1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of cumyluron in soil samples by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated to quantify the concentrations of cumyluron present in recovery samples prepared in sandy loam soil and loam soil. 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 sandy loam soil and loam soil by fortification with cumyluron at concentrations of 0.0500 (LOQ) and 0.500 (10X LOQ) mg/kg. Samples were extracted twice with 80/20 acetonitrile/purified reagent water (v/v). The recovery samples were further diluted into the calibration range with 20/80 acetonitrile/purified reagent water (v/v). All samples were analyzed using 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 30 to 31 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 Soil 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 Milli-Q 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.	Balances:	Mettler Toledo XSE205DU; Mettler Toledo PG-2002-S
3.	Moisture balance:	Sartorius MA-45
4.	Shaker table:	VWR Standard Analog 3500STD
5.	Centrifuges:	Thermo Scientific Sorvall Legend XFR; Beckman Coulter 367160
6.	Laboratory equipment:	Positive displacement pipets, graduated cylinders, volumetric flasks, disposable glass pipets, stir bars, stir plate, vortex mixer, 50-mL centrifuge tubes, 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 sandy loam soil and loam soil.

Characterization of the sandy loam soil and loam soil was performed by Agvise Laboratories, Northwood, North Dakota. The characterization for each matrix is summarized in the following table.

Parameter	Sandy Loam Soil	Loam Soil
Smithers Batch No.:	09Oct19Soil-D	09Oct19Soil-A
Collection location:	Northwood, North Dakota	Hanford, California
Percent organic matter:	3.6%	1.6%
USDA textural class:	Sandy loam	Loam
Particle size distribution:	74% sand 15% silt 11% clay	30% sand 49% silt 21% clay
pH (1/1 matrix/water ratio):	6.8	6.8
Percent water holding capacity (at 1/3 bar):	15.5%	23.4%
Bulk density (gm/cc):	1.05	1.17

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.

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

A 20/80 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by adding 200 mL of acetonitrile to 800 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	10X LOQ-level recovery samples and 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	1.00	10.0	Acetonitrile	Tech Stk 1	1.00	LOQ-level recovery samples
8302Q-1	10.0	0.0200	20.0	Acetonitrile	Ana Stk 1	0.0100	Calibration standards and 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 0.00500, 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.

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 0.0100 mg/L sub-stock solution to yield a concentration of 0.0500 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-SL-Std A, B, & C	Matrix-matched calibration standard (sandy loam soil)	0.0100	0.0250	5.00[a]	0.0500
MM-L-Std A, B, & C	Matrix-matched calibration standard (loam soil)		0.0250	5.00[b]	0.0500
Std A, B, & C	Solvent-based calibration standard		0.0250	5.00[c]	0.0500

[a] Diluted with control sample final fraction (Sample ID: 14102.6121-02)
[b] Diluted with control sample final fraction (Sample ID: 14102.6121-15)
[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 (sandy loam soil and loam soil) by fortification with stock solutions of cumyluron at concentrations of 0.0500 (LOQ) and 0.500 (10X LOQ) mg/kg. Recovery samples for both matrixes were prepared separately ("de novo") at these concentrations using 5.00 g (dry weight) aliquots of each soil type. Five replicates were produced for each concentration level. 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 tables below.

Sandy loam soil recovery samples

Sample ID 14102-6121-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
01	Reagent Blank	NA[a]	NA	NA	NA	0.00
02 & 03	Control	NA	NA	5.91	5.00	0.00
04, 05, 06, 07, & 08	LOQ	1.00	0.250	5.91	5.00	0.0500
09, 10, 11, 12, & 13	10X LOQ	10.0	0.250	5.91	5.00	0.500

[a] NA = Not Applicable

Loam soil recovery samples

Sample ID 14102-6121-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
14	Reagent Blank	NA[a]	NA	NA	NA	0.00
15 & 16	Control	NA	NA	5.22	5.00	0.00
17, 18, 19, 20, & 21	LOQ	1.00	0.250	5.22	5.00	0.0500
22, 23, 24, 25, & 26	10X LOQ	10.0	0.250	5.22	5.00	0.500

[a] NA = Not Applicable

2.11 Extraction of Samples

Samples were extracted twice with 80/20 acetonitrile/purified reagent water (v/v). A 20-mL aliquot of 80/20 acetonitrile/purified reagent water (v/v) was added to each soil recovery sample (5.00 g dry weight), which were sonicated for 10 minutes and then placed on a shaker table for 30 minutes at 250 rpm. Samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50-mL volumetric flasks. The extraction and centrifugation procedures were repeated one more time with an additional 20-mL aliquot of 80/20 acetonitrile/purified reagent water (v/v). The extracts were combined, taken to volume (50 mL) with 80/20 acetonitrile/purified reagent water (v/v) and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v). The extraction and dilution procedures for each separate matrix are outlined in the following tables.

Sandy loam soil recovery samples

Sample ID 14102-6121-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
01	Reagent Blank	0.00	NA[c]	50.0	0.100	10.0	1000
02	Control	0.00	5.00	50.0	0.500	50.0[d]	1000
03	Control	0.00	5.00	50.0	0.100	10.0	1000
04, 05, 06, 07, & 08	LOQ	0.0500	5.00	50.0	0.100	10.0	1000
09, 10, 11, 12, & 13	10X LOQ	0.500	5.00	50.0	0.0200	10.0	5000

[a] Extraction solvent: 80/20 acetonitrile/purified reagent water (v/v)
[b] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects.

Loam soil recovery samples

Sample ID 14102-6121-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
14	Reagent Blank	0.00	NA[c]	50.0	0.100	10.0	1000
15	Control	0.00	5.00	50.0	0.500	50.0[d]	1000
16	Control	0.00	5.00	50.0	0.100	10.0	1000
17, 18, 19, 20, & 21	LOQ	0.0500	5.00	50.0	0.100	10.0	1000
22, 23, 24, 25, & 26	10X LOQ	0.500	5.00	50.0	0.0200	10.0	5000

[a] Extraction solvent: 80/20 acetonitrile/purified reagent water (v/v)
[b] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects.

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: 100 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 eight 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 (mg/kg), 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., 1000)
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 (0.00500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 1000)
MDL	=	method detection limit reported for the analysis (0.00500 µg/L × 1000 mL/g = 0.00500 mg/kg)