Final Report

Study Title	Independent Laboratory Validation of Analytical Method 14166.6105 for the Determination of Dicamba Acid and DCSA Degradate in Soil
Study Guideline(s)	OCSPP 850.6100 (2012) SANCO/3029/99 rev 4 (2000)

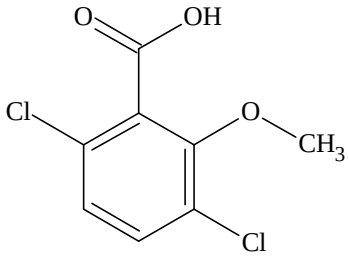
SMITHERS

MATERIALS AND METHODS

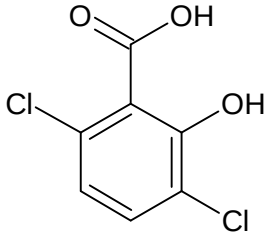
This study was conducted in accordance with the protocol with no deviations.

Throughout this report Dicamba acid is referred to as Dicamba, and DCSA degradate is referred to as DCSA.

Test Substances
The following test substances were used to fortify the samples, as per the analytical method validated by Smithers ERS, Wareham:

Test Substance Name:	**Dicamba (Technical)**
IUPAC Name:	3,6-Dichloro-2-methoxybenzoic acid
CAS Number:	1918-00-9
Structure:	
Molecular Formula:	$C_8H_6Cl_2O_3$
Molecular Weight:	221.04
Sponsor Lot Number:	DMBT01612B
Purity:	99.0%
Storage Conditions:	Room temperature (15-30°C)
Recertification Date:	08 September 2019[1]

[1] The retest date for Dicamba (Technical) was certified as 1 year after analysis, however the Dicamba reference substance had an expiry date 4 years after certification. Therefore purity of Dicamba (Technical) is unlikely to have significantly changed before the assigned 1 year recertification date and was judged to be suitable for use throughout this study (last used on 21 August 2019).

Test Substance Name:	**DCSA (Technical)**
Sponsor Lot Number:	60815CPU9
IUPAC Name:	3,6-Dichloro-2-hydroxybenzoic acid
CAS Number:	3401-80-7
Structure:	
Molecular Formula:	$C_7H_4Cl_2O_3$
Molecular Weight:	207.01
Purity:	$\geq$ 95%[1]
Storage Conditions:	Room temperature (15-30°C)
Recertification Date:	11 February 2022

[1] 95% was used for purity correction of primary stocks.

Reference Substances
The following reference substances were used to prepare calibration standards, as per the analytical method validated by Smithers ERS, Wareham:

Reference Substance Name:	**Dicamba**
Sponsor Lot Number:	7996600
Purity:	99.5%
Storage Conditions:	Room temperature (15-30°C)
Expiry Date:	30 September 2022

Reference Substance Name:	**DCSA**
Sponsor Lot Number:	60815C
Purity:	98.8%
Storage Conditions:	Room temperature (15-30°C)
Recertification Date:	11 February 2022

Test Matrices
Control sandy loam and silt loam soil were sourced by Smithers ERS, Harrogate. The soils used were CS 30/18 (sandy loam) and CS 17/18 (silt loam).

Soil characterisation data are listed in the following table:

Soil Name	Textural class[1]	% Sand, Silt, Clay[2]	CEC (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
ReferSol 01-A	sandy loam	74, 20, 6	5.3	0.9	6.4	5.3
Newhaven	silt loam	25, 51, 24	17.4	3.2	6.0	5.4

[1, 2] USDA classification.

Reagents
- Acetonitrile — HPLC grade, Fisher
- Water — Milli-Q with LCPAK polisher, In House
- Concentrated hydrochloric acid (37%) — ACS reagent, Sigma
- 0.1% Formic acid in water — MS grade, Honeywell
- 0.1% Formic acid in acetonitrile — MS grade, Honeywell

Equivalent reagents may have been used.

Equipment
- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.
- HPLC column: Agilent EC-C18 Poroshell 120, 100 × 3 mm, 2.7 µm
- Analytical balance
- Orbital shaker: Edmund Buhler SM 30 A
- Centrifuge: Beckman Coulter Allegra X-15R
- Nalgene centrifuge tubes
- Positive displacement pipettes
- Volumetric flasks
- Glass jars
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method
Analytical method 14166.6105 was supplied by Smithers ERS, Wareham on behalf of the sponsor. The method was re-written in Smithers ERS, Harrogate format as draft method SMV 3202424-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202424-02V when validation was complete. The complete analytical procedure is presented in Appendix 3.

Preparation of Reagents

0.1M hydrochloric acid in water (0.1M HCl)

1.7 mL concentrated hydrochloric acid was mixed with 200 mL Milli-Q water.

Acetonitrile: 0.1M hydrochloric acid (4:1 v/v)

800 mL acetonitrile was mixed with 200 mL 0.1M HCl.

Acetonitrile: water (25:75 v/v)

50 mL acetonitrile was mixed with 150 mL Milli-Q water

Reagents were stored at room temperature and given a nominal expiry date of one month.

Preparation of Stock Solutions

Primary Stock Solutions

Primary stock solutions of Dicamba and DCSA *test substance* (for sample fortification) were prepared under Smithers ERS, Harrogate GLP study no. 3202423 (Independent Laboratory Validation of Analytical Method 14166.6104 for the determination of Dicamba Acid and DCSA Degradate in Water) as described in the following table.

Stock ID	Test Substance	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Stock 1	Dicamba	12.91	99.0	Acetonitrile	12.781	1000
Stock 2		10.21			10.108	1000
Stock 5	DCSA	12.18	95[2]		11.571	1000
Stock 6		10.91			10.365	1000

[1] Corrected for Purity.

[2] Although the certified purity was ≥ 95%, 95% was used for purity correction.

Duplicate stocks were prepared for correlation purposes. Only stocks 1 and 5 were used in this study.

Primary stock solutions of Dicamba and DCSA *reference substance* (for calibration standard preparation) were prepared under Smithers ERS, Harrogate GLP study no. 3202423 as described in the following table:

Stock ID	Test Substance	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Stock 3	Dicamba	12.26	99.5	Acetonitrile	12.199	1000
Stock 4		11.99			11.930	1000
Stock 7	DCSA	12.50	98.8		12.351	1000
Stock 8		12.68			12.528	1000

[1] Corrected for Purity.

Duplicate stocks were prepared for correlation purposes. Only stocks 3 and 7 were used in this study.

Sub Stock Solutions
Sub stock solutions of Dicamba and DCSA *test substance* (for sample fortification) were prepared as described in the following table:

Test Substance	Fortifying Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Dicamba	1000	0.2	Acetonitrile	10	20
DCSA	1000	0.2			
Mixed	20	1		10	2

Sub-stock solutions of Dicamba and DCSA *reference substance* (for matrix standard and calibration standard preparation) were prepared as described in the following table:

Reference Substance	Fortifying Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Dicamba	1000	0.2	Acetonitrile	10	20
DCSA	1000	0.2			
Mixed	20	0.05		10	0.1[1]

[1] Equivalent to 100 µg/L.

Sub-stock solutions were prepared on the day of use and stored refrigerated until the corresponding analysis was complete.

Preparation of Matrix Matched Standards for Matrix Assessment
Matrix matched standards of Dicamba and DCSA *reference substance* were prepared in control soil final extract.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.05	Sandy loam soil final extract	5	1
100	0.05		5	1
100	0.05		5	1
100	0.05	Clay loam soil final extract	5	1
100	0.05		5	1
100	0.05		5	1

Preparation of Non-Matrix Matched Standards for Matrix Assessment
Non-matrix standards of Dicamba and DCSA *reference substance* were prepared in acetonitrile: water (25:75 v/v) for comparison with matrix matched standards.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.05	Acetonitrile: water (25:75 v/v)	5	1
100	0.05		5	1
100	0.05		5	1

The three matrix matched standards for each soil were analysed alternately with the three non-matrix matched standards and their peak areas compared.

Preparation of Calibration Standards

Mixed calibration standards of Dicamba and DCSA *reference substance* were prepared in as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.5	Acetonitrile: water (25:75 v/v)	10	5
5	0.8		1	4
5	0.6		1	3
5	0.4		1	2
5	0.2		1	1
5	0.12		1	0.6
5	0.08		1	0.4
5	0.04		1	0.2

A single set of calibration standards was prepared for each validation batch, which was analysed twice during the batch, interspersed with the samples.

Sample Fortification

10±0.05 g dry weight equivalent of soil was weighed into a Nalgene centrifuge tube. Quintuplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with a mixed stock solution of Dicamba and DCSA *test substance*. Duplicate control soil samples and a reagent blank (acetonitrile: water 25:75 v/v) were also prepared, as described in the following tables:

Sandy loam soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank A[1]	N/A	N/A	N/A	N/A
Control A[2]	10	N/A	N/A	N/A
Control C-D	10	N/A	N/A	N/A
F50 A-E	10	2	0.25	50
F500 A-E	10	20	0.25	500

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control A was used for the matrix assessment.

Silt loam soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank B[1]	N/A	N/A	N/A	N/A
Control B[2]	10	N/A	N/A	N/A
Control E-F	10	N/A	N/A	N/A
F50 F-J	10	2	0.25	50
F500 F-J	10	20	0.25	500

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control B was used for the matrix assessment.

Sample Extraction

The samples were extracted three times with 20 mL acetonitrile: 0.1M HCl (4:1 v/v) by shaking at 200 rpm for 30 minutes and centrifuging at 3000 rpm for 10 minutes. The supernatant was transferred into a glass jar and made to 100 mL with acetonitrile: 0.1M HCl (4:1 v/v). The sample extract was diluted into calibration range with acetonitrile: water (25:75 v/v) and transferred into an HPLC vial for analysis.
The extraction and dilution procedure is summarised in the following tables:

Sandy loam soil

Sample ID	Fortified Concentration (µg/kg)	Sample Weight (g)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank A	N/A	N/A	100	0.2-1	50
Control A	N/A	10	100	1-5[1]	50
Control C-D	N/A	10	100	0.2-1	50
F50 A-E	50	10	100	0.2-1	50
F500 A-E	500	10	100	0.05-1	200

N/A = Not applicable.
[1] Three aliquots of Control A final extract were used to prepare matrix matched standards for the matrix assessment.

Silt loam soil

Sample ID	Fortified Concentration (µg/kg)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank B	N/A	N/A	100	0.2-1	50
Control B	N/A	10	100	1-5[1]	50
Control E-F	N/A	10	100	0.2-1	50
F50 F-J	50	10	100	0.2-1	50
F500 F-J	500	10	100	0.05-1	200

N/A = Not applicable.
[1] Three aliquots of Control B final extract were used to prepare matrix matched standards for the matrix assessment.

Instrument Conditions

LC-MS/MS analysis was performed using the following instrument conditions:

LC Parameters:

Instrument	Shimadzu Nexera series HPLC system		
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		
Flow Rate	0.5 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	75	25
	0.20	75	25
	5.50	5	95
	7.00	5	95
	7.01	75	25
	9.00	75	25
Run Time	9.0 minutes		
Column Temperature	40°C		
Autosampler Temperature	5°C		
Injection Volume	25 µL		
Retention Time	Approx. 3.2 minutes (Dicamba) Approx. 2.6 minutes (DCSA)		
Valco Valve Diverter	Time (min)	Position	
	0	A (to waste)	
	1	B (to MS)	
	8	A (to waste)	

MS/MS Parameters:

Instrument	AB Sciex API 5000 Triple Quadrupole Mass Spectrometer			
Ionisation Type#	Electrospray (ESI)			
Polarity#	Negative			
Scan Type#	Multiple reaction monitoring (MRM)			
Resolution Q1:	Low			
Resolution Q3:	Low			
Ion Spray Voltage	-4500V			
Collision Gas (CAD)	5			
Curtain Gas (CUR)	30			
Gas Flow 1 (GS1)	40			
Gas Flow 2 (GS2)	40			
Vaporiser Temperature (TEM)	400°C			
Interface Heater (ihe)	On			
Entrance Potential (EP)	-10			
Collision Exit Potential (CXP)	-11			
Compound Name	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
Dicamba (Primary)	218.9/174.4	-70	-10	250
Dicamba (Confirmatory)	220.9/176.7	-63	-10	250
DCSA (Primary)	205.0/161.0	-40	-17	50
DCSA (Confirmatory)	205.0/125.0	-40	-31	50

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

LC-MS/MS data were collected and processed using Analyst 1.6.2.

Calculation of Results
When the calibration fit is linear as in this study, Analyst uses the following formula to calculate the concentration of test substance present in the sample:

$$x = \frac{(y - c)}{m} \times DF$$

Where:

x = concentration of test substance in sample extract (µg/L)
y = peak area due to test substance
c = *y* intercept on calibration graph
m = gradient of the calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$Recovery\ (\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/kg)
S = concentration added to fortified sample (µg/kg)

The Limit of Detection (LOD) based upon the sample concentration equivalent to three times the baseline noise of a control sample was calculated as follows:

LOD (µg/kg) = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (µg/L) / height of calibration standard peak

The Method Detection Limit (MDL) based upon the sample concentration equivalent to the lowest calibration standard was calculated as follows:

MDL (µg/kg) = lowest calibration standard (µg/L) × control sample dilution factor

Validation Pass Criteria
The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored for each compound:

Mean Recovery and Precision
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 110% and a % RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if no significant interferences at the retention time of Dicamba or DCSA were found in the control samples at > 30% of the LOQ.

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract concentration (after dilution). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

LOD (Limit of Detection) Assessment
An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions for both compounds.

MDL (Method Detection Limit)
The MDL was calculated as the initial sample concentration equivalent to the lowest calibration standard (based upon a lowest standard concentration of 0.2 µg/L and a dilution factor of 50).

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in acetonitrile: water (25:75 v/v) and in each control soil final extract. This was assessed for both compounds and for the primary and confirmatory transitions

Results were presented as a % difference from the mean non-matrix standard value.

A difference of ≥ 20% was considered significant.

If matrix effects were determined to be significant, matrix matched calibration standards would be used for method validation.

PERFORMANCE 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: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.995.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (<20% difference from non-matrix standards), therefore non-matrix calibration standards were used for method validation batches.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 50 µg/kg:	LOQ, 50 µg/kg:
		10×LOQ, 500 µg/kg:	10×LOQ, 500 µg/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 50 and 500 µg/kg; 50 µg/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, 50 µg/kg:	LOQ, 50 µg/kg:
		10×LOQ, 500 µg/kg:	10×LOQ, 500 µg/kg:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analysed 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 (50 µg/kg).	All blank sample values were <30% of the LOQ (50 µg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	2.53 µg/kg	4.77 µg/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.	10 µg/kg	10 µg/kg
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 218.9/174.4 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 220.9/176.7 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: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.995.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (<20% difference from non-matrix standards), therefore non-matrix calibration standards were used for method validation batches.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 50 and 500 µg/kg; 50 µg/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, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 (50 µg/kg).	All blank sample values were <30% of the LOQ (50 µg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	2.18 µg/kg	1.33 µg/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.	10 µg/kg	10 µg/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 in silt 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: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.995.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (<20% difference from non-matrix standards), therefore non-matrix calibration standards were used for method validation batches.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 50 and 500 µg/kg; 50 µg/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, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 (50 µg/kg).	All blank sample values were <30% of the LOQ (50 µg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	1.11 µg/kg	5.79 µg/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.	10 µg/kg	10 µg/kg
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 218.9/174.4 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 220.9/176.7 amu Meets all method and guideline specifications outlined in this table.

The method validation for DCSA in silt 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: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.995.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (<20% difference from non-matrix standards), therefore non-matrix calibration standards were used for method validation batches.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 50 and 500 µg/kg; 50 µg/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, 50 µg/kg: 10×LOQ, 500 µg/kg:	LOQ, 50 µg/kg: 10×LOQ, 500 µg/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 (50 µg/kg).	All blank sample values were <30% of the LOQ (50 µg/kg).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	1.59 µg/kg	1.38 µg/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.	10 µg/kg	10 µg/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.

Appendix 3
Analytical Procedure

Analytical Procedure

Procedure Title	Determination of Dicamba and DCSA in Soil by LC-MS/MS
Procedure Code	SMV 3202424-02V
Issue Date	24 October 2019
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The methodology described in this procedure has been validated in RefeSol 01-A (sandy loam) and Newhaven (silt loam) soil at 0.05 and 0.5 mg/kg.

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining concentrations of Dicamba and DCSA in soil by LC-MS/MS. Soil is extracted three times with acetonitrile: 0.1M hydrochloric acid (4:1 v/v) and diluted into calibration range with acetonitrile: water (25:75 v/v). The extracts are quantified by LC-MS/MS.

Matrix effects for Dicamba and DCSA in sandy loam and silt loam soil will be determined by comparing peak areas of calibration standards prepared in control soil final extract and in acetonitrile: water (22:75 v/v). Matrix effects are considered significant if the matrix matched standard area is ≥ 20% different from the non-matrix standard area. If matrix effects are significant, matrix matched calibration standards should be used.

Dicamba and DCSA *reference substance* should be used to prepare calibration standards and matrix matched standards.

Dicamba and DCSA *test substance* should be used for sample fortification.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.
- HPLC column: Agilent EC-C18 Poroshell 120, 100 × 3 mm, 2.7 µm
- Analytical balance
- Centrifuge: Beckman Coulter Allegra X-15R
- Orbital shaker: Edmund Buhler SM 30 A
- Nalgene centrifuge tubes
- Glass Jars
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Equivalent equipment may be used if required

Materials

• Acetonitrile	HPLC grade, VWR
• Water	Milli-Q (with LCPAK polisher)
• Concentrated hydrochloric acid (37%)	ACS reagent, Sigma
• 0.1% Formic acid in water	LC-MS grade, Honeywell
• 0.1% Formic acid in acetonitrile	LC-MS grade, Honeywell

Equivalent materials may be used if required

Reagents

0.1M hydrochloric acid in water (0.1M HCl)
Mix 1.7 mL concentrated hydrochloric acid with 200 mL Milli-Q water.

Acetonitrile: 0.1M hydrochloric acid (4:1 v/v)
Mix 200 mL 0.1M HCl with 800 mL acetonitrile.

Acetonitrile: water (25:75 v/v)
Mix 50 mL acetonitrile with 150 mL water.

Reagent volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stock
Separately prepare duplicate stock solutions of each Dicamba and DCSA *test substance* and *reference substance* at 1000 µg/mL in acetonitrile. Accurately weigh ≥ 10 mg *test/reference* substance, corrected for purity and transfer into a 10 mL volumetric flask. Adjust the volume to give exactly 1000 µg/mL. Transfer into amber glass bottles. The primary stocks should be stored refrigerated and given a nominal expiry date of 3 months.

Standard Correlation
Dilute the duplicate primary stocks to the mid-point of the calibration line. Correlate the standard solutions by injecting each of the two calibration standards 5 times into the LC-MS/MS. Ensure that the two solutions are injected alternately in the run sequence. The results for the correlation should be ± 5% of the overall mean calculated by peak areas.

Review of Results
Review the data and document the correlation calculations. If the correlation is out of specification, either repeat the injections, re-dilute, or prepare two new stock standards and repeat the procedures in sections <<*Initial Weighing of Stock Solutions*>> to <<*Review of Results*>>.

If the acceptance criteria from section <<*Standard Correlation*>> have been met, then the calibration solutions are acceptable for use. If required, fortification solutions for method validation will be made from the same stock standard, or its dilutions, from which the calibration line has been prepared.

Sub-Stocks
Prepare sub-stock solutions Dicamba and DCSA *test substance* as described in the following table:

Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub Stock Concentration (µg/mL)
Dicamba	1000	0.2	Acetonitrile	10	20
DCSA	1000	0.2			
Mixed	20	1		10	2

Prepare sub-stock solutions of Dicamba and DCSA *reference substance* as described in the following table:

Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub Stock Concentration (µg/mL)
Dicamba	1000	0.2	Acetonitrile	10	20
DCSA	1000	0.2			
Mixed	20	0.05		10	0.1[1]

[1] Equivalent to 100 µg/L.

Transfer into disposable glass vials. The sub-stock solutions should be prepared on the day of use.

Matrix Matched Standards for Matrix Assessment

Prepare mixed sandy loam and clay loam soil matrix matched standards of Dicamba and DCSA *reference substance* in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.05	Soil final extract	5	1
100	0.05		5	1
100	0.05		5	1

Non-Matrix Matched Standards for Matrix Assessment

Prepare mixed non-matrix matched standards of Dicamba and *DCSA reference substance* in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.05	Acetonitrile: water (25:75 v/v)	5	1
100	0.05		5	1
100	0.05		5	1

Calibration Standards

Prepare mixed calibration standards of Dicamba and DCSA *reference substance* as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.5	Acetonitrile: water (25:75 v/v)[1]	10	5
5	0.8		1	4
5	0.6		1	3
5	0.4		1	2
5	0.2		1	1
5	0.12		1	0.6
5	0.08		1	0.4
5	0.04		1	0.2

[1] If matrix effects are significant use soil final extract as the solvent.

A single set of calibration standards should be prepared for each validation batch and injected twice, interspersed with and bracketing the samples.

PROCEDURES

All procedures will be carried out in compliance with departmental SOPs, following departmental safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]
Weigh 10±0.05 g dry weight of either sandy loam or clay loam soil into a Nalgene centrifuge tube. Fortify with Dicamba and DCSA *test substance* using a mixed standard in acetonitrile as shown in the following table:

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Weight (g)	Fortified Concentration (mg/kg)
1	Reagent blank	N/A	N/A	N/A	N/A
2	Control	N/A	N/A	10	N/A
5	LOQ	2	0.25	10	0.05
5	10 × LOQ	20	0.25	10	0.5

N/A = Not Applicable.

Sample Extraction [1b, 4a]

1. Measure 10±0.05 g dry weight of soil into a Nalgene centrifuge tube.
2. Add 20 mL of acetonitrile: 0.1M HCl (4:1 v/v) to the soil.
3. Shake at 200 rpm for 30 minutes.
4. Centrifuge at 3000 rpm for 10 minutes.
5. Transfer the supernatant into a glass jar.
6. Repeat steps 2 to 5 twice more, combining the three extracts.
7. Make to 100 mL volume with acetonitrile: 0.1M HCl (4:1 v/v).
8. Dilute into calibration range with acetonitrile: water (25:75 v/v).
9. Transfer into an HPLC vial for analysis.

The recommended dilution procedure is given in the following table:

Sample type	Fortified Concentration (mg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank[1]	N/A	N/A	100	0.2-1	50
Control[2]	N/A	10	100	0.2-1	50
LOQ	0.05	10	100	0.2-1	50
10 × LOQ	0.5	10	100	0.05-1	200

N/A = Not Applicable.
[1] Use 0.1M HCl (4:1 v/v) with no soil as the reagent blank sample extract.
[2] Dilute additional control sample extract for matrix matched standards, if required.

LC-MS/MS CONDITIONS

HPLC Parameters:

Instrument:	Shimadzu Nexera series HPLC system
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
Flow Rate:	0.5 mL/min

Gradient:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	75	25
0.20	75	25
5.50	5	95
7.00	5	95
7.01	75	25
9.00	75	25

Run Time:	9.0 minutes
Column Temperature:	40°C
Autosampler Temperature:	5°C
Injection Volume:	25 µL
Retention Time:	Approx. 3.15 minutes (Dicamba) Approx. 2.65 minutes (DCSA)

Valco Valve Diverter:

Time (min)	Position
0	A (to waste)
1	B (to MS)
8	A (to waste)

MS/MS Parameters:

Instrument:	AB Sciex API 5000 Triple Quadrupole Mass Spectrometer
Ionisation Type#:	Electrospray (ESI)
Polarity#:	Negative
Scan Type#:	Multiple reaction monitoring (MRM)
Resolution Q1:	Low
Resolution Q3:	Low
Ion Spray Voltage:	-4500 V
Collision Gas (CAD):	5
Curtain Gas (CUR):	30
Gas Flow 1 (GS1):	40
Gas Flow 2 (GS2):	40
Vaporiser Temperature (TEM):	400°C
Interface Heater (ihe):	On
Entrance Potential (EP):	-10
Collision Exit Potential (CXP):	-11

Transition Name:	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
Dicamba (Primary):	218.9/174.4	-70	-10	250
Dicamba (Confirmatory):	220.9/176.7	-63	-10	250
DCSA (Primary):	205.0/161.0	-40	-17	50
DCSA (Confirmatory):	205.0/125.0	-40	-31	50

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

CALCULATION OF RESULTS

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where the calibration fit is linear as in this study, Analyst uses the following formula to calculate the concentration of test substance present in the sample extract.

$$x = \frac{(y - c)}{m} \times DF$$

Where:-

x = concentration of test substance in sample (µg/kg)
y = area of peak due to test substance
m = gradient
c = Y intercept on calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/kg)
S = concentration added to fortified sample (µg/kg)

METHOD CRITERIA

For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.995 with a 1/x weighting.
- All sample extracts will be within the appropriate range of calibration standards.
- Mean recovery from fortified samples will be considered acceptable within the range of 70 to 110% with a relative standard deviation (RSD) ≤ 20%.
- The control sample should not contain interference > 30% of the LOQ.

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main	Division	CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shield to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON – ensure antidote is available and is within its expiry date (must specify details)