1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of cumyluron in aqueous samples by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated (28 to 29 January 2020) to quantify the concentrations of cumyluron 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 cumyluron at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Recovery samples were diluted with 20/80 acetonitrile/purified reagent water (v/v) for a final composition of 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 27 January 2020, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted from 28 to 29 January 2020 at Smithers, located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers' archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers protocol entitled "Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Cumyluron in Groundwater and Surface water by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practice (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed

the SANCO/3029/99 rev. 4 guidance document (EC, 2000) and OCSPP 850.6100 guideline (U.S. EPA, 2012).

2.2 Test Substance

The test substance, cumyluron, was received on 6 June 2016 from Helena Chemical Company, Memphis, Tennessee. The following information was provided:

Name:	Cumyluron
Lot No.:	PLK0014E
CAS No.:	99485-76-4
Purity:	99.96% (Certificate of Analysis, Appendix 2)
Expiration Date:	21 July 2022

Upon receipt at Smithers Viscient, the test substance (SMV No. 8302) was stored at room temperature in a dark, ventilated cabinet in a 3-L Nalgene bottle. 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.	Acetonitrile:	EMD, reagent grade
2.	Methanol:	EMD, reagent grade
3.	0.1% formic acid in water:	Fisher, reagent grade
4.	0.1% formic acid in acetonitrile:	Fisher, 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 MDS Sciex API 4000 QTRAP mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACHT autosampler Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.6.2 software for data acquisition
2.	Balance:	Mettler Toledo XSE205DU
3.	Centrifuge:	Beckman Coulter 367160
4.	Laboratory equipment:	Positive displacement pipets, graduated cylinders, volumetric flasks, disposable glass pipets, stir bars, stir plate, vortex mixer, 1.5-mL polypropylene centrifuge tubes, disposable glass vials with PTFE-lined caps, autosampler vials, and amber glass bottles with Teflon-lined caps

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

2.5 Test Matrixes

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

The groundwater used for this method validation consists of unadulterated water from a 100-meter bedrock well collected from Smithers, Wareham, Massachusetts, prepared by filtering to remove any potential organic contaminants. The surface water used for this method validation analysis was collected from the Taunton River (Smithers Lot No. 05Feb19Wat-A) in Bridgewater, Massachusetts. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water. All documentation relating to the preparation, storage, and handling is maintained by Smithers.

Characterization of the groundwater and surface water was performed by Agvise Laboratories, Northwood, North Dakota. This analysis is summarized in the following table.

Parameter	Groundwater	Surface Water
Sample ID:	Groundwater 2019	05FEB19-WAT A
pH:	7.6	7.3
Calcium (ppm):	24	9.2
Magnesium (ppm):	7.8	2.3
Sodium (ppm):	92	42
Hardness (mg equivalent to $CaCO_3$/L):	92	33
Conductivity (mmhos/cm):	0.70	0.38
Sodium Adsorption Ratio (SAR):	4.19	3.24
Total Dissolved Solids (ppm):	228	10
Turbidity (NTU):	0.15	1.03

2.6 Preparation of Liquid Reagent Solutions

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

A 20/80 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by adding 300 mL of acetonitrile to 1200 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

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

2.7 Preparation of Stock Solutions

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

A primary stock solution was 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
8302Q	0.0502	0.0502	Acetonitrile	50.0	1000	Secondary stock solution

A secondary stock solution was 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
8302Q	1000	0.500	50.0	Acetonitrile	8302Q-1	10.0	Sub-stock solutions

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8302Q-1	10.0	0.0200	20.0	Acetonitrile	Tech Stk 1	0.0100	LOQ- and 10X LOQ-level recovery samples
8302Q-1	10.0	0.0200	20.0	Acetonitrile	Ana Stk 1	0.0100	Calibration standards and sub-stock solution
Ana Stk 1	0.0100	1.00	10.0	Acetonitrile	Ana Stk 2	0.00100	Matrix effects investigation 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

Calibration standards were prepared in 20/80 acetonitrile/purified reagent water (v/v) by fortifying with the 0.0100 mg/L sub-stock solution to yield concentrations of 0.00200, 0.00500, 0.00750, 0.0100, 0.0200, 0.0500, 0.100, 0.150, and 0.200 µg/L. Calibration standards were prepared according to the table below. Following fortification, each solution was mixed using a vortex mixer for 15 seconds.

Fortifying Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.0100	0.0200	100	0.00200	Std 1-1
		0.0200	40.0	0.00500	Std 1
		0.0300	40.0	0.00750	Std 2
		0.0200	20.0	0.0100	Std 3
		0.0200	10.0	0.0200	Std 4
		0.0500	10.0	0.0500	Std 5
		0.100	10.0	0.100	Std 6
		0.150	10.0	0.150	Std 7
		0.200	10.0	0.200	Std 8

2.9 Matrix Effect Investigation

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based calibration standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate. One set was prepared in control sample final fraction for each matrix (see Section 2.11) and a second set was prepared in 20/80 acetonitrile/purified reagent water (v/v) by fortifying with the 0.00100 mg/L sub-stock solution to yield a concentration of 0.0100 µg/L. The preparation procedure for each separate matrix is outlined in the following table.

Sample ID	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
MM-GW-Std A, B, & C	Matrix-matched calibration standard (groundwater)	0.00100	0.0500	5.00[a]	0.0100
MM-SW-Std A, B, & C	Matrix-matched calibration standard (surface water)		0.0500	5.00[b]	0.0100
Std A, B, & C	Solvent-based calibration standard		0.0500	5.00[c]	0.0100

[a] Diluted with control sample final fraction (Sample ID: 14102-6120-02)
[b] Diluted with control sample final fraction (Sample ID: 14102-6120-15)
[c] Diluted with 20/80 acetonitrile/purified reagent water (v/v)

2.10 Sample Fortification and Preparation

The recovery samples were prepared in two different matrixes (groundwater and surface water) by fortification with cumyluron at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Recovery samples for both matrixes were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level in each matrix. Two samples of each matrix were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared for each sample set and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the following tables.

Groundwater recovery samples

Sample ID 14102-6120-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
01	Reagent Blank	NA[a]	NA	5.00[b]	0.00
02 & 03	Control	NA	NA	5.00	0.00
04, 05, 06, 07, & 08	LOQ	0.0100	0.0500	5.00	0.100
09, 10, 11, 12, & 13	10X LOQ		0.500	5.00	1.00

[a] NA = Not Applicable
[b] Dilution solvent: purified reagent water

Surface water recovery samples

Sample ID 14102-6120-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
14	Reagent Blank	NA[a]	NA	5.00[b]	0.00
15 & 16	Control	NA	NA	5.00	0.00
17, 18, 19, 20, & 21	LOQ	0.0100	0.0500	5.00	0.100
22, 23, 24, 25, & 26	10X LOQ		0.500	5.00	1.00

[a] NA = Not Applicable
[b] Dilution solvent: purified reagent water

2.11 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 20/80 acetonitrile/purified reagent water (v/v) 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 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). Following dilution, samples were vortex mixed for 15 seconds and centrifuged at 13,000 rpm for 5 minutes. The dilution procedures are outlined in the following tables.

Groundwater recovery samples

Sample ID 14102-6120-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
01	Reagent Blank	0.00	5.00	50.0	10.0
02[b] & 03	Control	0.00	5.00	50.0	10.0
04, 05, 06, 07, & 08	LOQ	0.100	5.00	50.0	10.0
09, 10, 11, 12, & 13	10X LOQ	1.00	5.00	50.0	10.0

[a] Diluted with 20/80 acetonitrile/purified reagent water (v/v)
[b] Aliquots of this diluted control sample were used to prepare matrix effects investigation samples for the groundwater validation.

Surface water recovery samples

Sample ID 14102-6120-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
14	Reagent Blank	0.00	5.00	50.0	10.0
15[b] & 16	Control	0.00	5.00	50.0	10.0
17, 18, 19, 20, & 21	LOQ	0.100	5.00	50.0	10.0
22, 23, 24, 25, & 26	10X LOQ	1.00	5.00	50.0	10.0

[a] Diluted with 20/80 acetonitrile/purified reagent water (v/v)
[b] Aliquots of this diluted control sample were used to prepare matrix effects investigation samples for the surface water validation.

2.12 Analysis

2.12.1 Instrumental Conditions

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

LC parameters:

Column: Waters Xbridge BEH C18, 2.5 µm, 2.1 × 50 mm
Mobile Phase A: 0.1% formic acid in reagent grade water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.350	75.0	25.0
0.50	0.350	75.0	25.0
4.00	0.350	0.00	100
6.00	0.350	0.00	100
6.10	0.350	75.0	25.0
7.50	0.350	75.0	25.0

Run Time: 7.5 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 3.6 minutes

MS parameters:

Instrument:	AB MDS Sciex API 4000 QTRAP mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	4500 V
Scan Type:	MRM
Dwell Time:	500 milliseconds
Source Temperature:	550 °C
Curtain Gas:	20.0
Ion Source – Gas 1 / Gas 2:	30.0 / 80.0
Collision Gas:	Medium
Entrance Potential:	10.0
Declustering Potential:	50.0
Resolution Q1/Q3:	Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	303.0/184.9	303.4/125.0
Collision Energy:	17.0	41.0
Collision Cell Exit Potential:	24.0	14.0

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

2.12.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, approximately every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.13 Evaluation of Specificity, Linearity, Accuracy, and Precision

The specificity of the method was determined by examination of the control samples for peaks at the same retention times as cumyluron 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. 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. RSD values less than or equal to 20% were considered acceptable.

2.14 Limit of Quantitation (LOQ)

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

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

The LOD was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

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

3.0 CALCULATIONS

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

recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

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

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

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

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (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:

$$(4) \qquad LOD = ((3 \times (N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
LOD	=	limit of detection for the analysis

The 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.

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

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.00200 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
MDL	=	method detection limit reported for the analysis (0.00200 µg/L × 10.0 = 0.0200 µg/L)

[redacted]