1.0 INTRODUCTION

The purpose of this study was to validate an environmental chemistry method used to determine the content of cycloate in groundwater and surface water. The method was validated (26 and 28 January 2018) to quantify the concentrations of cycloate present in recovery samples prepared in groundwater and surface water. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in groundwater and surface water by fortification with cycloate at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. Recovery samples were diluted with methanol for a final composition of 20/80 methanol/test matrix (v/v). Recovery samples were further diluted into the calibration range, as necessary, with 20/80 methanol/test matrix (v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 17 January 2018, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted on 26 and 28 January 2018 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers Viscient's archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of an Environmental Chemistry Method for the Determination of Cycloate in Groundwater and Surface Water by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160

(U.S. EPA, 1989) and as accepted by OECD principles on GLP (OECD, 1998), and followed the guidance documents SANCO/3029/99 rev 4 (EC, 2000) and OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substance

The test substance, cycloate, was received on 30 November 2016 from Chem Service Inc., West Chester, Pennsylvania. The following information was provided:

Name:	cycloate
Lot No.:	5608300
CAS No.:	1134-23-2
Purity:	98.1%
Recertification Date:	18 January 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8624) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

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

2.3 Reagents

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

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	AB Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACRX autosampler Shimadzu DGU-20A5R vacuum degassers Shimadzu CBM-20A communications bus Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column oven Analyst version 1.6 software for data acquisition
2.	Balance:	Mettler Toledo XS205DU
3.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, graduated cylinders, Pasteur pipets, autosampler vials, and amber glass bottles with Teflon-lined caps

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

2.5 Test Matrices

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

Groundwater:

Groundwater used in the study was filtered well water collected from Smithers Viscient, Wareham, Massachusetts and was prepared by filtering to remove any potential organic contaminants. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

Surface water information:

The surface water used for this method validation analysis was collected from the Weweantic River (SMV Lot No.12 Jul 17 Water-A, collected on 12 July 2017) in Taunton, Massachusetts. The water was collected from an area of the river with approximately 60 cm of overlying water and was determined to have a pH of 6.2 (using a YSI pH100 meter) and a dissolved oxygen

concentration of 5.92 mg/L (using a YSI Pro 20 dissolved oxygen meter). All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

2.6 Preparation of Liquid Reagents

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

A 20/80 methanol/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 20.0 mL of methanol and 80.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 20/80 methanol/test matrix (v/v) liquid reagent solution was typically prepared by combining 100 mL of methanol and 400 mL of test matrix. The solution was mixed well using a stir bar and stir plate for five minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water.

2.7 Preparation of Stock Solutions

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

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

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8624-3C	0.0512	0.0502	Acetonitrile	50.0	1000	Secondary stock solution

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8624-3C	1000	0.500	50.0	Acetonitrile	8624-3C-1	10.0	Sub-stock solution

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8624-3C-1	10.0	0.0500	50.0	Methanol	Stk 1	0.0100	Calibration standards and LOQ and High-level recovery samples

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

2.8.1 Matrix-Matched Standard Solutions

Matrix-matched standard solutions were prepared in 20/80 methanol/test matrix (v/v) by fortifying with the 0.0100 mg/L sub-stock solution to yield test substance concentrations of 0.0500, 0.100, 0.200, 0.300, 0.400, and 0.500 µg/L. This procedure is detailed in the table below.

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Stk 1	0.0100	0.0500	10.0	0.0500	Std 1
		0.100	10.0	0.100	Std 2
		0.200	10.0	0.200	Std 3
		0.300	10.0	0.300	Std 4
		0.400	10.0	0.400	Std 5
		0.500	10.0	0.500	Std 6

2.8.2 Matrix Effects Investigation

Standards used to assess possible matrix effects were prepared as follows by fortifying with the 0.0100 mg/L sub-stock solution to yield a test substance concentration of 0.0800 µg/L.

2.8.2.1 Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Stk 1	0.0100	0.0800	10.0	0.0800	MM-Std A
		0.0800	10.0	0.0800	MM-Std B
		0.0800	10.0	0.0800	MM-Std C

[a] Dilution solvent: 20/80 methanol/test matrix (v/v)

2.8.2.2 Non Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Stk 1	0.0100	0.0800	10.0	0.0800	Sol-Std A
		0.0800	10.0	0.0800	Sol-Std B
		0.0800	10.0	0.0800	Sol-Std C

[a] Dilution solvent: 20/80 methanol/purified reagent water (v/v)

2.9 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (groundwater and surface water) with cycloate at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. Recovery samples for the two matrices were prepared separately ("de novo") at these concentrations. Seven replicates were produced for the LOQ concentration and five replicates were produced for the High concentration. Two samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Groundwater recovery samples

Sample ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
Reagent Blank A	NA[a]	NA	8.00[b]	0.00
Control A & B	NA	NA	8.00	0.00
LOQ A, B, C, D, E, F, & G	0.0100	0.0800	8.00	0.100
High A, B, C, D, & E	0.0100	0.800	8.00	1.00

[a] NA = Not Applicable
[b] Dilution solvent: Methanol

Surface water recovery samples

Sample ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
Reagent Blank A	NA[a]	NA	8.00[b]	0.00
Control A & B	NA	NA	8.00	0.00
LOQ A, B, C, D, E, F, & G	0.0100	0.0800	8.00	0.100
High A, B, C, D, & E	0.0100	0.800	8.00	1.00

[a] NA = Not Applicable
[b] Dilution solvent: Methanol

2.10 Dilution of Samples

To minimize the potential for losses of the test substance during processing, the aqueous test samples were not sub-sampled prior to dilution. The first dilution with methanol was performed by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 20/80 methanol/test matrix (v/v). Recovery samples were subsequently diluted into the calibration standard range with 20/80 methanol/test matrix (v/v) prior to analysis. The dilution procedures are outlined in the tables below.

Groundwater recovery samples

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blank A	0.00	8.00	10.0	NA[c]	NA	1.25
Control A & B	0.00	8.00	10.0	NA	NA	1.25
LOQ A, B, C, D, E, F, & G	0.100	8.00	10.0	NA	NA	1.25
High A, B, C, D, & E	1.00	8.00	10.0	4.00	10.0	3.13

[a] Dilution solvent: Methanol
[b] Dilution solvent: 20/80 methanol/groundwater (v/v)
[c] NA = Not Applicable

Surface water recovery samples

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blank A	0.00	8.00	10.0	NA[c]	NA	1.25
Control A & B	0.00	8.00	10.0	NA	NA	1.25
LOQ A, B, C, D, E, F, & G	0.100	8.00	10.0	NA	NA	1.25
High A, B, C, D, & E	1.00	8.00	10.0	4.00	10.0	3.13

[a] Dilution solvent: Methanol
[b] Dilution solvent: 20/80 methanol/surface water (v/v)
[c] NA = Not Applicable

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC parameters:

Column:	Waters Atlantis T3, 3.0 µm, 4.6 × 100 mm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.800	95.0	5.00
0.50	0.800	95.0	5.00
0.60	0.800	15.0	85.0
4.50	0.800	0.00	100
5.00	0.800	0.00	100
5.10	0.800	95.0	5.00
6.00	0.800	95.0	5.00

Run Time: 6.0 minutes
Autosampler Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 15 °C
Injection Volume: 50.0 µL
Retention Time: approximately 4.1 minutes

MS parameters:

Instrument: AB Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Dwell Time: 200 milliseconds
Source Temperature: 600 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 50.0 / 50.0
Collision Gas: 4.00
Collision Cell Entrance Potential: 4.00
Collision Cell Exit Potential: 9.00
Declustering Potential: 80.0
Resolution Q1/Q3: Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	216.18/82.97	216.18/54.95
Collision Energy:	23.0	40.0

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

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to each analysis of the recovery samples, and the second set of each immediately following the analysis of the recovery samples. Injection of samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 110% (for the individual mean concentrations) were acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples and retention times. RSD values less than 20% were considered acceptable for the recovery samples (with less than 10% considered ideal) and RSD values less than 2% were considered acceptable for the retention times. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as cycloate, which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.13 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

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

The LOD was calculated using the standard deviation of the average recovery in units of concentration of the seven samples fortified at the LOQ, multiplied by one-tailed t-statistic at the 99% confidence level for n-1 replicates. Representative calculations for the LOD can be found in Section 3.0.

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

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

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

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

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

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample

DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The LOD was calculated using the following equation (U.S. EPA, 2016):

$$(4) \quad LOD = t_{0.99} \times SD + \text{average residue in UTC}$$

where:

$t_{0.99}$	=	One-tailed t-statistic at the 99% confidence level for n-1 replicates (i.e., 3.143 for seven replicates)
SD	=	Standard deviation of the detected concentrations of n samples spiked at the estimated LOQ
LOD	=	Limit of detection for the analysis
UTC	=	Untreated controls

The method detection limit (MDL) is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (Equation 5) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(5) \quad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.0500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 1.25)
MDL	=	method detection limit reported for the analysis (0.0500 µg/L × 1.25 = 0.0625 µg/L)