MRID 50931310

Final Report

Study Title	Independent Laboratory Validation of Analytical Method 14166.6104 for the Determination of Dicamba Acid and DCSA Degradate in Water
Study Guideline(s)	OCSPP 850.6100 (2012) SANCO/3029/99 rev 4 (2000)

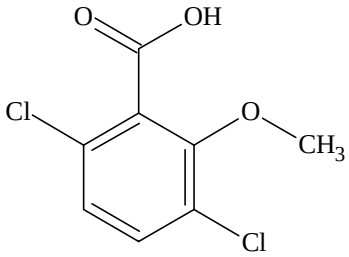
SMITHERS

MATERIALS AND METHODS

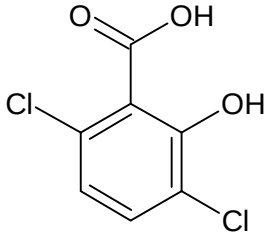
This study was conducted in accordance with the protocol with no deviations.

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 and matrix assessment, 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

Analytical Method

Analytical method 14166.6104 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 3202423-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202423-02V when validation was complete. The complete analytical procedure is presented in Appendix 3.

Preparation of Reagents

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

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months.

Secondary Stock Solutions
Secondary 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	0.1	Acetonitrile	10	10
DCSA	1000	0.1		10	10

Secondary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of one month.

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	10	0.2	Acetonitrile	10	0.2[1]
DCSA	10	0.2			
Mixed	0.2	1		10	0.02[2]

[1] Equivalent to 200 µg/L.
[2] Equivalent to 20 µg/L.

Sub-stock solutions of Dicamba and DCSA *reference substance* (for calibration standard preparation) 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	0.1		10	0.2[1]

[1] Equivalent to 200 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 water final extract.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
200	0.025	Surface water final extract	2.5	2
200	0.025		2.5	2
200	0.025		2.5	2
200	0.025	Ground water final extract	2.5	2
200	0.025		2.5	2
200	0.025		2.5	2

Preparation of Non-Matrix Matched Standards for Matrix Assessment
Non-matrix matched 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)
200	0.025	Acetonitrile: water (25:75 v/v)	2.5	2
200	0.025		2.5	2
200	0.025		2.5	2

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

Preparation of Calibration Standards

Non-matrix matched calibration standards of Dicamba and DCSA *reference substance* (for ground water validation) were prepared as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
200	0.25	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

Matrix matched calibration standards of Dicamba and DCSA *reference substance* (for surface water validation) were prepared as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
200	0.125	Surface water final extract	5	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

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

50 mL of water was measured into a glass separating funnel. Quintuplicate water samples were fortified at the LOQ (0.1 µg/L) and at 10 × LOQ (1 µg/L) with mixed stock solutions of Dicamba and DCSA *test substance*. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Control A, C & E[1]	50	N/A	N/A	N/A
Reagent Blank B[2]	50	N/A	N/A	N/A
Control I-P[3]	50	N/A	N/A	N/A
Control Q-R	50	N/A	N/A	N/A
F0.1 F-J	50	20	0.25	0.1
F1 F-J	50	200	0.25	1

N/A = Not applicable.
[1] Control A, C & E were used for the matrix assessment.
[2] Milli-Q water was used for the reagent blank.
[3] Control I-P were used for matrix matched calibration standards.

Borehole ground water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Control B, D & F[1]	50	N/A	N/A	N/A
Reagent Blank A[2]	50	N/A	N/A	N/A
Control G-H	50	N/A	N/A	N/A
F0.1 A-E	50	20	0.25	0.1
F1 A-E	50	200	0.25	1
Reagent Blank C[2]	50	N/A	N/A	N/A
Control S-T	50	N/A	N/A	N/A
F1 K-O	50	200	0.25	1

N/A = Not applicable.
[1] Control B, D & F were used for the matrix assessment.
[2] Milli-Q water was used for the reagent blanks.
The first validation attempt was unsuccessful at 1 µg/L (F1 A-E), so was repeated (F1 K-O) with a second set of controls (Control S-T) and a reagent blank (Reagent Blank C).

Sample Extraction

50 mL of water was measured into a glass separating funnel. 0.16 mL and 0.17 mL of concentrated hydrochloric acid was added to the ground water and surface water respectively and mixed, in order to achieve a pH in the range of 1.62 to 1.66 (the pH was checked using a control sample for each water). The water was extracted three times with 50 mL DCM by shaking vigorously for approximately 1 minute. The three DCM extracts were combined in a round bottom flask and evaporated to approximately 3 mL using a rotary evaporator set to 30°C. The 3 mL extract was transferred into a glass tube and the flask was rinsed using a further 3 mL of DCM, which was also transferred to the glass tube. The DCM was evaporated to dryness under a gentle stream of nitrogen, without heating. The extract was reconstituted with acetonitrile: water (25:75 v/v), according to the final volumes given in the following tables, then vortex-mixed and ultrasonicated for approximately 5 minutes.
The extraction procedure is summarised in the following tables:

Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Control A, C & E	N/A	50	2.5	N/A	0.05
Reagent Blank B	N/A	50	2.5	N/A	0.05
Control I-P	N/A	50	2.5	N/A	0.05
Control Q-R	N/A	50	2.5	N/A	0.05
F0.1 F-J	0.1	50	2.5	N/A	0.05
F1 F-J	1	50	2.5	0.2-1[1]	0.25

N/A = Not applicable.
[1] Sample dilutions were performed with control water extract (matrix matched).

Borehole ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Control B, D & F	N/A	50	2.5	N/A	0.05
Reagent Blank A	N/A	50	2.5	N/A	0.05
Control G-H	N/A	50	2.5	N/A	0.05
F0.1 A-E	0.1	50	2.5	N/A	0.05
F1 A-E	1	50	12.5	N/A	0.25
Reagent Blank C	N/A	50	2.5	N/A	0.05
Control S-T	N/A	50	2.5	N/A	0.05
F1 K-O	1	50	12.5	N/A	0.25

N/A = Not applicable.

Instrument Conditions

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

LC Parameters:

Instrument	Shimadzu Nexera series HPLC system		
Column#	Agilent EC-C-18 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 minutes		
Column Temperature	40°C		
Autosampler Temperature	5°C		
Injection Volume	15 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			
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/L)
S = concentration added to fortified sample (µg/L)

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/L) = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (µg/mL) / 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/L) = 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 120% 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.

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 0.4 µg/L and a dilution factor of 0.05).

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 matrix 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 $\underline{\geq}$ 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 in surface water (using matrix matched calibration 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: 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), however matrix matched calibration standards were used for method validation due to a significant matrix effect for DCSA confirmatory transition.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 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 0.1 and 1 µg/L; 0.1 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, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 µg/L:
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 (0.1 µg/L).	All blank sample values were <30% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.0243 µg/L	0.0309 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.	0.02 µg/L	0.02 µg/L
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 surface water (using matrix matched calibration 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: 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) for the primary transition, but significant for the confirmatory transition, therefore matrix matched calibration standards were used for method validation.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 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 0.1 and 1 µg/L; 0.1 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, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 µg/L:
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 (0.1 µg/L).	All blank sample values were <30% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.0129 µg/L	0.0613 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.	0.02 µg/L	0.02 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 in ground water (using non-matrix matched calibration 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: 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 matched calibration standards were used for method validation.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×	10× LOQ, 1 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 0.1 and 1 µg/L; 0.1 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, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 µg/L:
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 (0.1 µg/L).	All blank sample values were <30% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.00442 µg/L	0.0430 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.	0.02 µg/L	0.02 µg/L
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 ground water (using non-matrix matched calibration 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: 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 matched calibration standards were used for method validation.	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 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 0.1 and 1 µg/L; 0.1 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, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10× LOQ, 1 µg/L:	10× LOQ, 1 µg/L:
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 (0.1 µg/L).	All blank sample values were <30% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.00420 µg/L	0.00262 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.	0.02 µg/L	0.02 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 3
Analytical Procedure

Analytical Procedure

Procedure Title	Determination of Dicamba and DCSA in Ground Water and Surface Water by LC-MS/MS
Procedure Code	SMV 3202423-02V
Issue Date	12 September 2019
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The methodology described in this procedure has been validated in Borehole Ground Water and Fountains Abbey Surface Water at 0.1 and 1 µg/L.

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
- Rotary evaporator
- Sample concentrator
- Glass separating funnels (150 mL)
- Round bottom flask (250 mL)
- Glass tubes with screw caps
- 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
• Dichloromethane (DCM)	ACS reagent, Sigma
• Water	Milli-Q (with LCPAK polisher)
• Concentrated hydrochloric acid	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

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.

Secondary Stocks
Prepare secondary stock solutions of Dicamba and DCSA *test substance* and *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	0.1	Acetonitrile	10	10
DCSA	1000	0.1		10	10

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

Substance	Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub Stock Concentration (µg/mL)
Dicamba	10	0.2	Acetonitrile	10	0.2[1]
DCSA	10	0.2			
Mixed	0.2	1		10	0.02[2]

[1] Equivalent to 200 µg/L.
[2] Equivalent to 20 µg/L.

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	10	0.2	Acetonitrile	10	0.2[1]
DCSA	10	0.2			

[1] Equivalent to 200 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 ground water and surface water 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)
200	0.025	Water final extract	2.5	2
200	0.025		2.5	2
200	0.025		2.5	2

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)
200	0.025	Acetonitrile: water (25:75 v/v)	2.5	2
200	0.025		2.5	2
200	0.025		2.5	2

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)
200	0.25	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

[1] If matrix effects are significant use water 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]
Measure 50 mL of either ground water or surface water into a glass separating funnel. Fortify samples 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/L)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
1	Reagent blank[1]	N/A	N/A	50	N/A
2	Control	N/A	N/A	50	N/A
5	LOQ	20	0.25	50	0.1
5	10 × LOQ	200	0.25	50	1

N/A = Not Applicable.
[1] Use Milli-Q water for the reagent blank.

Sample Extraction [1b, 4a]

1. Measure 50 mL of water into a glass separating funnel.
2. Add 0.16 mL or 0.17 mL concentrated hydrochloric acid to ground and surface water respectively and mix well (critical step).
3. Check the pH of a control sample using a pH meter, which should be approximately pH 1.62 to pH 1.66.
4. Add 50 mL DCM and shake vigorously for approximately 1 minute.
5. Transfer the bottom layer (DCM) into a 250 mL round bottom flask.
6. Repeat steps 4-5 twice more, combining the three extracts.
7. Place on a rotary evaporator set to 30°C until approximately 3 mL remains.
8. Transfer to a glass tube, rinsing the flask into the tube with 3 mL DCM.
9. Evaporate to dryness under a gentle stream of nitrogen without heating.
10. Reconstitute with acetonitrile: water (25:75 v/v), according to the final volume given in the following table.
11. Vortex mix and then ultrasonicate for 5 minutes.
12. Transfer into an HPLC vial for analysis.

The recommended dilution procedure is given in the following table:

Sample type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Reagent blank	N/A	N/A	2.5	0.05
Control[1]	N/A	50	2.5	0.05
LOQ	0.1	50	2.5	0.05
10 × LOQ	1	50	12.5[2]	0.25

N/A = Not Applicable.
[1] Use additional control extracts for matrix matched calibration standards, if required.
[2] If matrix-matched dilutions are required, make to a final volume of 2.5 mL in acetonitrile: water (25:75 v/v) then dilute 0.2 mL to 1 mL with control extract.

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:	15 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 G3	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/L)
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/L)
S = concentration added to fortified sample (µg/L)

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)