Study Title

Validation of the Analytical Method for the Determination of Dicamba Acid and DCSA Degradate in Ground Water and Surface Water by LC-MS/MS

Test Guidelines

OCSPP 850.6100 (2012)
SANCO/3029/99 rev. 4 (2000)

1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of dicamba acid and DCSA degradate in aqueous samples by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated (28 March to 2 April 2019) to quantify the concentrations of dicamba acid and DCSA present in recovery samples prepared in ground water and surface water. The analytical method was validated with regards to accuracy, precision, specificity, linearity, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in ground water and surface water by fortification with dicamba acid and DCSA at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Samples were acidified with hydrochloric acid, extracted three times with dichloromethane, concentrated, and reconstituted with 25/75 acetonitrile/purified reagent water (v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 8 March 2019, 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 validation was conducted on 28 March to 2 April 2019 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers Viscient's archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of the Analytical Method for the Determination of Dicamba Acid and DCSA degradate in Ground Water and Surface Water by LC-MS/MS" (Appendix 1). The study

was conducted under Good Laboratory Practice (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the SANCO/3029/99 rev. 4 guidance document (EC, 2000) and OCSPP 850.6100 guideline (U.S. EPA, 2012).

2.2 Test and Reference Substances

2.2.1 Test Substances

The test substance, dicamba acid, was received on 19 September 2018 from EAG Labs, Columbia, Missouri. The following information was provided:

Name:	dicamba acid
Lot No.:	DMBT01612B
CAS No.:	1918-00-9
Purity:	99.0% ± 0.4
Recertification Date:	8 September 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 9632) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples during testing.

The test substance, 3,6-dichloro-2-hydroxybenzoic acid, was received on 8 February 2019 from AK Scientific, Union City, California. The following information was provided:

Name:	3,6-dichloro-2-hydroxybenzoic acid
Synonym(s):	DCSA; 3,6-dichlorosalicylic acid
Lot No.:	60815CPU9
CAS No.:	3401-80-7
Purity:	≥95%
Retest Date:	11 February 2022

Upon receipt at Smithers Viscient, the test substance (SMV No. 9835) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples during testing.

2.2.2 Reference Substances

The reference substance, dicamba, was received on 12 February 2019 from Chem Service, West Chester, Pennsylvania. The following information was provided:

Name:	dicamba
Lot No.:	7996600
CAS No.:	1918-00-9
Purity:	99.5%
Expiration Date:	30 September 2022

Upon receipt at Smithers Viscient, the reference substance (SMV No. 9847) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the reference substance. This sample of reference substance was used to prepare the calibration standards during analysis.

The reference substance, 3,6-dichloro-2-hydroxybenzoic acid, was received on 8 February 2019 from AK Scientific, Union City, California. The following information was provided:

Name:	3,6-dichloro-2-hydroxybenzoic acid
Synonym(s):	DCSA; 3,6-dichlorosalicylic acid
Lot No.:	60815C
CAS No.:	3401-80-7
Purity:	98.8%
Retest Date:	11 February 2022

Upon receipt at Smithers Viscient, the reference substance (SMV No. 9836) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted

for the purity of the reference substance. This sample of reference substance was used to prepare the calibration standards during analysis.

Determination of stability and characterization, verification of the test and reference substance identities, maintenance of records on the test and reference substances, and archival of a sample of the test and reference substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
2.	Dichloromethane:	EMD, reagent grade
3.	12 N hydrochloric acid:	EMD, reagent grade
4.	Methanol:	EMD, reagent grade
5.	0.1% formic acid in water:	Fisher, reagent grade
6.	0.1% formic acid in acetonitrile:	Fisher, reagent grade
7.	Purified reagent water:	Prepared from a Millipore MilliQ Direct 8 water purification system (meets ASTM Type II requirements)

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACHT autosampler Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balance:	Mettler Toledo XSE205DU
3.	pH meter:	YSI Ecosense pH100A
4.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, Teflon centrifuge tubes, graduated cylinders, Whatman filter paper, Pasteur pipets, autosampler vials, and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used in future testing but may require optimization to achieve the desired separation and sensitivity.

2.5 Test Matrices

The matrices used during this method validation were ground water and surface water.

Ground water information:

The ground water used in the study was filtered well water collected on site at Smithers Viscient, located in Wareham, Massachusetts. The ground water was filtered to remove any potential organic contaminants. All documentation relating to the preparation, storage, and handling of the ground water is maintained by Smithers Viscient.

Surface water information:

The surface water used for this study was collected from Taunton river in Massachusetts (SMV Lot No. 03Jan19 Wat-C, collected on 3 January 2019). The water was collected from an area of the river with approximately 30 to 60 cm depth of overlying water and was determined to have a pH of 5.96 (measured using a Yellow Springs Instrument YSI, pH100 pH meter) and dissolved oxygen concentration of 10.3 mg/L (measured using a Thermo Scientific Orion Versa Star Pro water quality meter). All documentation relating to the preparation, storage, and handling of the surface water is maintained by Smithers Viscient.

2.6 Preparation of Liquid Reagent Solutions

The volumes listed in this section were those used during the validation. For future testing, the actual volumes used may be scaled up or down as necessary.

A 25/75 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 125 mL of acetonitrile and 375 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five 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. The solution was mixed well before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

Primary stock solutions were typically prepared as described in the table below:

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
Test Substances						
9632G (Dicamba)	0.02533	0.02508	Acetonitrile	25.0	1000	Secondary stock solution
9835-1A (DCSA)	0.02632	0.02500	Acetonitrile	25.0	1000	Secondary stock solution
Reference Substances						
9847-1A (Dicamba)	0.02513	0.02500	Acetonitrile	25.0	1000	Secondary stock solution
9836-1B (DCSA)	0.02530	0.02500	Acetonitrile	25.0	1000	Secondary stock solution

Secondary 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 (mg/L)	Stock Use
Test Substances							
9632G (Dicamba)	1000	0.500	50.0	Acetonitrile	9632G-1	10.0	Mixed sub-stock solution
9835-1A (DCSA)	1000	0.500	50.0	Acetonitrile	9835-1A-1	10.0	Mixed sub-stock solution
Reference Substances							
9847-1A (Dicamba)	1000	0.500	50.0	Acetonitrile	9847-1A-1	10.0	Mixed sub-stock solution
9836-1B (DCSA)	1000	0.500	50.0	Acetonitrile	9836-1B-1	10.0	Mixed sub-stock solution

Sub-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[a] (µg/L)	Stock Use
Test Substances							
9632G-1 (Dicamba)	10.0	0.200	10.0	Acetonitrile	Tech-Mix-Stk 1	200 / 200	10X LOQ-level recovery samples and mixed sub-stock solution
9835-1A-1 (DCSA)	10.0	0.200					
Tech-Mix-Stk 1	0.200[b]	1.00	10.0	Acetonitrile	Tech-Mix-Stk 2	20.0 / 20.0	LOQ-level recovery samples
	0.200[c]						
Reference Substances							
9847-1A-1 (Dicamba)	10.0	0.200	10.0	Acetonitrile	Ana-Mix-Stk (28-Mar-19)	200 / 200	Calibration standards (ground water validation)
9836-1B-1 (DCSA)	10.0	0.200					
9847-1A-1 (Dicamba)	10.0	2.00	10.0	Acetonitrile	Ana-Mix-Stk (1-Apr-19)	2000 / 2000	Calibration standards (surface water validation)
9836-1B-1 (DCSA)	10.0	2.00					

[a] Concentrations are expressed as dicamba acid / DCSA.
[b] Concentration as dicamba acid
[c] Concentration as DCSA

All primary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Tech-Mix-Stk sub-stock solutions were prepared fresh on the day of use and stored refrigerated for future use. Ana-Mix-Stk sub-stock solutions were prepared fresh on the day of use.

2.8 Preparation of Calibration Standards

2.8.1 Ground Water Validation - Calibration Standards

Calibration standards were prepared in 25/75 acetonitrile/purified reagent water (v/v) by dosing with the 200 µg/L mixed sub-stock solution to yield concentrations of 0.400, 0.600, 1.00, 2.00,

3.00, 4.00, and 5.00 µg/L. Calibration standard solutions were prepared according to the table below.

Fortifying Stock ID	Mixed Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Calibration Standard Concentration (µg/L)	Stock ID
Ana-Mix-Stk (28-Mar-19)	200	0.0200	10.0	0.400	Mix-Std 1
		0.0300	10.0	0.600	Mix-Std 2
		0.0500	10.0	1.00	Mix-Std 3
		0.100	10.0	2.00	Mix-Std 4
		0.150	10.0	3.00	Mix-Std 5
		0.200	10.0	4.00	Mix-Std 6
		0.250	10.0	5.00	Mix-Std 7

2.8.2 Surface Water Validation - Calibration Standards

Calibration standards were prepared as both solvent-based and matrix-matched calibration standards. This was done by first preparing spiking solutions in 25/75 acetonitrile/purified reagent water (v/v) by dosing with the 2000 µg/L mixed sub-stock solution to yield concentrations of 4.00, 6.00, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/L. Calibration standard spiking solutions were prepared according to the table below.

Fortifying Stock ID	Mixed Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Stock Concentration (µg/L)	Stock ID
Ana-Mix-Stk (1-Apr-19)	2000	0.0200	10.0	4.00	Mix-Std 1
		0.0300	10.0	6.00	Mix-Std 2
		0.0500	10.0	10.0	Mix-Std 3
		0.100	10.0	20.0	Mix-Std 4
		0.150	10.0	30.0	Mix-Std 5
		0.200	10.0	40.0	Mix-Std 6
		0.250	10.0	50.0	Mix-Std 7

The mixed standard spiking solutions were then used to fortify matrix-matched and solvent-based calibrations standards according to the tables below.

Matrix-Matched Calibration Standards

Stock ID	Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Calibration Standard Concentration (µg/L)	Sample ID
Mix-Std 1	4.00	0.250	2.50	0.400	SW-MM-Std 1
Mix-Std 2	6.00	0.250	2.50	0.600	SW-MM-Std 2
Mix-Std 3	10.0	0.250	2.50	1.00	SW-MM-Std 3
Mix-Std 4	20.0	0.250	2.50	2.00	SW-MM-Std 4
Mix-Std 5	30.0	0.250	2.50	3.00	SW-MM-Std 5
Mix-Std 6	40.0	0.250	2.50	4.00	SW-MM-Std 6
Mix-Std 7	50.0	0.250	2.50	5.00	SW-MM-Std 7

[a] Diluted with matrix blank samples 14166-6104-MB4B, MB5B, MB6B, MB7B, MB8B, MB9B, and MB10B.

Solvent-Based Calibration Standards

Stock ID	Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Calibration Standard Concentration (µg/L)	Sample ID
Mix-Std 1	4.00	0.250	2.50	0.400	SW-Sol-Std 1
Mix-Std 2	6.00	0.250	2.50	0.600	SW-Sol-Std 2
Mix-Std 3	10.0	0.250	2.50	1.00	SW-Sol-Std 3
Mix-Std 4	20.0	0.250	2.50	2.00	SW-Sol-Std 4
Mix-Std 5	30.0	0.250	2.50	3.00	SW-Sol-Std 5
Mix-Std 6	40.0	0.250	2.50	4.00	SW-Sol-Std 6
Mix-Std 7	50.0	0.250	2.50	5.00	SW-Sol-Std 7

[a] Diluted with 25/75 acetonitrile/purified reagent water (v/v)

2.9 Matrix Effect Investigation

2.9.1 Ground Water

The effects of matrix enhancement or suppression in ground water were evaluated through the assessment of matrix-matched and solvent-based standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate in matrix blank samples ( ) and 25/75 acetonitrile/purified reagent water (v/v) by fortifying with the 200 µg/L mixed sub-stock solution to yield a concentration of 2.00 µg/L.

Sample ID	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
GW MM-Std A1, B1, & C1	Matrix-matched standard	200	0.0250	2.50[a]	2.00
Sol-Std A1, B1, & C1	Solvent Standard	200	0.0250	2.50[b]	2.00

[a] Diluted with matrix bank samples 14166-6104-MB1A, MB2A, and MB3A
[b] Diluted with 25/75 acetonitrile/purified reagent water (v/v)

2.9.2 Surface Water

During method development, it was observed a matrix interference peak was present for surface water. Therefore, effects of matrix enhancement or suppression in surface water were evaluated through the assessment of matrix-matched and solvent-based calibration standards as described in Section 2.8.2 above.

2.10 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (ground water and surface water) with dicamba acid and DCSA at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Recovery samples for both matrices were prepared separately ("de novo") at these concentrations. Thirteen 50-mL aliquots of each control matrix were transferred into individual separatory funnels and fortified according to table below. Five replicates were prepared for each concentration level. Two samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, a reagent blank was prepared and processed in the same manner as the control samples. Additional samples (three for ground water and seven for surface water) were prepared to serve as matrix blanks to prepare matrix effects or matrix-matched standards. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water recovery samples

Sample ID 14166-6104-	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
1A	Reagent Blank	NA[a]	NA	NA	0.00
2A & 3A	Control	NA	NA	50.0	0.00
MB1A	Matrix Blank	NA	NA	50.0	0.00
MB2A	Matrix Blank	NA	NA	50.0	0.00
MB3A	Matrix Blank	NA	NA	50.0	0.00
4A, 5A, 6A, 7A, & 8A	LOQ	20.0	0.250	50.0	0.100
9A, 10A, 11A, 12A, & 13A	10X LOQ	200	0.250	50.0	1.00

[a] NA = Not Applicable; Matrix blank MB1A, MB2A and MB3A were prepared to assess matrix effects.

Surface water recovery samples

Sample ID 14166-6104-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
14B	Reagent Blank	NA[a]	NA	NA	0.00
15B & 16B	Control	NA	NA	50.0	0.00
MB4B	Matrix Blank	NA	NA	50.0	0.00
MB5B	Matrix Blank	NA	NA	50.0	0.00
MB6B	Matrix Blank	NA	NA	50.0	0.00
MB7B	Matrix Blank	NA	NA	50.0	0.00
MB8B	Matrix Blank	NA	NA	50.0	0.00
MB9B	Matrix Blank	NA	NA	50.0	0.00
MB10B	Matrix Blank	NA	NA	50.0	0.00
17B, 18B, 19B, 20B, & 21B	LOQ	20.0	0.250	50.0	0.100
22B, 23B, 24B, 25B, & 26B	10X LOQ	200	0.250	50.0	1.00

[a] NA = Not Applicable. Matrix blanks (MB4B through MB10B) were used to prepare matrix matched standards.

2.11 Extraction of Samples

Samples were first acidified by adding a 0.150- or 0.250-mL aliquot of 12 N hydrochloric acid to the ground and surface water samples, respectively, and shaken to mix well (critical step). The pH of the solution was then measured using a pH meter and confirmed to be pH 1.62 and 1.66 for ground water and surface water, respectively. Following acidification, the samples were extracted three times with 50.0-mL aliquots of dichloromethane by shaking vigorously for

one minute per extraction. The bottom layer (dichloromethane) was transferred into separate 250-mL round bottom flasks. The three extracts were combined and taken to low volume (approximately 3 mL) by rotary evaporation using minimal heating (30 °C). The concentrated extracts were then transferred to glass centrifuge tubes by rinsing the round bottom flasks with additional 3-mL aliquots of dichloromethane and transferred into corresponding glass centrifuge tubes. The samples were then evaporated to dryness under a gentle stream of nitrogen at room temperature. Samples were reconstituted with 25/75 acetonitrile/purified reagent water (v/v), vortex mixed, and sonicated for five minutes to mix well and aid in dissolution of the concentrated extract. The following table summarizes the extraction procedure for each sample.

Ground water recovery samples

Sample ID 14166-6104-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Extract Volume[a] (mL)	Final Volume[b] (mL)	Dilution Factor
1A	Reagent Blank	0.00	NA[c]	150	2.50	0.0500
2A & 3A	Control	0.00	50.0	150	2.50	0.0500
MB1A	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB2A	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB3A	Matrix Blank	0.00	50.0	150	2.50	0.0500
4A, 5A, 6A, 7A, & 8A	LOQ	0.100	50.0	150	2.50	0.0500
9A, 10A, 11A, 12A, & 13A	10X LOQ	1.00	50.0	150	12.5	0.250

[a] Extraction solvent: dichloromethane
[b] Reconstituted with 25/75 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable

Surface water recovery samples

Sample ID 14166-6104-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Extract Volume[a] (mL)	Final Volume[b] (mL)	Dilution Factor
14B	Reagent Blank	0.00	NA[c]	150	2.50	0.0500
15B & 16B	Control	0.00	50.0	150	2.50	0.0500
MB4B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB5B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB6B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB7B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB8B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB9B	Matrix Blank	0.00	50.0	150	2.50	0.0500
MB10B	Matrix Blank	0.00	50.0	150	2.50	0.0500
17B, 18B, 19B, 20B, & 21B	LOQ	0.100	50.0	150	2.50	0.0500
22B, 23B, 24B, 25B, & 26B	10X LOQ	1.00	50.0	150	12.5	0.250

[a] Extraction solvent: dichloromethane
[b] Reconstituted with 25/75 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable

2.12 Analysis

2.12.1 Instrumental Conditions

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

LC parameters:

Column: Agilent EC-C18 Poroshell 120, 100 × 3 mm, 2.7 µm
Mobile Phase A: 0.1% formic acid in reagent grade water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.20	0.500	75.0	25.0
5.50	0.500	5.00	95.0
7.00	0.500	5.00	95.0
7.01	0.500	75.0	25.0
9.00	0.500	75.0	25.0

Run Time: 9.0 minutes
Autosampler Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C

Injection Volume: 15.0 µL
Retention Times: approximately 3.6 minutes (dicamba acid)
approximately 3.2 minutes (DCSA)

MS parameters:

Instrument: AB MDS Sciex API 5000 mass spectrometer
Ionization Mode: Negative (-) ESI
Ion Spray Voltage: -4500 V
Scan Type: MRM
Dwell Time: 50.0 milliseconds
Source Temperature: 550 °C
Curtain Gas: 30.0
Ion Source – Gas 1 / Gas 2: 50.0 / 60.0
Collision Gas: 8.00
Collision Cell Entrance Potential: -10.0
Declustering Potential: -40.0
Resolution Q1/Q3: Low/Low

Analyte	Analysis	Q1/Q3 Masses (amu)	Collision Energy	Collision Cell Exit Potential
Dicamba acid	Primary	219.0/175.0	-12.0	-16.0
	Confirmatory	221.0/177.0	-12.0	-16.0
DCSA	Primary	205.0/161.0	-17.0	-21.0
	Confirmatory	205.0/125.0	-31.0	-15.0

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.12.2 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.

2.13 Evaluation of Accuracy, Precision, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 110% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples and retention times. RSD values less than 20% were considered acceptable for the recovery samples and RSD values less than 2% were considered acceptable for the retention times. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as dicamba acid and DCSA which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.14 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level (0.100 µg/L). Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ for surface water. One of the control replicates (Sample ID 14166-6104-2A) resulted in a concentration greater than the MDL for ground water. During method development, the control ground water sample was shown to be clean. Additionally, the reagent blank and the other control replicate (Sample ID 14166.6104-3A) were shown to be free of analyte, which indicated that contamination was not a result of the extraction process. Therefore, it was concluded as an isolated incident of contamination and does not impact the overall conclusion of the results.

2.15 Limit of Detection (LOD) and Method Detection Limit (MDL)

The LOD was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

The MDL was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Section 3.0.

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.
A 1/x-weighted linear regression was used to quantify the recovery samples. The equation for the line including the slope and intercept was generated using Analyst version 1.6.3 software (Sciex vendor software). 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, 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.

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

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

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

where:

x	=	analyte concentration
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)
A	=	analytical result (µg/L), concentration in the original sample

The MDL is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (Equation 4) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(4) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.400 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 0.0500)
MDL	=	method detection limit reported for the analysis (i.e., 0.400 µg/L × 0.0500 = 0.0200 µg/L)

The LOD was calculated using the following equation:

$$(5) \qquad LOD = ((3\times(N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 0.0500)
LOD	=	limit of detection for the analysis

5.0 VALIDITY CRITERIA

The method validation for dicamba acid in ground water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	Matrix-matched and solvent-based calibration standards were prepared and analyzed with the recovery samples. The matrix effect was <20% for ground water, therefore no significant matrix effect was observed and solvent-based calibration standards were used for quantitation.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.004 µg/L	0.008 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 219.0/175.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 221.0/177.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for DCSA in ground water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	Confirmatory
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	Matrix-matched and solvent-based calibration standards were prepared and analyzed with the recovery samples. The matrix effect was <20% for ground water, therefore no significant matrix effect was observed and solvent-based calibration standards were used for quantitation.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L: 10X LOQ, 1.00 µg/L:	LOQ, 0.100 µg/L: 10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L: 10X LOQ, 1.00 µg/L:	LOQ, 0.100 µg/L: 10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L) with the exception of one control replicate, which resulted in a concentration greater than the MDL. Please see Section 2.14 for additional discussion.	All blank sample values were <30% of the LOQ (0.100 µg/L) with the exception of one control replicate, which resulted in a concentration greater than the MDL. Please see Section 2.14 for additional discussion.
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.004 µg/L	0.004 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 205.0/161.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 205.0/125.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for dicamba acid in surface water utilizing matrix-matched standards met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	Matrix-matched standards were prepared and analyzed with the recovery samples. Results generated with both matrix-matched and solvent-based calibration standards met acceptance criteria and were not significantly different. This indicates that there are likely no matrix effects for surface water.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.008 µg/L	0.01 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 219.0/175.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 221.0/177.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for dicamba acid in surface water utilizing solvent-based standards met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	Solvent-based calibration standards were prepared and analyzed with the recovery samples. Results generated with both matrix-matched and solvent-based calibration standards met acceptance criteria and were not significantly different. This indicates that there are likely no matrix effects for surface water.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.009 µg/L	0.01 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 219.0/175.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 221.0/177.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for DCSA in surface water utilizing matrix-matched standards met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
Linearity: Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards.	Matrix-matched calibration standards were prepared and analyzed with the recovery samples. Results generated with both matrix-matched and solvent-based calibration standards met acceptance criteria and were not significantly different. This indicates that there are likely no matrix effects for surface water.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.006 µg/L	0.002 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 205.0/161.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 205.0/125.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for DCSA in surface water utilizing solvent-based standards met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Coefficient of Determination	The data should have a coefficient of determination (r^2) of not less than 0.990.		
		analyzed with the recovery samples. Results generated with both matrix-matched and solvent-based calibration standards met acceptance criteria and were not significantly different. This indicates that there are likely no matrix effects for surface water.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.100 µg/L:	LOQ, 0.100 µg/L:
		10X LOQ, 1.00 µg/L:	10X LOQ, 1.00 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analyzed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ.	All blank sample values were <30% of the LOQ (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.004 µg/L	0.002 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.0200 µg/L	0.0200 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 205.0/161.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 205.0/125.0 amu Meets all method and guideline specifications outlined in this table.

APPENDIX 1 - STUDY PROTOCOL

Study No.: 14166.6104

Validation of the Analytical Method for the Determination of Dicamba Acid and DCSA degradate in Ground Water and Surface Water by LC-MS/MS

1.0 INTRODUCTION

The purpose of this study is to validate an analytical method used to determine the content of Dicamba acid and DCSA degradate in two aqueous matrices (ground water and surface water) by LC-MS/MS. The analytical method will be validated with regards to accuracy, precision, specificity, linearity, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of identification.

2.0 JUSTIFICATION OF THE TEST SYSTEM

This study is conducted to support the registration of the test substance.

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 and SANCO/3029/99 rev.4: Guidance for generating and reporting methods of analysis in support of pre-registration data. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as accepted by OECD principles of GLP (OECD, 1998).

3.0 TEST SUBSTANCE

3.1 Test Substances

Upon arrival at Smithers Viscient, the test substances (and the reference substances) 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 and reference substances 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 test and reference substance 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, SDS, and safe handling procedures, and a verified expiration or reanalysis date.

Study No.: 14166.6104

3.2 Reference Standards

Residues of Dicamba Acid and DCSA degradate will be determined in surface water and groundwater. Current available information on the analytes is summarized below.

Name:	Dicamba Acid
Batch/Lot No.:	7996600
CAS No.:	1918-00-9
Purity:	99.5%
Expiration Date:	30 September 2022

Name:	3,6-Dichloro-2-hydroxybenzoic acid
Synonym:	Dichlorosalicylic acid (DCSA)
Batch/Lot No.:	60815C
CAS No.:	3401-80-7
Purity:	98.8%
Expiration Date:	11 February 2022

3.3 Test Matrices

3.3.1 Ground Water

The ground water used in the study will be filtered well water collected on site at Smithers Viscient, Wareham, MA. This will be prepared by filtering to remove any potential organic contaminants. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.

3.3.2 Surface Water

The surface water used for the method validation will be collected from river water in Massachusetts. All documentation relating to the collection, preparation, storage and handling will be maintained by Smithers Viscient.

3.4 Reagents

Highly pure reagents will be used throughout the study. The actual reagent grade will be depending on the manufacturer's designation. Generally these reagents will have grades, such as high purity solvent, ACS grade, or Select. The reagents used are recorded along with test chemical information at the time of preparation.

Study No.: 14166.6104

4.0 VALIDATION DESIGN

The test design will consist of two aqueous matrices (ground water and surface water) fortified with each test substance at two concentrations with five replications at the target LOQ and five replicates at 10x LOQ level for each matrix. The procedural blank will be reagent blank without matrix. The control matrix for the validation will be untreated matrix representing ground water or surface water. The validation study levels (approximate concentrations) for each test substance are:

1.	Procedural blank-reagent blank	0.0 µg/L
2.	Matrix blank-control matrix	0.0 µg/L
3.	Control matrix fortified at LOQ	0.10 µg/L
4.	Control matrix fortified at 10 x LOQ	1.0 µg/L

4.1 Accuracy and Precision

The accuracy of the analytical method will be determined by applying the method to five samples at the LOQ and five samples at 10X LOQ for each test substance. Accuracy will be reported as the mean recovery at each fortification level. Mean recoveries in the range 70 – 110% of nominal concentrations of the target analyte in the fortified samples will be considered acceptable.

The precision will be calculated for the fortified samples in terms of the 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 at each fortification level (n = 5 per level). 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% per level. The RSD of the recovery values should be less than or equal to 20% per level as well.

4.2 Specificity

The specificity of the method will be determined by applying the method to the appropriate number of reagent blank (n=1) and control matrix samples (n=2). Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte(s) peak of interest. Peaks attributable to the test substance(s) should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. Blank values (including procedural blanks and untreated samples) should not exceed 30% of the LOQ. If this is exceeded, detailed justification is required. Unequivocal identification of the target analyte will be achieved by LC-MS/MS primary and confirmatory analysis.

4.3 Regression Analysis

Quantitative analysis will be achieved with the aid of a calibration curve. The calibration curve will be constructed using a minimum of five analytical standards and will extend over a range appropriate to the lowest and highest nominal concentrations of the target analyte in relevant analytical solutions ± at least 20%.

The calibration data will be subjected to regression analysis; a plot of analyte concentration versus detector response will be included in the report along with the correlation coefficient (r) and the equation describing the curve. The linearity of the detector response will be assessed according

to the strength of the correlation coefficient: this should be ≥ 0.995 (or coefficient of determination, r^2 ≥ 0.990). If non-linear calibration is used an explanation will be provided.

4.4 Limits of Quantitation (LOQ)

The method will be validated at the limit of quantitation (LOQ). This will be defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. If this is exceeded, it will be discussed with the Sponsor and detailed justification provided.

4.5 Limits of Detection (LOD)

The Limits of Detection (LOD) will be calculated using three times the signal-to-noise value of the control samples. The method detection limit (MDL) will be set at the lowest concentration that can be detected in sample test solutions. The value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.

4.6 Matrix Effects Determination

Determination of LC/MS-MS matrix effects will be evaluated through the assessment of solvent-based and matrix-matched calibration standards for both primary and confirmatory transitions. Matrix effects should be evaluated at the LOQ level for each test substance. Only if experiments clearly demonstrate that matrix effects are not significant (i.e. <20%), calibration with standards in solvent may be used.

4.7 Confirmatory Analyses

Unequivocal identification of the target analytes will be achieved by LC-MS/MS using a primary quantitation ion and secondary quantitation/confirmatory ion. All of the required elements need to be met for this confirmatory method with full method validation results generated for both transitions. For triple-quad MS methods, the confirmation method would be where a confirmatory (secondary) product ion will be used for quantification. The confirmatory ion analysis will also adhere to the aforementioned method specifications (Sections 4.1 - 4.6 above)

5.0 PROCEDURE FOR THE INDENTIFICATION OF THE TEST SYSTEM

The test system will be defined as the fortified recovery samples. The fortified recovery samples will be labeled as defined in Section 4.0 and each sample replicate will be assigned a unique identifier. Processing of fortified recovery samples will be performed at a lab station labeled with the study number.

6.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples and replicate analysis.