INTRODUCTION

The objective of this study was to independently validate the analytical method in Study No. 14102.6121, for measuring residues of Cumyluron in soil, in accordance with EPA OCSPP 850.6100 (2012) and SANCO/3029/99 rev 4 (2000) guidelines.

The analytical method (Study No. 14102.6121) 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 3202654-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202654-01V when validation was complete.

Control samples of sandy loam and clay soil were fortified with Cumyluron at 50 and 500 µg/kg in quintuplicate and analysed. Samples were extracted twice with acetonitrile: water (80:20 v/v). An aliquot was diluted into calibration range with acetonitrile: water (20:80 v/v).

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

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

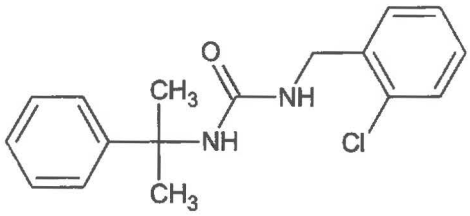
STUDY TIMETABLE

Study initiation:	07 July 2020 (date the protocol was signed by the Study Director).
Experimental start:	09 July 2020 (soil moisture).
Experimental completion:	29 July 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
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 sandy loam and clay soil were sourced by Smithers ERS. The soils used were CS04/20 (Speyer 2.3) and CS06/20 (South Witham).

Soil characterisation data are listed in the following table:

Soil Name	Unique ID	Textural class[1]	% Sand, Silt, Clay[2]	CEC (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
Speyer 2.3	CS04/20	sandy loam[3]	59, 33, 7[3]	6.8[3]	0.7[3]	7.0	6.1[3]
South Witham	CS06/20	clay	34, 23, 43	24.8	2.9	8.1	7.5

[1, 2] USDA classification.
[3] Soil characterisation data provided by LUFA Speyer.

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

The moisture contents of the soils were determined to be 9.89 % and 12.02 % of the dry soil weight for Speyer 2.3 and South Witham soil respectively.

Reagents

• Acetonitrile	HPLC grade, Honeywell
• Water	Milli-Q (with LC-PAK 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 × 50 mm
- Analytical balance
- Centrifuge: Beckman Coulter Allegra X-15R
- Centrifuge tubes
- Glass jars
- Orbital shaker: Edmund Buhler SM 30 A
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

Analytical method 14102.6121 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 3202654-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202654-01V 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 (80:20 v/v)

800 mL acetonitrile was mixed with 200 mL water.

Acetonitrile: water (20:80 v/v)

200 mL acetonitrile was mixed with 800 mL 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 as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]	Stock Use
Stock 1	10.17	99.96	Acetonitrile	10.166	1000	Secondary stock solution
Stock 2	10.46			10.456	1000	

[1] Corrected for Purity.

Duplicate stocks were prepared for correlation purposes.

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months.

Secondary stock solutions

Secondary stock solutions 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 and fortification at 10 × LOQ

Secondary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of one month.

Sub-Stocks

Sub-stock solutions of Cumyluron in acetonitrile 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	1	Acetonitrile	10	1	Fortification at LOQ
10	0.1		10	0.1	Sub-stock solution
0.1	0.1		1[1]	0.01[2]	Intermediate calibration standard

[1] The final volume of sub-stock solution was scaled as appropriate using the required volume and concentration of secondary stock solution.

[2] 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 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 as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.05	Acetonitrile: water (20:80 v/v)	10	0.05
10	0.05		10	0.05
10	0.05		10	0.05

Preparation of Matrix-Matched Standards for Matrix Assessment
Matrix-matched standards of Cumyluron were prepared in control soil final extract as shown in the table below:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.05	Sandy loam soil final extract	10	0.05
10	0.05		10	0.05
10	0.05		10	0.05
10	0.05	Clay soil final extract	10	0.05
10	0.05		10	0.05
10	0.05		10	0.05

The three matrix-matched standards for each soil were analysed alternately with the 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 sandy loam and clay soil 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
0.2	0.05		1	0.01
0.1	0.075		1	0.0075
0.1	0.05		1	0.005

Different stock concentrations and volumes may have been used to achieve the same final concentration.

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±0.05 g dry weight equivalent of soil was weighed into a Polypropylene centrifuge tube. Quintuplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with a stock solution of Cumyluron. Duplicate control soil samples and a reagent blank (no soil) were also prepared, as described in the following tables:

Sandy loam soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank A[1]	N/A	N/A	N/A	N/A
Control A[2]	5	N/A	N/A	N/A
Control C-D	5	N/A	N/A	N/A
F0.05 A-E	5	1	0.25	50
F0.5 A-E	5	10	0.25	500

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control A was used for matrix assessment.

Clay soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
Reagent Blank B[1]	N/A	N/A	N/A	N/A
Control B[2]	5	N/A	N/A	N/A
Control E-F	5	N/A	N/A	N/A
F0.05 F-J	5	1	0.25	50
F0.5 F-J	5	10	0.25	500

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control B was used for matrix assessment.

Sample Extraction

The samples were extracted twice with 20 mL acetonitrile: water (80:20 v/v) by sonicating for 10 minutes, shaking at 200 rpm for 30 minutes and centrifuging at 3000 rpm for 10 minutes. The supernatant was transferred into a glass jar and made to 50 mL with acetonitrile: water (80:20 v/v) in a volumetric flask. The sample extract was diluted into calibration range with acetonitrile: water (20:80 v/v). This was transferred into an HPLC vial for analysis. The extraction and dilution procedure is summarised in the following tables:

Sandy loam soil

Sample ID	Fortified Concentration (µg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank A	N/A	N/A	50	0.1-10	1000
Control A	N/A	5	50	0.1-10[1]	1000
Control C-D	N/A	5	50	0.1-10	1000
F0.05 A-E	50	5	50	0.1-10	1000
F0.5 A-E	500	5	50	0.02-10	5000

N/A = Not applicable.
[1] Three dilutions were prepared for matrix-matched standards.

Clay soil

Sample ID	Fortified Concentration (µg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank B	N/A	N/A	50	0.1-10	1000
Control B	N/A	5	50	0.1-10[1]	1000
Control E-F	N/A	5	50	0.1-10	1000
F0.05 F-J	50	5	50	0.1-10	1000
F0.5 F-J	500	5	50	0.02-10	5000

N/A = Not applicable.
[1] Three dilutions were prepared for matrix-matched standards.

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 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/kg)
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/kg)
S = concentration added to fortified sample (µg/kg)

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/kg) = 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/kg) = lowest calibration standard (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 final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract 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 0.005 µg/L and a dilution factor of 1000).

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 each control soil final extract. This was assessed for the primary and confirmatory transitions of Cumyluron.

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.