Study Title

Independent Laboratory Validation (ILV) of the Analytical Method
for the Determination of Benzobicyclon and its Metabolites 1315P-070, 1315P-076,
1315P-570, 1315P-683, 1315P-960, 1315P-962, and 1315P-966 in Water by LC-MS/MS

Data Requirements

OCSPP Guideline 850.6100
OCSPP Guideline 850.7100
OCSPP Guideline 860.1340

1.0 INTRODUCTION

This independent laboratory validation (ILV) study is required by the U.S. EPA under the Guideline for Environmental Chemistry Method and Associated Independent Laboratory Validations OCSPP No. 850.6100 (U.S. EPA, 2012), Residue Analytical Methods OCSPP No. 860.1340 (U.S. EPA, 1996b), and OCSPP 860.1000 (U.S. EPA, 1996c) as well as satisfies OECD guidance document ENV/JM/MONO(2007)17 (OECD, 2007) to confirm that the original analytical method, developed by one laboratory, can be independently validated by a second laboratory. This analytical method was validated by fortification of two water types with Benzobicyclon and metabolites at the limit of quantification (LOQ, 1.00 µg/L) and 10 × LOQ (10.0 µg/L) concentration levels.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

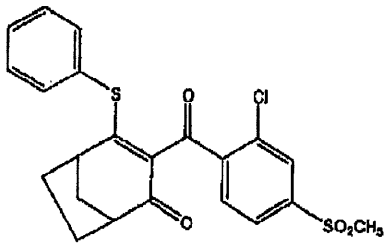
This study was performed following the Smithers Viscient protocol entitled "Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Benzobicyclon and its Metabolites 1315P-070, 1315P-076, 1315P-570, 1315P-683, 1315P-960, 1315P-962, and 1315P-966 in Water by LC-MS/MS" (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA, 2012), OCSPP Guideline 850.7100 (U.S. EPA, 1996a), SANCO/3029/99 rev. 4 (EC, 2000), SANCO/825/00 rev. 8.1 (EC, 2010), and OSCPP Guideline 860.1340: Residue Analytical Method (U.S. EPA, 1996b).

2.2 Test Substances

The test substance, Benzobicyclon, was received on 10 January 2017 from EAG Laboratories, Hercules, California. The following information was provided:

Name:	Benzobicyclon
Chemical Name:	3-[2-chloro-4-(methylsulfonyl)benzoyl]-4-(phenylthio)bicyclo [3.2.1] oct-3-en-2-one
Molecular Weight:	447.0 g/mole
Amount Received:	0.72 g
Lot No.:	1L0108
CAS No.:	156963-66-5
Purity:	99.3% (Certificate of Analysis, Appendix 2)
Expiration Date:	1 April 2018

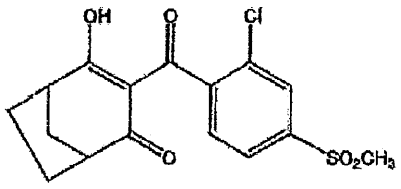
Chemical Structure:



Upon receipt at Smithers Viscient, the test substance (SMV No. 8696) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

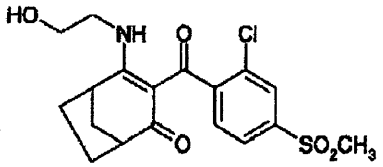
The test substance, 1315P-070, was received on 10 January 2017 from EAG Laboratories, Hercules, California. The following information was provided:

Name:	1315P-070
Synonym:	Benzobicyclon metabolite 1315P-070
Chemical Name:	3-[(2-Chloro-4-methylsulfonyl)benzoyl)]-bicyclo[3.2.1]octane-2,4-dione
Molecular Weight:	354.8 g/mole
Amount Received:	0.59 g
Lot No.:	95Z25
CAS No.:	126656-88-0
Purity:	98.8% (Certificate of Analysis, Appendix 2)
Recertification Date:	24 February 2018
Chemical Structure:	



Upon receipt at Smithers Viscient, the test substance (SMV No. 8697) was stored in a freezer in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-076, was received on 8 January 2016 from PTRL West, Hercules, California. The following information was provided:

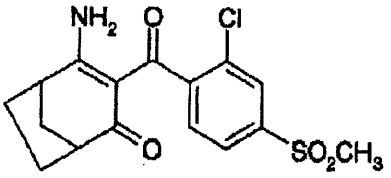
Name:	1315P-076
Synonym:	Benzobicyclon metabolite 1315P-076
Chemical Name:	(3-(2-chloro-4-(methylsulfonylbenzoyl)-4-(2-hydroxyethylamino))bicyclo[3.2.1]oct-3-en-2-one
Molecular Weight:	397.9 g/mole
Amount Received:	5 g
Lot No.:	TNA-10-074
CAS No.:	Not Listed
Purity:	99.5% (Certificate of Analysis, Appendix 2)
Expiration Date:	23 February 2019
Chemical Structure:	

Upon receipt at Smithers Viscient, the test substance (SMV No. 8028) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-570, was received on 8 January 2016 from PTRL West, Hercules, California. The following information was provided:

Name:	1315P-570
Synonym:	Benzobicyclon metabolite 1315P-570
Chemical Name:	(3-(2-Chloro-4-methylsulfonylbenzoyl)-4-amino)bicyclo[3.2.1]oct-3-en-2-one
Molecular Weight:	353.8 g/mole
Amount Received:	5 g
Lot No.:	TNA-9-186
CAS No.:	Not Listed
Purity:	99.8% (Certificate of Analysis, Appendix 2)
Recertification Date:	28 February 2019

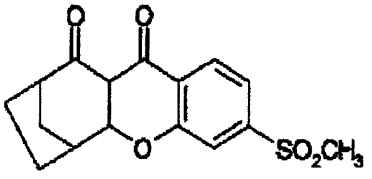
Chemical Structure:



Upon receipt at Smithers Viscient, the test substance (SMV No. 8029) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

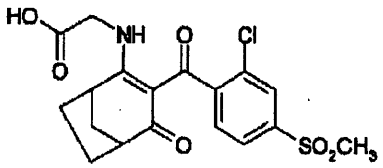
The test substance, 1315P-683, was received on 5 May 2016 from PTRL West, Hercules, California. The following information was provided:

Name:	1315P-683
Synonym:	Benzobicyclon metabolite 1315P-683
Chemical Name:	3,4-dihydro-2,4-ethylene-6-methylsulfonyl-1*H*-xanthene-1,9(2*H*)-dione
Molecular Weight:	318.35 g/mole
Amount Received:	4.50 g
Lot No.:	TM-8-198
CAS No.:	Not Listed
Purity:	99.77% (Certificate of Analysis, Appendix 2)
Recertification Date:	21 February 2019
Chemical Structure:	



Upon receipt at Smithers Viscient, the test substance (SMV No. 8256) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-960, was received on 2 February 2017 from EAG Laboratories, Hercules, California. The following information was provided:

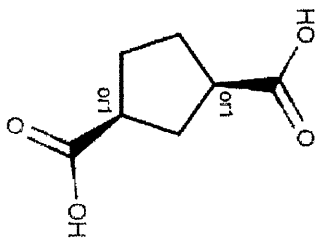
Name:	1315P-960
Synonym:	Benzobicyclon metabolite 1315P-960
Chemical Name:	4-(carboxymethylamino)-3-[2-chloro-4-(methylsulfonyl)benzoyl]bicyclo[3.2.1]oct-3-en-2-one
Molecular Weight:	411.86 g/mole
Amount Received:	0.5 g
Lot No.:	H/M-13-57-2
CAS No.:	Not Listed
Purity:	99.48% (Certificate of Analysis, Appendix 2)
Recertification Date:	24 March 2019
Chemical Structure:	

Upon receipt at Smithers Viscient, the test substance (SMV No. 8732) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-962, was received on 1 February 2017 from Gowan Company, Yuma, Arizona. The following information was provided:

Name:	1315P-962
Synonym:	Benzobicyclon metabolite 1315P-962
Chemical Name:	cis-1,3-Cyclopentanedicarboxylic acid
Molecular Weight:	158.16 g/mole
Amount Received:	1 g
Lot No.:	N21Q
CAS No.:	876-05-1
Purity:	99.63% (Certificate of Analysis, Appendix 2)
Recertification Date:	27 March 2019

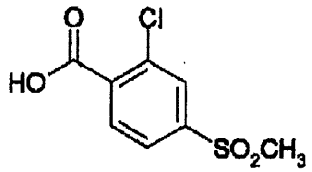
Chemical Structure:



Upon receipt at Smithers Viscient, the test substance (SMV No. 8731) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-966, was received on 5 May 2016 from PTRL West, Hercules, California. The following information was provided:

Name:	1315P-966
Synonym:	Benzobicyclon metabolite 1315P-966
Chemical Name:	2-Chloro-4-(methylsulfonyl)benzoic acid
Molecular Weight:	234.65 g/mole
Amount Received:	4.29 g
Lot No.:	H/M-12-55-1
CAS No.:	53250-83-2
Purity:	99.25% (Certificate of Analysis, Appendix 2)
Recertification Date:	20 February 2018
Chemical Structure:	



Upon receipt at Smithers Viscient, the test substance (SMV No. 8258) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substances, and archival of samples of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
2.	Formic acid:	BDH, reagent grade
3.	Methanol:	EMD, reagent grade
4.	Purified reagent water:	prepared from a Millipore Milli-Q Direct 8 system (meeting ASTM Type II requirements)
5.	Ultra-pure reagent water:	Fisher, reagent grade

2.4 Equipment

1.	Instruments:	AB Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACHT autoinjector Shimadzu DGU-20A3V vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.4.2 software for data acquisition
2.	Balance:	Mettler Toledo XSE205DU
3.	Laboratory equipment:	Volumetric flasks, disposable glass pipets, positive displacement pipets, stir bars, stir plates, vortexers, sonicator, 20-mL disposable glass vials, autosampler vials, and amber glass bottles with Teflon-lined caps

2.5 Test Systems

The test systems evaluated during this study were waters representative of the type of matrix this method was intended to analyze. The waters used for this ILV analysis were Ground water (in-house well water) and Surface water (Wewantic River, West Wareham, Massachusetts, Lot No. 17Oct16Wat-A-3). The in-house well water is unadulterated water from a 100-meter

bedrock well which is considered soft with a typical hardness of < 160 mg (as $CaCO_3$). The surface water was determined have a pH of 6.9 and a dissolved oxygen concentration of 9.3 mg/L (Agvise Laboratories, Northwood, North Dakota).

2.6 Preparation of Stock Solutions

Primary stock solutions were typically prepared as described in the table below. All volumes and masses may be scaled up or down as necessary.

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8696D	0.0504	0.0500	Acetonitrile	50.0	1000	Fortification stock solution
8697D	0.0508	0.0502		50.0	1000	Fortification stock solution
8028Q	0.0503	0.0500		50.0	1000	Fortification stock solution
8029Q	0.0501	0.0500		50.0	1000	Fortification stock solution
8256D	0.0502	0.0501		50.0	1000	Fortification stock solution
8732D	0.0503	0.0500		50.0	1000	Fortification stock solution
8731D	0.0503	0.0501		50.0	1000	Fortification stock solution
8258D	0.0506	0.0502		50.0	1000	Fortification stock solution

Fortification and intermediate stock solutions were typically prepared as described in the table below.

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (µg/L)	Stock Use
8696D	1000	0.500	50.0	Acetonitrile	Aq. Mixed Sol. 1	10,000	Fortification stock solutions
8697D	1000	0.500					
8028Q	1000	0.500					
8029Q	1000	0.500					
8256D	1000	0.500					
8732D	1000	0.500					
8731D	1000	0.500					
8258D	1000	0.500					
Aq. Mixed Sol. 1	10.0	0.500	50.0	30/70 Acetonitrile/purified reagent water (v/v)	Aq. Ana Sol. 2	100	Calibration standards for Benzobicyclon, 1315P-070, 1315P-076, 1315P-570, 1315P-683, and 1315P-960
Aq. Mixed Sol. 1	10.0	0.500	50.0	10/90 Acetonitrile/purified reagent water (v/v)	Aq. Ana Sol. 3	100	Calibration standards for 1315P-962, and 1315P-966
Aq. Mixed Sol. 1	10.0	5.00	50.0	Acetonitrile	Aq. Fort Sol. 4	1000	Fortification of High-level (10 × LOQ) recovery samples
Aq. Mixed Sol. 1	10.0	0.500	50.0	Acetonitrile	Aq. Fort Sol. 5	100	Fortification of LOQ-level recovery samples

All stock solutions were stored in a freezer (-25 to -10 °C) in amber glass bottles fitted with Teflon-lined caps.

2.7 Liquid Reagent Preparation

All volumes and masses may be scaled up or down as necessary.

A 10/90 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 100 mL of acetonitrile and 900 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 30/70 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 300 mL of acetonitrile and 700 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 0.2% formic acid in ultra-pure reagent water mobile phase solution was typically prepared by adding 4.00 mL of formic acid to 2000 mL of ultra-pure reagent water. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for ten minutes.

A 0.2% formic acid in acetonitrile mobile phase solution was typically prepared by adding 4.00 mL of formic acid to 2000 mL of acetonitrile. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for 10 minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water.

2.8 Preparation of Calibration Standards

Standards were prepared in the appropriate dilution solvent using the 100 µg/L stock solution according to the tables below. Following fortification, each solution was vortex-mixed for 15 seconds, then standards were transferred to amber glass bottles with Teflon-lined caps to be stored frozen prior to analysis.

For analysis of Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960:

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Aq. Ana Sol. 2	100	0.125	50.0	0.250	Pos - Std 1
		0.250	50.0	0.500	Pos - Std 2
		0.500	50.0	1.00	Pos - Std 3
		1.25	50.0	2.50	Pos - Std 4
		2.50	50.0	5.00	Pos - Std 5
		5.00	50.0	10.0	Pos - Std 6

[a] Dilution solvent = 30/70 acetonitrile/purified reagent water (v/v).

For analysis of 1315P-962 and 1315P-966:

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Aq. Ana Sol. 3	100	0.125	50.0	0.250	Neg - Std 1
		0.250	50.0	0.500	Neg - Std 2
		0.500	50.0	1.00	Neg - Std 3
		1.25	50.0	2.50	Neg - Std 4
		2.50	50.0	5.00	Neg - Std 5
		5.00	50.0	10.0	Neg - Std 6

[a] Dilution solvent = 10/90 acetonitrile/purified reagent water (v/v).

2.9 Sample Fortification and Preparation

Twelve aliquots of ground water (10.0 mL) were transferred to 20.0-mL disposable glass vials with PTFE-lined caps. Five replicates were dosed with the 100 µg/L aqueous fortification solution and five aliquots were dosed with the 1000 µg/L aqueous fortification solution to obtain concentrations of 1.00 and 10.0 µg/L (ppb), respectively. Two aliquots were left unfortified to serve as controls and an additional sample was extracted using only purified reagent water as a reagent blank. The dosing procedure is detailed in the following table.

Ground water:

Sample ID	Stock ID	Fortifying Stock Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Nominal Concentration (µg/L)
Reagent Blk 1-G	NA[a]	NA	NA	10.0[b]	0.00
Control 1-G & 2-G	NA	NA	NA	10.0	0.00
LOQ 1-G, 2-G, 3-G, 4-G, & 5-G	Aq. Fort. Sol. 5	100	0.100	10.0	1.00
High 1-G, 2-G, 3-G, 4-G, & 5-G	Aq. Fort. Sol. 4	1000	0.100	10.0	10.0

[a] NA = Not Applicable
[b] Purified reagent water is utilized for the reagent blank.

Twelve aliquots of surface water (10.0 mL) were transferred to 20.0-mL disposable glass vials with PTFE-lined caps. Five replicates were dosed with the 100 µg/L aqueous fortification solution and five aliquots were dosed with the 1000 µg/L aqueous fortification solution to obtain concentrations of 1.00 and 10.0 µg/L (ppb), respectively. Two aliquots were left unfortified to serve as controls and an additional sample was extracted using only purified reagent water as a reagent blank. The dosing procedure is detailed in the following table.

Surface water:

Sample ID	Stock ID	Fortifying Stock Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Nominal Concentration (µg/L)
Reagent Blk 1-S	NA[a]	NA	NA	10.0[b]	0.00
Control 1-S & 2-S	NA	NA	NA	10.0	0.00
LOQ 1-S, 2-S, 3-S, 4-S, & 5-S	Aq. Fort. Sol. 5	100	0.100	10.0	1.00
High 1-S, 2-S, 3-S, 4-S, & 5-S	Aq. Fort. Sol. 4	1000	0.100	10.0	10.0

[a] NA = Not Applicable
[b] Purified reagent water is utilized for the reagent blank.

2.10 Dilution of Fortified Recovery Samples

Fortified ground water recovery samples were mixed well by vortexing. After vortexing, samples were sub-sampled and diluted with acetonitrile. Samples were diluted in two different ways: one to yield a final diluent of 30/70 acetonitrile/purified reagent water (v/v) to be analyzed for Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960 and another to yield a final diluent of 10/90 acetonitrile/purified reagent water (v/v) to be analyzed for 1315P-962 and 1315P-966. High-level samples were further diluted with the appropriate solvent into the calibration curve range. Samples and calibration standards were transferred to clear autosampler vials for LC-MS/MS analysis.

Ground water samples for analysis of Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960 were prepared as described in the following table:

Sample ID	Nominal Concentration (µg/L)	Sample Volume (mL)	Sub-Sample Volume (mL)	Final Volume[a] (mL)	Sub-Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blk 1-G-P	0.00	10.0	3.50	5.00	NA[c]	NA	1.43
Control 1-G-P & 2-G-P	0.00	10.0	3.50	5.00	NA	NA	1.43
LOQ 1-G-P, 2-G-P, 3-G-P, 4-G-P, & 5-G-P	1.00	10.0	3.50	5.00	NA	NA	1.43
High 1-G-P, 2-G-P, 3-G-P, 4-G-P, & 5-G-P	10.0	10.0	3.50	5.00	1.00	10.0	14.3

[a] Diluted with acetonitrile.
[b] Diluted with 30/70 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

Ground water samples for analysis of 1315P-962 and 1315P-966 were prepared as described in the following table:

Sample ID	Nominal Concentration (µg/L)	Sample Volume (mL)	Sub-Sample Volume (mL)	Final Volume[a] (mL)	Sub-Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blk 1-G-N	0.00	10.0	4.50	5.00	NA[c]	NA	1.11
Control 1-G-N & 2-G-N	0.00	10.0	4.50	5.00	NA	NA	1.11
LOQ 1-G-N, 2-G-N, 3-G-N, 4-G-N, & 5-G-N	1.00	10.0	4.50	5.00	NA	NA	1.11
High 1-G-N, 2-G-N, 3-G-N, 4-G-N, & 5-G-N	10.0	10.0	4.50	5.00	1.00	10.0	11.1

[a] Diluted with acetonitrile.
[b] Diluted with 10/90 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

Fortified surface water recovery samples were mixed well by vortexing. After vortexing, samples were sub-sampled and diluted with acetonitrile. Samples were diluted in two different ways: one to yield a final diluent of 30/70 acetonitrile/purified reagent water (v/v) to be analyzed for Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960 and another to yield a final diluent of 10/90 acetonitrile/purified reagent water (v/v) to be analyzed for 1315P-962 and 1315P-966. High-level samples were further diluted with the appropriate solvent into the calibration curve range. Samples and calibration standards were transferred to clear autosampler vials for LC-MS/MS analysis.

Surface water samples for analysis of Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960 were prepared as described in the following table:

Sample ID	Nominal Concentration (µg/L)	Sample Volume (mL)	Sub-Sample Volume (mL)	Final Volume[a] (mL)	Sub-Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blk 1-S-P	0.00	10.0	3.50	5.00	NA[c]	NA	1.43
Control 1-S-P & 2-S-P	0.00	10.0	3.50	5.00	NA	NA	1.43
LOQ 1-S-P, 2-S-P, 3-S-P, 4-S-P, & 5-S-P	1.00	10.0	3.50	5.00	NA	NA	1.43
High 1-S-P, 2-S-P, 3-S-P, 4-S-P, & 5-S-P	10.0	10.0	3.50	5.00	1.00	10.0	14.3

[a] Diluted with acetonitrile.
[b] Diluted with 30/70 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

Surface water samples for analysis of 1315P-962 and 1315P-966 were prepared as described in the following table:

Sample ID	Nominal Concentration (µg/L)	Sample Volume (mL)	Sub-Sample Volume (mL)	Final Volume[a] (mL)	Sub-Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blk 1-S-N	0.00	10.0	4.50	5.00	NA[c]	NA	1.11
Control 1-S-N & 2-S-N	0.00	10.0	4.50	5.00	NA	NA	1.11
LOQ 1-S-N, 2-S-N, 3-S-N, 4-S-N, & 5-S-N	1.00	10.0	4.50	5.00	NA	NA	1.11
High 1-S-N, 2-S-N, 3-S-N, 4-S-N, & 5-S-N	10.0	10.0	4.50	5.00	1.00	10.0	11.1

[a] Diluted with acetonitrile.
[b] Diluted with 10/90 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

2.11 LC-MS/MS Instrumental Conditions

The LC-MS/MS analysis for Benzobicyclon, 1315P-970, 1315P-076, 1315P-570, 1315P-683, and 1315P-960 was conducted using the following instrumental conditions:

LC Parameters:

Column: Phenomenex Luna C18, 3 µm, 30 × 2 mm
Mobile Phase A: 0.2% formic acid in ultra-pure reagent water
Mobile Phase B: 0.2% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.500	90.0	10.0
7.00	0.500	5.00	95.0
8.00	0.500	5.00	95.0
8.10	0.500	90.0	10.0
9.50	0.500	90.0	10.0

Run time: 9.50 minutes
Injector Wash solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column temperature: 40 °C
Sample temperature: 5 °C
Injection volume: 10.0 µL
Approximate Retention Times:

Analyte	Retention Time (min)
Benzobicyclon	4.57
1315P-070	3.49
1315P-076	2.20
1315P-570	2.29
1315P-683	2.12
1315P-960	2.28

MS Parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Resolution Q1/Q3: Unit/Unit
Ion Spray Voltage: 5500 V
Scan type: MRM
Source Temperature: 550 °C
Curtain Gas: 20.00
Ion Source – Gas 1 / Gas 2: 80.00 / 80.00
Collision Gas: 12.00
Collision Cell Entrance Potential: 10.00
Declustering Potential: 100.00

Analyte	Q1/Q3 *m/z*	Dwell Time (msec)	Collision Energy	Collision Cell Exit Potential
Benzobicyclon	447.44/257.07 (Primary)	40.00	52.00	17.00
	447.4/229.10 (Confirmatory)	20.00	58.00	17.00
1315P-070	355.35/165.09 (Primary)	20.00	36.20	20.00
	355.35/183.09 (Confirmatory)	20.00	33.50	20.00
1315P-076	398.30/208.14 (Primary)	40.00	41.50	20.00
	398.30/319.10 (Confirmatory)	20.00	42.80	20.00
1315P-570	354.34/164.10 (Primary)	40.00	42.40	20.00
	354.34/318.10 (Confirmatory)	20.00	37.00	20.00
1315P-683	319.33/240.12 (Primary)	40.00	53.00	20.00
	319.33/212.10 (Confirmatory)	20.00	57.00	20.00
1315P-960	412.33/176.15 (Primary)	40.00	54.50	20.00
	412.33/222.12 (Confirmatory)	20.00	38.60	20.00

The LC-MS/MS analysis for 1315P-962 and 1315P-966 was conducted using the following instrumental conditions:

LC Parameters:

Column: Phenomenex Luna C18, 3 µm, 30 × 2 mm
Mobile Phase A: 0.2% formic acid in ultra-pure reagent water
Mobile Phase B: 0.2% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.500	100	0.0
7.00	0.500	5.00	95.0
8.00	0.500	5.00	95.0
8.10	0.500	100	0.0
9.50	0.500	100	0.0

Run time: 9.50 minutes
Injector Wash solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column temperature: 40 °C
Sample temperature: 5 °C
Injection volume: 20.0 µL
Approximate Retention Times:

Analyte	Retention Time (min)
1315P-962	2.02
1315P-966	2.16

MS Parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Negative (-) ESI

Resolution Q1/Q3:	Unit/Unit
Ion Spray Voltage:	-4500 V
Scan type:	MRM
Source Temperature:	500 °C
Curtain Gas:	20.00
Ion Source – Gas 1 / Gas 2:	60.00 / 40.00
Collision Gas:	3.00
Collision Cell Entrance Potential:	-10.00
Declustering Potential:	-30.00
Collision Cell Exit Potential:	-12.00

Analyte	Q1/Q3 *m/z*	Dwell Time (msec)	Collision Energy
1315P-962	157.1/113.1 (Primary)	200	-22.00
	157.1/71.0 (Confirmatory)	100	-35.00
1315P-966	233.1/189.0 (Primary)[a]	200	-13.00

[a] Please see Appendix 3 for sponsor confirmation that there is no confirmatory ion transition for this substance.

2.11.1 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; calibration standards were interspersed among analysis of the recovery samples, every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11.2 Method Differences

The analytical method used for Benzobicyclon and its metabolites in this independent laboratory validation followed the procedures described in the original method validation. The analytical method used for Benzobicyclon and its metabolites in this independent laboratory validation required the following minor modifications from the original method validation.

- Mass spectrometer parameters were optimized for sensitivity and linearity, as necessary.
- The validated method suggested bracketed standards with a curve check standard every two to six sample injections. In this study, acceptable results were obtained

when calibration curves were generated from calibration standards run interspersed among recovery samples.

- The primary transition for 1315P-966 utilized in this study was 233.1/189.0.
- Column temperature for this analysis was 40 °C.

2.12 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the LOQ and 10 × LOQ recovery samples. Recoveries of 70.0 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 LOQ and 10 × LOQ recovery samples. 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 time as Benzobicyclon and metabolites which might interfere with the quantitation of the analytes. Interferences with peak areas that are less than 50% of the LOQ are not considered significant. Linearity of the method was determined by the correlation coefficient (r), y-intercept, and slope of the regression line. A 1/x weighted linear regression was used for the LC-MS/MS analysis. The calibration curves were evaluated based on the correlation coefficient and the recoveries of the calibration standards. The signal response data should have an intercept close to zero and a correlation coefficient (r) not less than 0.995. The precision of the method at the LOQ was reported in terms of the coefficient of variation of the observed recovery values.

2.13 Communications

Communications occurred with the Study Monitor to discuss items including: approval of the protocol and method, challenges in regards to calibration curves, possible reasons for the failure during the first attempt of the ILV, and the results of the second attempt of the ILV.

A complete summary list of communications is provided in Appendix 3.

2.14 Time Required for Analysis

There were two water matrices investigated in this ILV. Each water matrix investigation included two sets of samples used for LC-MS/MS analysis. Each set of samples consisted of ten fortified, two unfortified samples, one reagent blank, and twelve calibration standards (25 samples total). A single analyst completed a set of 50 samples in one working day (8 hours) with LC-MS/MS analysis performed overnight (9 hours). Two calendar days were required for sample dilution through data analysis for one set of samples.

2.15 Critical Steps

With analyses organized around bracketed standards (calibration standards before and after a complete set of recovery samples), calibration curves for some analytes failed to meet acceptance criteria. Calibration curves generated from solvent-based standards interspersed among recovery samples met acceptance criteria and provided for quantification of analyte. Thus, it is recommended that the analysis be run with interspersed calibration standards.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis with 1/x weighting, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1)\quad y = mx + b$$

$$(2)\quad DC\,(x) = \frac{(y - b)}{m}$$

$$(3)\quad A = DC \times DF$$

where:

x	=	analyte concentration (µg/L)
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume (mL/g))
A	=	analytical result (µg/L), concentration in the original sample

NOTE: A 1/x weighting was used for calibration curves and sample quantitation using Analyst software, version 1.4.

The Instrument LOD was calculated using the following equation:

$$(4)\ LOD = (3 \times SN_{ctl})/Resp_{LS} \times Conc_{LS}$$

where:

SN_{ctl}	=	Mean signal to noise in height of the control samples (or blanks)
$Resp_{LS}$	=	Mean response in height of the two low calibration standards (0.250 µg/L)
$Conc_{LS}$	=	Concentration of the low calibration standard (µg/L)
LOD	=	Instrument Limit of Detection for the analysis (µg/L)

The Overall Method LOD was calculated using the following equation:

$$(5)\ LOD_{Overall} = LOD \times DF_{Ctl}$$

where:

LOD	=	Limit of Detection calculated from signal to noise ratio (µg/L)
DF_{Ctl}	=	Dilution factor for control sample
$LOD_{Overall}$	=	Overall Method Limit of Detection

APPENDIX 1 – STUDY PROTOCOL

Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Benzobicyclon and its Metabolites 1315P-070, 1315P-076, 1315P-570, 1315P-683, 1315P-960, 1315P-962, and 1315P-966 in Water by LC-MS/MS

1.0 INTRODUCTION

The purpose of this study is to confirm that an analytical method, developed by one group, can be independently validated by a second group in the absence of major interaction between the two. This study is required by EPA under Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [EPA 712-C-001], Guideline OCSPP 850.7100: Data Reporting for Environmental Chemistry Methods [EPA 712-C-96-348], and Guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174], as well as satisfies OECD guidance document ENV/JM/MONO(2007)17, EC guidance documents SANCO/3029/99 REV 4(2000) and SANCO/825/00 REV 8.1(2010). Independent labs are allowed to analyze three sample sets in order to validate the method as written. A complete set of samples should consist of, at a minimum, a reagent blank, two un-spiked matrix control samples, five matrix control samples fortified at the limit of quantification (LOQ), and five matrix control samples fortified at 10X LOQ for each distinct matrix. A complete set may include more than thirteen samples depending on the number of reagents, and un-fortified and fortified control matrix samples. It may be necessary, however, to divide a complete set into two subsets for efficient handling. Each subset should contain a reagent blank, two un-fortified matrix control samples, and five matrix control samples fortified at the LOQ or 10X LOQ.

If the performance data on the first set of samples at any of the required spiking levels is unsuccessful, the independent laboratory may contact the registrant to clarify the directions given in the method. Any contact with the registrant or developers during the method validation must be documented in writing in the final report submitted by the independent laboratory. If the independent laboratory cannot generate performance data that is similar to the registrant's or developers' after the second set of spiked samples, the independent laboratory may contact the registrant to further clarify the directions given in the method. If the independent laboratory cannot generate performance data that is similar to the registrant's or developers' after the third set, the method should be failed and a report will be sent to the registrant explaining why the method failed. The registrant should then decide whether to repeat the independent laboratory validation at another laboratory, further develop the method or withdraw it. This ILV trial will be conducted under FIFRA Good Laboratory Practice (GLP) standards as specified in 40 CFR part 160. A maximum of three sample sets are used by an independent laboratory to validate the method as written. A successful ILV trial will require adequate results on at least one complete set of samples on a given matrix.

The purpose of this protocol is to perform an ILV for the analytical method used to determine the test substance in ground and surface water (identified in the raw data and final report). The analytical method will be validated with regards to accuracy, precision, signal response, selectivity, and limits of quantitation.

2.0 OBJECTIVE

The objective of this study is to confirm that the analytical method for Benzobicyclon and its metabolites in water, developed by one group, can be independently validated by a second group in the absence of major interaction between the two.

3.0 JUSTIFICATION OF THE TEST SYSTEM

The method validations described in this protocol are designed to conform to EPA Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [EPA 712-C-001], Guideline OCSPP 850.7100: Data Reporting for Environmental Chemistry Methods [EPA 712-C-96-348], and Guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174].

4.0 MATERIALS

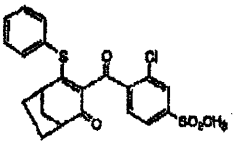
4.1 Test Substance

Upon arrival at Smithers Viscient, the test and reference substance(s) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

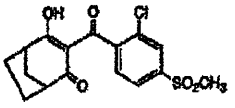
Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

4.1.1 Test Substance Information

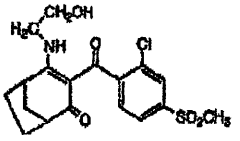
1. Name: Benzobicyclon
   Purity: 99.3%
   Batch or Lot #: 1L0108



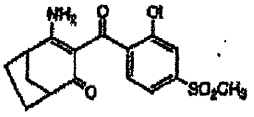
2. Name: 1315P-070
Purity: ~~99.7%~~ 98.8% updated per COA. KRB 14APR17
Batch or Lot #: 95Z25



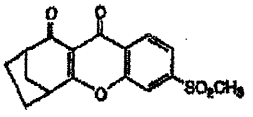
3. Name: 1315P-076
Purity: 99.5%
Batch or Lot #: TNA-10-074



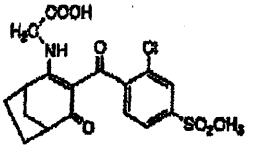
4. Name: 1315P-670
Purity: 99.8%
Batch or Lot #: TNA-9-186



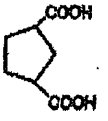
5. Name: 1315P-683
Purity: 99.77%
Batch or Lot #: TM-8-198



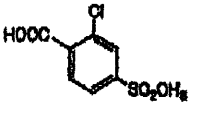
6. Name: 1315P-960
Purity: 99.48%
Batch or Lot #: H/M-13-57-2



7. Name: 1315P-962
Purity: ~~97%~~ 99.63% updated per COA. KRB 14Apr17
Batch or Lot #: N21Q



8. Name: 1315P-966
Purity: 99.25%
Batch or Lot #: H/M-12-55-1



5.0 TEST SYSTEM IDENTIFICATION

Test solution preparation will be documented on data forms which include the amount of test substance, the volume or mass of the test solution, lot, batch or other sample designation of the test substance and date the solution was prepared. Individual sample containers will be labeled with a unique ID number.

6.0 ANALYTICAL METHOD

The analytical method used for this ILV is, "Analytical Method for the Determination of Benzobicyclon and its Metabolites 1315P-070, 1315P-076, 1315P-570, 1315P-683, 1315P-960, 1315P-962, and 1315P-966 in Water by LC-MS/MS (M. Boatwright, GPL-MTH-087 Revision 2, effective July 29, 2015).

7.0 VALIDATION DESIGN

The standard curve will be comprised of at least five concentrations. The anticipated concentration range is 0.250-10.0 ppb. A smaller, larger, or shifted range may be necessary if achievable. The range will be documented in the study records and final report.

The limit of detection (LOD) will be established by evaluating the signal-to-noise (S/N) ratio from samples of known concentration and blank samples to establish the lowest level at which the analyte can be reliably detected. A S/N ratio of 3:1 is generally considered the minimum acceptable ratio for reliable detection.

7.1 Accuracy and Precision

The accuracy of the analytical method will be determined by applying the method to five samples of two water types (ground and surface) at the LOQ (1.0 ppb) and five samples at 10X LOQ (10.0 ppb) for each test substance. The accuracy will be reported in terms of percent recovery and the difference between the mean determined and the theoretical value. Recoveries of 70.0 to 120% of nominal are acceptable.

The precision will be calculated for the fortified samples in terms of the standard deviation (SD) and relative standard deviation (RSD or coefficient of variation (CV)) calculated for the retention time, peak area based quantitation (i.e., µg/L), and the observed recovery values. The retention time should have a RSD of less than or equal to 2%. The RSD of the peak area based quantitation (i.e., µg/L) should be less than or equal to 20%. The RSD of the recovery values should be less than or equal to 20% as well.

7.2 Specificity

The specificity of the method will be determined by applying the method to two un-fortified matrix control samples for each matrix. Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte peak of interest. Peaks attributable to test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. Interferences with peak areas that are less than 50% at the limit of detection (LOD) are not considered significant.

7.3 Signal Response

The signal response of the method will be determined by preparing a calibration curve with a minimum of five standards to encompass approximately 70.0 to 120% of the test concentration.

The calibration data will be subjected to a regression analysis; a plot of the analyte concentration versus the detector response will be included in the report along with the correlation coefficient, y-intercept, and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient (r) not less than 0.995 (r^2 not less than 0.990). The responses of the standards shall be inserted into the regression equation, and a calculated concentration value calculated. This calculated value shall be within ±20% of the theoretical value. Deviations from these criteria will be addressed by reevaluating the calibration range, such that the calculated values meet these criteria.