INTRODUCTION

The objective of this study was to independently validate the analytical method in Study No. 14102.6120, for measuring residues of Cumyluron in ground and surface water, in accordance with EPA OCSPP 850.6100 (2012) and SANCO/3029/99 rev. 4 (2000) guidelines.

The analytical method (Study No. 14102.6120) was provided by Smithers ERS, Wareham on behalf of the sponsor. The method was re-written in Smithers ERS, Harrogate format as draft method SMV 3202655-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202655-01V when validation was complete.

Control samples of ground and surface water were fortified with Cumyluron at 0.1 and 1 µg/L in quintuplicate and analysed. Samples were diluted with acetonitrile: water (20:80 v/v).

To assess matrix effects, triplicate standards were prepared in acetonitrile: water (20:80 v/v) and in control water final extract.

Samples were analysed for Cumyluron 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 water for Cumyluron. One primary and one confirmatory LC-MS/MS transition were analysed for Cumyluron.

STUDY TIMETABLE

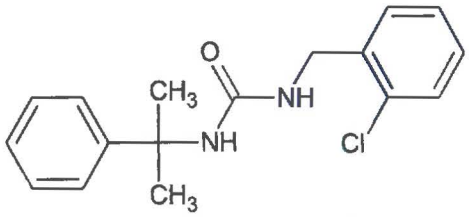
Study initiation:	07 July 2020 (date the protocol was signed by the Study Director).
Experimental start:	18 July 2020 (matrix assessment).
Experimental completion:	13 August 2020 (LC-MS/MS analysis).
Study completion:	Date the final report was signed by the Study Director.

MATERIALS AND METHODS

Protocol Adherence

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

Test Substance

Test Substance Name:	**Cumyluron**
IUPAC Name:	1-(2-chlorobenzyl)-3-(1-methyl-1-phenylethyl)urea
CAS Number:	99485-76-4
Structure:	
Molecular Formula:	$C_{17}H_{19}ClN_2O$
Molecular Weight:	302.8 g/mol
Batch Number:	PLK0014E
Purity:	99.96%
Storage Conditions:	Room temperature (15-25°C)
Retest Date:	21 July 2022

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

Test Matrices

Control ground and surface water were sourced by Smithers ERS. The waters used were CS 38/20 Borehole ground water and CS 01/20 Fountains Abbey surface water.

Water characterisation data are listed in the following table:

Water Name	Unique ID	Water Type	Suspended Solids (mg/L)	Conductivity (µS/cm)	Hardness (mg/L $CaCO_3$)	pH	Dissolved Organic Carbon (mg/L)
Borehole	CS38/20	Ground	1	631	312	8.4	3.68
Fountains Abbey	CS01/20	Surface	5	140	132	7.51	8.53

The certificates of analysis for each test matrix are presented in Appendix 2.

Reagents

• Acetonitrile	HPLC grade, Honeywell
• Acetonitrile	HPLC grade, Fisher
• Water	Milli-Q (with LCPAK polisher)
• 0.1% Formic acid in water	LC-MS grade, Honeywell
• 0.1% Formic acid in acetonitrile	LC-MS grade, Honeywell

Equipment

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector
- HPLC column: Waters Xbridge BEH C18, 2.5 µm, 2.1 x 50 mm
- Analytical balance
- Positive displacement pipettes
- Glass jars
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

Analytical method 14102.6120 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 3202655-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202655-01V when validation was complete. The complete analytical procedure is presented in Appendix 3. A typical batch of thirteen samples can be completed by a skilled analyst within one working day (8 hours).

Preparation of Reagents

Acetonitrile: water (20:80 v/v)

200 mL acetonitrile was mixed with 800 mL Milli-Q 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 Cumyluron were prepared at 1000 µg/mL in acetonitrile under Smithers ERS GLP Study No. 3202654 (Independent Laboratory Validation of Analytical Method 14102.6121 for the Determination of Cumyluron in Soil). Primary stock solutions were stored refrigerated in amber glass bottles for up to three months.

Secondary Stock Solutions
Secondary stock solution of Cumyluron were prepared as described in the following table:

Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)	Stock Use
1000	0.1	Acetonitrile	10	10	Sub-stock solution

Secondary stock solutions were stored refrigerated in amber glass bottles for up to one month.

Sub-Stock Solutions
Sub-stock solutions of Cumyluron were prepared as described in the following table:

Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)	Stock Use
10	0.1	Acetonitrile	10	0.1[1]	Fortification at 10 × LOQ
0.1	0.1		1	0.01[2]	Fortification at LOQ and intermediate calibration standard
0.01	0.1		1	0.001[3]	Matrix assessment

[1] Equivalent to 100 µg/L.
[2] Equivalent to 10 µg/L.
[3] Equivalent to 1 µg/L.
Volumes may have been scaled as appropriate.

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

Preparation of Non-Matrix Matched Standards for Matrix Assessment
Non-matrix matched standards of Cumyluron were prepared in acetonitrile: water (20:80 v/v) for comparison with matrix-matched standards.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.1	Acetonitrile: water (20:80 v/v)	10	0.01
1	0.1		10	0.01
1	0.1		10	0.01

Preparation of Matrix Matched Standards for Matrix Assessment
Matrix-matched standards of Cumyluron were prepared in control water final extract.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.1	Ground water final extract	10	0.01
1	0.1		10	0.01
1	0.1		10	0.01
1	0.1	Surface water final extract	10	0.01
1	0.1		10	0.01
1	0.1		10	0.01

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

Preparation of Calibration Standards
Non-matrix matched calibration standards of Cumyluron were prepared for the validation of ground water and surface water as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.2	Acetonitrile: water (20:80 v/v)	10	0.2
0.2	0.75		1	0.15
0.2	0.5		1	0.1
0.2	0.25		1	0.05
0.2	0.1		1	0.02
0.1	0.1		1	0.01
0.1	0.075		1	0.0075
0.1	0.05		1	0.005
0.02	0.1		1	0.002

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

Sample Preparation and Fortification
5 mL of water was measured into a glass vial. Quintuplicate water samples were fortified at the LOQ (0.1 µg/L) and at 10 × LOQ (1 µg/L) with stock solutions of Cumyluron. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Borehole ground water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank C[1]	5	N/A	N/A	N/A
Control F[2]	5	N/A	N/A	N/A
Control G-H	5	N/A	N/A	N/A
F0.1 K-O	5	0.01	0.05	0.1
F1 K-O	5	0.1	0.05	1

N/A = Not applicable.
[1] Milli-Q water was used as the reagent blank.
[2] Control F was used for matrix assessment.

Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B[1]	5	N/A	N/A	N/A
Control A[2]	5	N/A	N/A	N/A
Control D-E	5	N/A	N/A	N/A
F0.1 F-J	5	0.01	0.05	0.1
F1 F-J	5	0.1	0.05	1

N/A = Not applicable.
[1] Milli-Q water was used as the reagent blank.
[2] Control A was used for matrix assessment.
An additional set of samples was prepared but not reported, due to suspected contamination of the dilution solvent.

Sample Dilution

45 mL of acetonitrile: water (20:80 v/v) was added to the 5 mL of water and mixed. Samples were vortex mixed for 15 seconds and centrifuged at 13,000 rpm for 5 minutes.

Borehole ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Reagent Blank C	N/A	5	50	10
Control F	N/A	5	50	10
Control G-H	N/A	5	50	10
F0.1 K-O	0.1	5	50	10
F1 K-O	1	5	50	10

N/A = Not applicable.

Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Reagent Blank B	N/A	5	50	10
Control A	N/A	5	50	10
Control D-E	N/A	5	50	10
F0.1 F-J	0.1	5	50	10
F1 F-J	1	5	50	10

N/A = Not applicable.
An additional set of samples was prepared but not reported, due to suspected contamination of the dilution solvent.

Instrument Conditions

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

LC Parameters:

Instrument:	Shimadzu Nexera series HPLC system		
Column#:	Waters XBridge BEH C18, 2.5 µm, 2.1 × 50 mm		
Mobile Phase A#:	0.1% Formic acid in water		
Mobile Phase B#:	0.1% Formic acid in acetonitrile		
Flow Rate:	0.35 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	75	25
	0.50	75	25
	4.00	0	100
	6.00	0	100
	6.10	75	25
	7.50	75	25
Run Time:	7.5 minutes		
Column Temperature:	40°C		
Autosampler Temperature:	4°C		
Injection Volume:	50 µL		
Retention Time:	Approx. 3.0 minutes		
Valco Valve Diverter:	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	6.5	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		
Declustering Potential (DP):	50		
Collision Exit Potential (CXP)	15		
Resolution Q1/Q3:	Unit/Unit		
Transition Name:	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Cumyluron (Primary):	303.0/185.3	17	250
Cumyluron (Confirmatory):	303.0/125.2	41	250

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

When the calibration fit is linear as in this study, Analyst 1.6.2 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 (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 sample dilution factor × calibration standard concentration (µg/L) / 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 concentration (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 Cumyluron:

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) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if no significant interferences at the retention time of Cumyluron were found in the control samples at > 30% of the LOQ peak height response.

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ concentration (after dilution). The highest calibration standard concentration was ≥ 120% of the 10 × LOQ concentration (after dilution). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

LOD (Limit of Detection) Assessment
An estimate of the LOD was made at 3 × baseline noise of the control samples for primary and confirmatory transitions for Cumyluron.

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 0.002 µg/L and a dilution factor of 10).

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in acetonitrile: water (20:80 v/v) and in control water final extract. This was assessed for Cumyluron for both the primary and confirmatory transitions.

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

A difference of ≥ 20% was considered significant.

If matrix effects were determined to be significant, matrix-matched calibration standards would be used for method validation.