

INTRODUCTION

The objective of this study was to independently validate the analytical method used in Smithers Viscient, Wareham Study No. 14113.6130 (Validation of an Environmental Chemistry Method for the Determination of Cycloate in Groundwater and Surface Water by LC-MS/MS). The analytical method was validated with regards to accuracy and precision, linearity, specificity and limit of quantification.

This study was conducted to support the registration of the test substances.

The method validation described in this report is designed to conform to SANCO 3029/99 rev 4 (2000) Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414, OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.

Analytical method 14113.6130 was supplied by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3202260-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was followed for method validation, and re-issued as SMV 3202260-01V when validation was complete.

The supplied method was followed with only minor changes (equipment and reagents were substituted for suitable equivalents).

Control samples of ground water and surface water were fortified with Cycloate at 0.1 µg/L in septuplicate and at 1 µg/L in quintuplicate and analysed. Samples were diluted with methanol. An aliquot was diluted into calibration range with methanol: test matrix (20:80 v/v).

To assess matrix effects, calibration standards were prepared in methanol: test matrix (20:80 v/v) and in methanol: water (20:80 v/v).

Samples were analysed for Cycloate using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy were calculated at each validation level in each test matrix. One primary and one confirmatory LC-MS/MS transition were analysed for Cycloate.

The study was initiated on 30 August 2018 (date the protocol was signed by the Study Director) and completed on the date the final report was signed by the Study Director. The practical phase of the study was conducted by Smithers Viscient (ESG) and was started on 03 September 2018 (reagent preparation) and completed on 10 September 2018 (LC-MS/MS analysis).

MATERIALS AND METHODS

Test Substance

Test Substance Name:	Cycloate
CAS Number	1134-23-2
Molecular Formula:	C ₁₁ H ₂₁ NOS
Molecular Weight:	215.39 g/mol
Sponsor Lot Number:	5608300
Purity:	98.1%
Storage Conditions:	Room temperature (15-30°C)
Recertification Date:	18 January 2019

The Certificate of Analysis for the test substance is presented in Appendix 1.

Test System

Control ground water was sourced and characterised by Smithers Viscient Harrogate. Ground water was collected from the bore hole at Smithers Rapra, UK. It was given the unique identification CS 13/18 and was stored refrigerated. The following characteristics were determined:

Suspended Solids (mg/L)	Electrical Conductivity (µS/cm)	pH	Hardness (mg/L CaCO ₃)	Dissolved Organic Carbon (mg/L)
2	436	8.0	349	0.00

The Certificate of Analysis for the ground water is presented in Appendix 2.

Control surface water was sourced and characterised by Smithers Viscient, Wareham. Characterisation data was provided in the primary method study report, 14113.6130. Surface water was collected from the Weweantic River in Taunton, Massachusetts (ID# 19Mar18 WAT-B). Surface water was collected from an area of the river with approximately 60 cm of overlying water. On receipt at Smithers Viscient Harrogate, it was given the unique identification 18/0000000/18 and stored refrigerated.

The following characteristics were provided:

pH	Dissolved Oxygen (mg/L)
6.2	5.92

Reagents

Acetonitrile	HPLC grade, Honeywell
Methanol	HPLC grade, Honeywell
Water	Milli-Q with LCPAK polisher, In House

0.1% Formic acid in water MS grade, Honeywell
0.1% Formic acid in acetonitrile MS grade, Honeywell

Equivalent or better reagents may have been used.

Equipment

Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.

Analytical Method

Analytical method 14113.6130 was supplied by the Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3202260-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was followed for method validation, and re-issued as SMV 3202260-01V when validation was complete. The completed analytical procedure is presented in Appendix 6. One person can complete the extraction of 15 samples in 1 day (8 hour working period). Automated LC-MS/MS analysis of 15 sample extracts can be completed in a further 5 hours from submission. A schematic diagram of the analytical method is presented in Appendix 7.

Preparation of Reagents

Methanol: water (20:80 v/v) was prepared by mixing 20 mL HPLC grade methanol with 80 mL Milli-Q water.

Methanol: test matrix (20:80 v/v) was prepared by mixing 100 mL methanol with 400 mL test matrix (either ground water or surface water).

Preparation of Stock Solutions

Primary stock solutions of Cycloate at 1000 µg/mL in acetonitrile and secondary stocks at 10 µg/mL in acetonitrile were prepared under Smithers Viscient (Harrogate) Study No. 3202259 (Independent Laboratory Validation of Analytical Method 14113.6131 for the Determination of Cycloate in Soil).

Sub-stock solutions were prepared as described in the following table:

Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL) ¹
10	0.025	Methanol	25	0.01

¹ Equivalent to 10 µg/L.

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

Preparation of Calibration Standards

Matrix matched calibration standards of Cycloate were prepared as described in the following tables:

Ground water

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.5	Methanol: ground water (20:80 v/v)	10	0.5
0.5	0.8		1	0.4
0.5	0.6		1	0.3
0.5	0.4		1	0.2
0.5	0.2		1	0.1
0.5	0.1		1	0.05

Surface water

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.5	Methanol: surface water (20:80 v/v)	10	0.5
0.5	0.8		1	0.4
0.5	0.6		1	0.3
0.5	0.4		1	0.2
0.5	0.2		1	0.1
0.5	0.1		1	0.05

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

Preparation of Matrix Matched Standards for Matrix Assessment

Matrix matched standards of Cycloate were prepared in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.2	Methanol: ground water (20:80 v/v)	10	0.2
10	0.2		10	0.2
10	0.2		10	0.2
10	0.2	Methanol: surface water (20:80 v/v)	10	0.2
10	0.2		10	0.2
10	0.2		10	0.2

Preparation of Non-Matrix Matched Standards for Matrix Assessment

Non-matrix matched standards of Cycloate were prepared in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.2	Methanol: water (20:80 v/v)	10	0.2
10	0.2		10	0.2
10	0.2		10	0.2

Sample Fortification

8 mL water was measured into a disposable glass vial. Water samples were fortified with a stock solution of Cycloate at 0.1 µg/L in septuplicate and at 1 µg/L in

quintuplicate. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Ground water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank A	N/A	N/A	N/A	N/A
Control A-B	8	N/A	N/A	N/A
F0.1 A-G	8	10	0.08	0.1
F1 A-E	8	10	0.8	1

N/A = Not applicable.

Surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B	N/A	N/A	N/A	N/A
Control C-D	8	N/A	N/A	N/A
F0.1 H-N	8	10	0.08	0.1
F1 F-J	8	10	0.8	1

N/A = Not applicable.

Sample Extraction

2 mL methanol was added to the water and mixed well. A portion of extract was diluted into calibration range with methanol: test matrix (20:80 v/v) for the samples at 10 × LOQ. The final extract was transferred into an HPLC vial for analysis. Sample extracts were stored refrigerated in case further analysis was required. The extraction procedure is summarised in the following tables:

Ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Extract Volume (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank A	N/A	N/A	10	N/A	1.25
Control A-B	N/A	8	10	N/A	1.25
F0.1 A-G	0.1	8	10	N/A	1.25
F1 A-E	1	8	10	0.4-1	3.125

N/A = Not applicable.

Surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Volume of Extract (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank B	N/A	N/A	10	N/A	1.25
Control C-D	N/A	8	10	N/A	1.25
F0.1 H-N	0.1	8	10	N/A	1.25
F1 F-J	1	8	10	0.4-1	3.125

N/A = Not applicable.

Instrument Conditions

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

HPLC Parameters:

Instrument	Shimadzu Nexera series HPLC system		
Column#	Waters Atlantis T3, 3 µm, 4.6 × 100 mm		
Mobile Phase A#	0.1% Formic acid in water		
Mobile Phase B#	0.1% Formic acid in acetonitrile		
Flow Rate	0.8 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	95	5
	0.5	95	5
	0.6	15	85
	4.5	0	100
	5.0	0	100
	5.1	95	5
	6.0	95	5
Run Time	6.0 minutes		
Column Temperature	40°C		
Autosampler Temperature	15°C		
Injection Volume	50 µL		
Retention Time	Approx. 3.8 minutes (Cycloate)		
Valco Valve Diverter	Time (min)		Position
	0		A (to waste)
	1		B (to MS)
	5		A (to waste)

MS/MS Parameters:

Instrument	AB Sciex API 5000 Triple Quadrupole Mass Spectrometer			
Ionisation Type#	Electrospray (ESI)			
Polarity#	Positive			
Scan Type#	Multiple reaction monitoring (MRM)			
Ion Spray Voltage	5500 V			
Collision Gas (CAD)	5			
Curtain Gas (CUR)	25			
Gas Flow 1 (GS1)	40			
Gas Flow 2 (GS2)	40			
Vaporiser Temperature (TEM)	550°C			
Interface Heater (ihe)	On			
Entrance Potential (EP)	10			
Collision Exit Potential (CXP)	13			
Compound Name	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
Cycloate (Primary)	215.90/83.00	80	22.26	100
Cycloate (Confirmatory)	215.90/54.95	80	40.00	100

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

Calculation of Results

LC-MS/MS data were calculated using Analyst 1.6.2. 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 extract:

$$x = (y - c) / m$$

Where:

x = concentration of test substance in sample extract ($\mu\text{g/L}$)

y = peak area due to test substance

c = y intercept on calibration graph

m = gradient of the calibration graph

The concentration of test substance in the sample was calculated as follows:

Sample concentration ($\mu\text{g/L}$) = Extract concentration ($\mu\text{g/L}$) \times Dilution factor

Dilution factor = Final extract volume (mL) / volume of water in final extract (mL)

Procedural recovery from fortified samples is calculated as follows:

Recovery (%) = Sample concentration / Fortified concentration \times 100

The Limit of Detection (LOD) was calculated according to the following equation (U.S. EPA, 2016, 1994, Definition and Procedure for the Determination of the Method Detection Limit, Revision 1.11 and Revision 2):

$\text{LOD} = (t_{0.99} \times \text{SD}) + \text{mean apparent residue in the control samples}$

Where SD = standard deviation for 7 replicate samples at the LOQ

$t_{0.99}$ = one-tailed statistic at the 99% confidence interval for 7 replicates (3.143)

The Method Detection Limit (MDL) was calculated as the lowest calibration standard concentration multiplied by the dilution factor for the control.

Validation Pass Criteria

The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored.

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) \leq 20%.

Specificity/Selectivity

Specificity was acceptable if the amounts found in blank samples were \leq 30% of the LOQ.

Linearity

Linearity was acceptable if the lowest calibration standard concentration was $\leq 80\%$ of the equivalent LOQ final extract concentration. The highest calibration standard concentration was $\geq 120\%$ of the $10 \times$ LOQ extract concentration (after dilution if applicable). Matrix matched calibration standards were used for consistency with the primary method. The correlation coefficient (r) was acceptable if it was ≥ 0.995 .

LOD (Limit of Detection) Assessment

The LOD was calculated according to the U.S. EPA, 2016, 1994, Definition and Procedure for the Determination of the Method Detection Limit, Revision 1.11 and Revision 2.

MDL (Method Detection Limit)

The MDL was calculated as the sample concentration equivalent to the lowest calibration standard.

Matrix Assessment

An assessment of matrix effects was made by comparison of peak areas from triplicate standards prepared in blank solvent and in each control matrix final extract. This was assessed for the primary and confirmatory transitions.

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

A difference of $\geq 20\%$ was considered significant.

Cycloate was analysed using matrix matched calibration standards for consistency with the primary method.

Appendix 7
Schematic Diagram of the Analytical Method

