Study Title

Validation of the Analytical Method for the Determination of Dicamba Acid and DCSA Degradate in Soil 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 soil samples by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated (18 to 26 March 2019) to quantify the concentrations of dicamba acid and DCSA present in recovery samples prepared in sandy loam soil and loamy sand soil. 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 sandy loam soil and loamy sand soil by fortification with dicamba acid and DCSA at concentrations of 0.0500 (LOQ) and 0.500 (10X LOQ) mg/kg. Samples were extracted three times with 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v). The recovery samples were further diluted into the calibration range 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 from 18 to 26 March 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 Soil 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 calibration standards during testing.

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 calibration standards during testing.

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.	12 N hydrochloric acid:	EMD, reagent grade
3.	Methanol:	EMD reagent grade
4.	0.1% formic acid in water:	Fisher, reagent grade
5.	0.1% formic acid in acetonitrile:	Fisher, reagent grade
6.	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:	AB MDS Sciex 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 binary pumps Shimadzu CTO-20A column oven Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo Top Loader PG-2002-S; Mettler Toledo XSE205DU
3.	Shaker table:	VWR Standard Analog 3500STD
4.	Centrifuge:	Thermo Scientific Sorvall Legend XFR
5.	Moisture balance:	Mettler Toledo HB43-S

6. Laboratory equipment: Positive displacement pipets, graduated cylinders, volumetric flasks, disposable glass pipets, stir bars, stir plate, vortex mixer, 50-mL Nalgene centrifuge tubes, amber bottles, clear vials with snap caps, amber vials with crimp caps, 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 sandy loam soil and loamy sand soil.

Characterization of the sandy loam soil and loamy sand soil was performed by Agvise Laboratories, Northwood, North Dakota.

Parameter	Sandy Loam Soil	Loamy Sand Soil
Smithers Viscient Batch No.:	24Oct18Soil-A	05Oct18Soil-D
Collection location:	Grand Forks, ND	Grand Forks, ND
Percent organic matter:	3.7%	3.6%
USDA textural class:	Sandy loam	Loamy sand
Particle size distribution:	64% sand 17% silt 19% clay	83% sand 10% silt 7% clay
pH (1:1 matrix:water ratio):	6.6	6.9
Percent water holding capacity (at 1/3 bar):	23.6%	15.2%
Bulk Density (gm/cc):	1.05	1.10

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 0.1 N hydrochloric acid in purified reagent water liquid reagent solution was typically prepared by adding 1.66 mL of hydrochloric acid (12 N) to 100 mL of purified reagent water and

adjusting to final volume of 200 mL with purified reagent water. The solution was mixed well using a vortex mixer.

A 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v) liquid reagent solution was typically prepared by adding 200 mL of 0.1 N hydrochloric acid solution to 800 mL of acetonitrile. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 25/75 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by adding 50.0 mL of acetonitrile to 150 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 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						
9632F (Dicamba)	0.02530	0.02505	Acetonitrile	25.0	1000	Sub-stock solution (loamy sand soil)
9632G (Dicamba)	0.02533	0.02508	Acetonitrile	25.0	1000	Sub-stock solution (sandy loam soil)
9835-1A (DCSA)	0.02632	0.02500	Acetonitrile	25.0	1000	Sub-stock solutions
Reference Substances						
9836-1A (DCSA)	0.02536	0.02506	Acetonitrile	25.0	1000	Sub-stock solution (loamy sand soil)
9836-1B (DCSA)	0.02530	0.02500	Acetonitrile	25.0	1000	Sub-stock solution (sandy loam soil)
9847-1A (Dicamba)	0.02513	0.02500	Acetonitrile	25.0	1000	Sub-stock solutions

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] (mg/L)	Stock Use
Test Substances							
9632F (Dicamba)	1000	0.200	10.0	Acetonitrile	Tech-Mix-Stk 1 (18 Mar 19)	20.0 / 20.0	10X LOQ-level recovery samples and sub-stock solution (loamy sand soil)
9835-1A (DCSA)	1000	0.200					
Tech-Mix-Stk 1	20.0	1.00	10.0	Acetonitrile	Tech-Mix-Stk 2 (18 Mar 19)	2.00 / 2.00	LOQ-level recovery samples (loamy sand soil)
9632G (Dicamba)	1000	0.200	10.0	Acetonitrile	Tech-Mix-Stk 1 (25 Mar 19)	20.0 / 20.0	10X LOQ-level recovery samples and sub-stock solution (sandy loam soil)
9835-1A (DCSA)	1000	0.200					
Tech-Mix-Stk 1	20.0	1.00	10.0	Acetonitrile	Tech-Mix-Stk 2 (25 Mar 19)	2.00 / 2.00	LOQ-level recovery samples (sandy loam soil)
Reference Substances							
9847-1A (Dicamba)	1000	0.100	50.0	Acetonitrile	Ana-Mix-Stk (18 Mar 19)	2.00 / 2.00	Calibration standards (loamy sand soil)
9836-1A (DCSA)	1000	0.100					
9847-1A (Dicamba)	1000	0.100	50.0	Acetonitrile	Ana-Mix-Stk (25 Mar 19)	2.00 / 2.00	Calibration standards (sandy loam soil)
9836-1B (DCSA)	1000	0.100					

[a] Concentrations are expressed as dicamba acid / 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 and discarded after use.

2.8 Preparation of Calibration Standards

Calibration standard spiking solutions were prepared in acetonitrile by dosing with the 2.00 mg/L Ana-Mix-Stk solution to yield concentrations of 2.00, 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 (mg/L)	Sample Volume (mL)	Final Volume (mL)	Stock Concentration (µg/L)	Stock ID
Ana-Mix-Stk	2.00	0.0200	20.0	2.00	Mix-Std 1
		0.0200	10.0	4.00	Mix-Std 2
		0.0300	10.0	6.00	Mix-Std 3
		0.0500	10.0	10.0	Mix-Std 4
		0.100	10.0	20.0	Mix-Std 5
		0.150	10.0	30.0	Mix-Std 6
		0.200	10.0	40.0	Mix-Std 7
		0.250	10.0	50.0	Mix-Std 8

Matrix-matched calibration standards were prepared using spiking solutions according to the table below in control blank final dilution (see Section 2.11). Following fortification, each solution was mixed using a vortex mixer for 15 seconds.

Sandy loam soil validation - matrix-matched calibration standards

Stock ID	Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Calibration Standard Concentration (µg/L)	Calibration Standard ID
Mix-Std 1	2.00	0.500	5.00	0.200	SL-Mix-Std 1
Mix-Std 2	4.00	0.500	5.00	0.400	SL-Mix-Std 2
Mix-Std 3	6.00	0.500	5.00	0.600	SL-Mix-Std 3
Mix-Std 4	10.0	0.500	5.00	1.00	SL-Mix-Std 4
Mix-Std 5	20.0	0.500	5.00	2.00	SL-Mix-Std 5
Mix-Std 6	30.0	0.500	5.00	3.00	SL-Mix-Std 6
Mix-Std 7	40.0	0.500	5.00	4.00	SL-Mix-Std 7
Mix-Std 8	50.0	0.500	5.00	5.00	SL-Mix-Std 8

[a] Diluted with the control blank 14166-6105-2A

Loamy sand soil validation - matrix-matched calibration standards

Stock ID	Stock Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Calibration Standard Concentration (µg/L)	Calibration Standard ID
Mix-Std 1	2.00	0.500	5.00	0.200	LS-Mix-Std 1
Mix-Std 2	4.00	0.500	5.00	0.400	LS-Mix-Std 2
Mix-Std 3	6.00	0.500	5.00	0.600	LS-Mix-Std 3
Mix-Std 4	10.0	0.500	5.00	1.00	LS-Mix-Std 4
Mix-Std 5	20.0	0.500	5.00	2.00	LS-Mix-Std 5
Mix-Std 6	30.0	0.500	5.00	3.00	LS-Mix-Std 6
Mix-Std 7	40.0	0.500	5.00	4.00	LS-Mix-Std 7
Mix-Std 8	50.0	0.500	5.00	5.00	LS-Mix-Std 8

[a] Diluted with the control blank 14166-6105-15A

2.9 Matrix Effect Investigation

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based calibration standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate in final control blank final dilution (see Section 2.11) and 25/75 acetonitrile/purified reagent water (v/v) by fortifying with the 10.0 µg/L Mix-Std-4 to yield a concentration of 1.00 µg/L.

Sandy loam soil validation

Sample ID	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
SL-MM-Std A1, B1, & C1	Matrix-matched calibration standard	10.0	0.500	5.00[a]	1.00
Sol-Std A1, B1, & C1	Solvent-based calibration standard	10.0	0.500	5.00[b]	1.00

[a] Diluted with the control matrix blank 14166-6105-2A
[b] Diluted with 25/75 acetonitrile/purified reagent water (v/v)

Loamy sand soil validation

Sample ID	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
LS-MM-Std D1, E1, & F1	Matrix-matched calibration standard	10.0	0.500	5.00[a]	1.00
Sol-Std D1, E1, & F1	Solvent-based calibration standard	10.0	0.500	5.00[b]	1.00

[a] Diluted with the control matrix blank 14166-6105-15A
[b] Diluted with 25/75 acetonitrile/purified reagent water (v/v)

2.10 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (sandy loam soil and loamy sand soil) by fortification with mixed stock solutions of dicamba acid and DCSA at concentrations of 0.0500 (LOQ) and 0.500 (10X LOQ) mg/kg. Recovery samples for both matrices were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples of each matrix were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared for each sample set and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Sandy loam soil recovery samples

Sample ID 14166-6105-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
1A	Reagent Blank	NA[a]	NA	NA	NA	0.00
2A & 3A	Control	NA	NA	11.7	10.0	0.00
4A, 5A, 6A, 7A, & 8A	LOQ	2.00	0.250	11.7	10.0	0.0500
9A, 10A, 11A, 12A, & 13A	10X LOQ	20.0	0.250	11.7	10.0	0.500

[a] NA = Not Applicable

Loamy sand soil recovery samples

Sample ID 14166-6105-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
14A	Reagent Blank	NA[a]	NA	NA	NA	0.00
15A & 16A	Control	NA	NA	11.2	10.0	0.00
17A, 18A, 19A, 20A, & 21A	LOQ	2.00	0.250	11.2	10.0	0.0500
22A, 23A, 24A, 25A, & 26A	10X LOQ	20.0	0.250	11.2	10.0	0.500

[a] NA = Not Applicable

2.11 Extraction of Samples

Samples were extracted three times with the extraction solvent, 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v). A 20-mL aliquot of 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v) was added to each soil recovery sample (10.0 g dry weight) and they were placed on a shaker table for 30 minutes at 200 rpm. Samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 100-mL volumetric flasks. The extraction and centrifugation procedures were repeated two more times with an additional 20-mL aliquot of 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v). The extracts were combined, taken to volume (100 mL) with 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v) and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 25/75 acetonitrile/purified reagent water (v/v). The extraction and dilution procedures for each separate matrix is outlined in the tables below.

Sandy loam soil recovery samples

Sample ID 14166-6105-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
1A	Reagent Blank	0.00	NA[c]	20.0	100	1.00	5.00	50.0
2A	Control	0.00	10.0	20.0	100	20.0	100[d]	50.0
3A	Control	0.00	10.0	20.0	100	1.00	5.00	50.0
4A, 5A, 6A, 7A, & 8A	LOQ	0.0500	10.0	20.0	100	1.00	5.00	50.0
9A, 10A, 11A, 12A, & 13A	10X LOQ	0.500	10.0	20.0	100	0.400	5.00[e]	125

[a] Extraction solvent: 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v)
[b] Dilution solvent: 25/75 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects and prepare matrix-matched calibration standards and dilution of 10X LOQ samples.
[e] Control matrix blank final dilution 14166-6105-2A

Loamy sand soil recovery samples

Sample ID 14166-6105-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume [a] (mL)	Final Volume [a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
14A	Reagent Blank	0.00	NA[c]	20.0	100	1.00	5.00	50.0
15A	Control	0.00	10.0	20.0	100	20.0	100[d]	50.0
16A	Control	0.00	10.0	20.0	100	1.00	5.00	50.0
17A, 18A, 19A, 20A, & 21A	LOQ	0.0500	10.0	20.0	100	1.00	5.00	50.0
22A, 23A, 24A, 25A, & 26A	10X LOQ	0.500	10.0	20.0	100	0.400	5.00[e]	125

[a] Extraction solvent: 4/1 acetonitrile/0.1 N hydrochloric acid solution (v/v)
[b] Dilution solvent: 25/75 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects and prepare matrix-matched calibration standards and dilution of 10X LOQ samples.
[e] Control matrix blank final dilution 14166-6105-15A

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 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/reagent grade water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 25.0 µL
Retention Times: approximately 3.6 minutes (dicamba acid)
approximately 3.1 minutes (DCSA)

MS parameters:

Instrument:	AB MDS Sciex 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
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 seven 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 LOQ. This was defined as the lowest fortification level (0.0500 mg/kg). Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

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 (mg/kg) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (mg/kg), 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 (0.200 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 50.0 mL/g)
MDL	=	method detection limit reported for the analysis (0.200 µg/L × 50.0 mL/g × 1 L/1000 mL = 0.0100 µg/g or mg/kg)

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., 50.0 mL/g)
LOD	=	limit of detection for the analysis

5.0 VALIDITY CRITERIA

The method validation for dicamba acid in sandy loam soil 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. 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 sandy loam soil.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 0.0500 and 0.500 mg/kg; 0.0500 mg/kg was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 (0.0500 mg/kg).	All blank sample values were <30% of the LOQ (0.0500 mg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.002 mg/kg	0.004 mg/kg
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.	0.0100 mg/kg	0.0100 mg/kg
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 sandy loam soil 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. 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 sandy loam soil.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 0.0500 and 0.500 mg/kg; 0.0500 mg/kg was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 (0.0500 mg/kg).	All blank sample values were <30% of the LOQ (0.0500 mg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.001 mg/kg	0.001 mg/kg
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.	0.0100 mg/kg	0.0100 mg/kg
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 loamy sand soil 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. 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 loamy sand soil.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 0.0500 and 0.500 mg/kg; 0.0500 mg/kg was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 (0.0500 mg/kg).	All blank sample values were <30% of the LOQ (0.0500 mg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.002 mg/kg	0.004 mg/kg
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.	0.0100 mg/kg	0.0100 mg/kg
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 loamy sand soil 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. 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 loamy sand soil.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 0.0500 and 0.500 mg/kg; 0.0500 mg/kg was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.0500 mg/kg:	LOQ, 0.0500 mg/kg:
		10X LOQ, 0.500 mg/kg:	10X LOQ, 0.500 mg/kg:
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 (0.0500 mg/kg).	All blank sample values were <30% of the LOQ (0.0500 mg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.0004 mg/kg	0.002 mg/kg
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.	0.0100 mg/kg	0.0100 mg/kg
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.