized water (v:v) liquid reagent solution was prepared by transferring a portion of deionized water to a 1000 mL volumetric flask. A 3.38 mL portion of phosphoric acid (14.8 M) was measured and transferred to the volumetric flask containing the deionized water. This solution was brought to a final volume of 1000 mL with deionized water and mixed well.

A 20:80 acetonitrile:50mM phosphoric acid in deionized water (v:v) liquid reagent solution was prepared by combining 20.0 mL of acetonitrile with 80 mL of 50mM phosphoric acid in deionized water. This reagent solution was mixed well using a stir bar and stir plate.

A 100 mM ammonium acetate in methanol liquid reagent solution was typically prepared by dissolving 0.77 g of ammonium acetate in 100 mL of methanol. This solution was shaken to mix well.

A 19:1 purified reagent water:100 mM ammonium acetate in methanol (v:v) mobile phase solution was prepared by combining 950 mL of purified reagent water with 50.0 mL of 100 mM ammonium acetate in methanol. The mobile phase solution was mixed well and degassed under vacuum with sonication.

A 5 mM ammonium acetate in methanol (v:v) mobile phase solution was prepared by combining 950 mL of methanol with 50.0 mL of 100 mM ammonium acetate in methanol. The mobile phase solution was mixed well and degassed under vacuum with sonication.

2.8 Preparation of Calibration Standards

Calibration standards were prepared in 50:50 acetonitrile:purified reagent water at concentrations of 0.500 and 1.00 µg/L using the 0.100 mg/L mixed sub-stock solution, at concentrations of 2.50 and 10.0 µg/L using the 1.00 mg/L mixed sub-stock solution and at concentrations of 50.0, 75.0 and 100 µg/L using the 10.0 mg/L mixed sub-stock solution.

2.9 Sample Fortification and Preparation

All soil recovery samples (10.0 g dry weight) were weighed into individual 250-mL HDPE centrifuge tubes for each soil type. Five replicates of each concentration were dosed with the appropriate test substance mixed sub-stock solutions at 0.00500 and 0.0500 ppm. The dosing procedure is detailed in the following table:

Sample ID	**Mixed Sub-Stock Concentration (mg/L)**	**Volume of Stock Solution (mL)**	**Soil Dry Weight (g)**	**Fortified Sample Concentration (ppm)**
Reagent Blank	NA[a]	NA	NA	0.00
Control A, & B	NA	NA	10.0	0.00
Low A, B, C, D and E	0.100	0.500	10.0	0.00500
High A, B, C, D and E	1.00	0.500	10.0	0.0500

[a] NA = Not Applicable

Two additional 10.0 g samples were prepared and left unfortified to serve as controls. One additional sample was extracted using only extraction solvents to serve as the reagent blank.

2.10 Soil Extraction and Dilution

Following fortification, a 90 mL aliquot of 80:20 actonitrile:0.5 M citric acid in deionized water (v:v) was added to each reagent blank and soil recovery sample (10.0 g dry weight). The samples were then placed on an orbital shaker table for 30 minutes at 150 rpm. After 30 minutes, the samples were vacuum filtered through Whatman #5 filter paper with a 1.0 cm thick layer of diatomaceous earth using a 70 mm Buchner funnel, into a 500 mL filter flask. The filter cake was then rinsed with 10.0 mL of 80:20 acetonitrie:0.5 M citric acid in deionized water (v:v), transferred to a graduated cylinder and brought to a final volume of 100 mL with 80:20 acetonitrie:0.5 M citric acid in deionized water (v:v). The extracts were transferred to separate 100-mL amber Wheaton bottles and mixed well.

Recovery samples were additionally processed using a solid phase extraction (SPE) sample clean-up step with Agilent Bond Elut C18 solid phase extraction columns (500 mg, 6-mL cartridge). A 4.00 mL aliquot of each recovery sample was transferred to separate 50.0-mL Nalgene® centrifuge tubes. An aliquot (20.0 mL) of 50 mM phosphoric acid in deionized water (v:v) was added to each centrifuge tube and inverted to mix. Each SPE column was conditioned first with acetonitrile (5.00 mL) followed by 50 mM phosphoric acid in deionized water (v:v, 5.0 mL). This wash was discarded and the cartridges were not allowed to go dry. The recovery samples (24.0 mL) were loaded onto the columns and eluted at a flow rate of approximately 2.0 mL/minute; this eluate was discarded. Columns were rinsed with 5.00 mL of 20:80 acetonitrile:50 mM phosphoric acid in deionized water (v:v). This rinse was discarded and the cartridges were not allowed to go dry. Finally, the columns were eluted with 2.00 mL of acetonitrile and the eluent was collected in glass conical tubes; vacuum was pulled briefly until droplets were no longer observed. Sample volumes were adjusted to 2.00 mL with acetonitrile as necessary. The extraction and dilution procedure is summarized in the table below.

Sample ID	**Fortified Concentration (ppm)**	**Dry weight (g)**	**Extract Volume[a] (mL)**	**Final Volume[a] (mL)**	**Sample Volume (mL)**	**Solvent Volume[b] (mL)**	**Rinse Volume[c] (mL)**	**Acetonitrile Final Volume (mL)**	**Dilution Factor**
Reagent Blank	0.00	NA[d]	90.0	100	4.00	20.0	5.00	2.00	5.00
Control A & B	0.00	10.0	90.0	100	4.00	20.0	5.00	2.00	5.00
Low A, B, C, D & E	0.00500	10.0	90.0	100	4.00	20.0	5.00	2.00	5.00
High A, B, C, D & E	0.0500	10.0	90.0	100	4.00	20.0	5.00	2.00	5.00

[a] Extracted/diluted with 80:20 acetonitrile:0.5 M citric acid in deionized water (v:v).
[b] Addition of 50 mM phosphoric acid in deionized water (v:v).
[c] Rinsed with 20:80 acetonitrile:50 mM phosphoric acid in deionized water (v:v).
[d] NA = Not Applicable.

Samples were transferred to amber GC vials with crimp caps and analyzed by LC/MS/MS.

2.11 Analysis

2.11.1 Instrumental Conditions

The LC/MS/MS analysis was conducted utilizing the following instrumental conditions;

Column:	Phenomenex Luna C18, 3µm, 150 x 2.0 mm
Temperature:	40 °C
Mobile Phase A:	19:1 purified reagent water:100 mM ammonium acetate in methanol, gradient analysis
Mobile Phase B:	5 mM ammonium acetate in methanol, gradient analysis
Flow Rate:	0.20 mL/minute
Run Time:	13 minutes
Injection Volume:	5.0 µL
Retention Time:	Approximately 7.8 minutes (benzobicyclon) Approximately 5.8 minutes (1315P-070)

Type	Condition	Parameter
MS	Source temperature	500 °C
	Scan type	MRM
	Ionization Mode	Positive
	Ion Source	Turbo Spray
	Ion spray voltage	5000.00
	Dwell time	150.00 msec
	Curtain Gas	10.00
	Ion source- gas 1/gas 2	20.00/20.00
	Collision Gas	4.00
	Collision Cell Entrance Potential	10.00
	Interface Heater	On

Transitions Monitored:

Compound	Ion[a] (amu)	Collision Energy	Collision Exit Potential	Declustering Potential
Benzobicyclon	447.08/257.20	35.00	16.00	76.00
Benzobicyclon	447.08/229.20	51.00	16.00	76.00
Benzobicyclon	447.08/139.10	107.00	8.00	76.00
1315P-070	355.14/165.20	33.00	10.00	101.00
1315P-070	355.18/183.10	31.00	12.00	101.00
1315P-070	355.18/319.00	31.00	12.00	101.00
1315P-070	355.18/69.20	77.00	12.00	101.00
1315P-070	355.18/81.10	63.00	14.00	101.00

[a] Ions are presented as Q1 mass/Q3 mass.

Gradient Table:

Time (min)	A (%)	B (%)
1.0	80.0	20.0
3.0	20.0	80.0
9.0	20.0	80.0
10.0	80.0	20.0
13.0	80.0	20.0

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11.3 Method Differences

The following were modifications from the original method, Meth-222 Revision #2:

- No platform shaker table speed was listed in the original method. Therefore, samples were shaken at 150 rpm.
- During the SPE profiling analysis, low signal-to-noise was observed for the 1315P-070 (Metabolite B) low concentration standards confirmation ions 3, 4 and 5. Therefore in order to gain additional sensitivity to increase signal-to-noise, the sample injection volume was increased to 5 µL.

2.12 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the low- and high-level recovery samples. Recoveries of 70 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the low- and high-level recovery samples for each analyte. The retention time should have an RSD of less than or equal to 2%. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as benzobicyclon and 1315P-070 which might interfere with the quantitation of analytes. Interferences with peak areas that are less than 50% at the limit of detection (LOD) are not considered significant. Linearity of the method was determined by the correlation coefficient (r^2), y-intercept and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient not less than 0.990. The precision of the method at the LOQ was reported in terms of the relative standard deviation or coefficient of variation of the observed recovery values.

2.13 Communications

Communications occurred with the Sponsor Monitor to discuss items such as 1) clarification/approval of the protocol and method, 2) acquisition of analytical standard and control sample, and 3) pre-validation evaluation and method establishment including calibration curve linearity. A complete list of communications is maintained in the study raw data.

2.14 Time Required for Analysis

A normal batch of samples consists of 10 fortified and 2 unfortified samples, 1 matrix-match blank and 7 solvent standards (20 samples total) for each matrix. A single analyst completed a set of 20 samples in one working day (8 hours) with LC/MS/MS analysis performed overnight.

3.0 Calculations

A calibration curve was constructed by plotting the analyte concentration (µg/L) in the calibration standards against the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the peak area of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = ax^2 + bx + c$$

$$(2) \qquad \mathrm{DC}\,(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

$$(3) \qquad A = \mathrm{DC} \times \mathrm{DF}$$

where:

y = detector response (peak area) for analyte
a, b and c = regression constants
DC (x) = detected concentration (µg/L) in the sample
C = constant c minus the peak area; C = (c - y)
DF = dilution factor (the final sample volume divided by the original sample volume)
A = concentration of the analyte in the original sample

The method limit of detection (LOD) was calculated using the following equation (U.S. EPA, 2000):

$$(4)\ \mathrm{LOD} = t_{0.99} \times s$$

where:

$t_{0.99}$ = t value for n-1 replicates at the 99% confidence interval
s = standard deviation (µg/L) for the method LOQ, where the LOQ = 0.005 ppm