Final Report

Study Title	Independent Laboratory Validation of Analytical Method 14125.6100 for the Determination of Novaluron Degradates CPU and CLA in Water
Study Guideline(s)	OCSPP 850.6100 OPPTS 860.1340

SMITHERS

INTRODUCTION

The objective of this study was to independently validate the analytical method in Study No. 14125.6100, for measuring residues of Novaluron degradates CPU and CLA in aqueous matrices, in accordance with EPA OCSPP 850.6100 (2012) and OPPTS 860.1340 (1996) guidelines.

The analytical method (Study No. 14125.6100) 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 3202770-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202770-01V when validation was complete.

Control samples of ground and surface water were fortified with CPU and CLA at 0.1 and 1 µg/L in quintuplicate and analysed. Samples were diluted 1:1 v/v with acetonitrile and then diluted into calibration range with acetonitrile: water (50:50 v/v).

To assess matrix effects, triplicate standards were prepared in acetonitrile: water (50:50 v/v) and in control water final extract.

Samples were analysed for CPU and CLA 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 CPU and CLA. One primary and one confirmatory LC-MS/MS transition were analysed for CPU and CLA.

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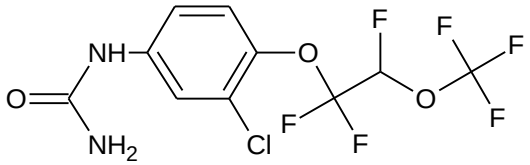
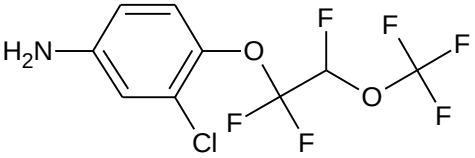
MATERIALS AND METHODS

Protocol Adherence

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

Test Substances

Test Substance Name:	**CPU**
IUPAC Name:	*N*-{3-chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]phenyl}urea
Structure:	
Molecular Formula:	$C_{10}H_7ClF_6N_2O_3$
Molecular Weight:	352.6 g/mol
Lot Number:	554-187-01
Purity:	99.50%
Storage Conditions	Room temperature (15-25°C)
Retest Date:	10 August 2022

Test Substance Name:	**CLA**
IUPAC Name:	3-chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]aniline
Structure:	
Molecular Formula:	$C_9H_6ClF_6NO_2$
Molecular Weight:	309.6 g/mol
Lot Number:	554-136-01
Purity:	97.33%
Storage Conditions:	Room temperature (15-25°C)
Retest Date:	10 August 2022

Test Matrices
Control ground and surface water were sourced by Smithers ERS. The waters used were CS38/20 Borehole ground water and CS01/20 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

Reagents
- Acetonitrile — HPLC grade, Honeywell
- 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 14125.6100 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 3202770-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMV 3202770-01V 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 (50:50 v/v)
250 mL acetonitrile was mixed with 250 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 CPU and CLA were prepared at 1000 µg/mL in acetonitrile as described in the following table:

Stock ID	Test substance	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]	Stock Use
Stock 1	CLA	10.43	97.33	Acetonitrile	10.156	1000	Secondary stock solution
Stock 2		10.45			10.175	1000	
Stock 1	CPU	10.19	99.5		10.139	1000	
Stock 2		10.23			10.179	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 CPU and CLA were prepared as described in the following table:

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

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

Sub-Stock Solutions

Sub-stock solutions of CPU and CLA were prepared as described in the following table:

Test substance name	Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)	Stock Use
CPU	10	0.01	Acetonitrile	10	0.01	Fortification at LOQ and 10 x LOQ
CLA	10	0.01				
Mixed	0.01	1		10	0.001	Intermediate calibration standard

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 CPU and CLA were prepared in acetonitrile: water (50:50 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)
1	0.25	Acetonitrile: water (50:50 v/v)	5	0.05
1	0.25		5	0.05
1	0.25		5	0.05

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

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.25	Ground water final extract	5	0.05
1	0.25		5	0.05
1	0.25		5	0.05
1	0.25	Surface water final extract	5	0.05
1	0.25		5	0.05
1	0.25		5	0.05

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 CPU and CLA 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)
1	0.5	Acetonitrile: water (50:50 v/v)	10	0.05
0.05	0.8		1	0.04
0.05	0.6		1	0.03
0.05	0.4		1	0.02
0.05	0.2		1	0.01
0.05	0.15		1	0.0075
0.05	0.1		1	0.005
0.05	0.06		1	0.003

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 CPU and CLA. 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/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank A	5	N/A	N/A	N/A
Control E+F	5	N/A	N/A	N/A
F0.1 A-E	5	10	0.05	0.1
F1 A-E	5	10	0.5	1

N/A = Not applicable.
Additional controls were prepared for the initial matrix assessment, which was not reported.

Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B	5	N/A	N/A	N/A
Control G+H	5	N/A	N/A	N/A
F0.1 F-J	5	10	0.05	0.1
F1 F-J	5	10	0.5	1

N/A = Not applicable.
Additional controls were prepared for the initial matrix assessment, which was not reported.

Sample Dilution

5 mL of acetonitrile was added to the 5 mL of water and mixed. Samples were diluted further into the calibration range using acetonitrile: water (50:50 v/v).

Borehole ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank A	N/A	5	10	N/A	10
Control E+F	N/A	5	10	0.2-1	10
F0.1 A-E	0.1	5	10	0.2-1	10
F1 A-E	1	5	10	0.08-1	25

N/A = Not applicable.

Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank B	N/A	5	10	N/A	10
Control G+H	N/A	5	10	0.2-1	10
F0.1 F-J	0.1	5	10	0.2-1	10
F1 F-J	1	5	10	0.08-1	25

N/A = Not applicable.

Instrument Conditions

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

LC Parameters:

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	0.3 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	70	30
	0.5	70	30
	1.5	40	60
	4.0	0	100
	5.0	0	100
	5.1	70	30
	6.1	70	30
Run Time	6.1 minutes		
Column Temperature	40°C		
Autosampler Temperature	4°C		
Injection Volume	20 µL		
Retention Time	Approx. 2.5 minutes (CPU) Approx. 2.9 minutes (CLA)		
Valco Valve Diverter	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.5	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	5000 V			
Collision Gas (CAD)	5			
Curtain Gas (CUR)	25			
Gas Flow 1 (GS1)	50			
Gas Flow 2 (GS2)	50			
Vaporiser Temperature (TEM)	500°C			
Interface Heater (ihe)	On			
Entrance Potential (EP)	10			
Collision Exit Potential (CXP)	13			
Compound Name	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
CPU (Primary)	353.0/275.1	90.0	35.0	400
CPU (Confirmatory)	353.0/108.0	90.0	55.0	200
CLA (Primary)	310.0/108.0	90.0	45.0	100
CLA (Confirmatory)	310.0/127.0	90.0	45.0	100

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

To optimise instrument sensitivity, the CPU and CLA transitions were monitored in a separate sequence. This was not specified in the method validated in study 14125.6100, however a more sensitive instrument was used in that study.

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/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 CPU and CLA:

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 were found in the control samples at > 20% of the LOQ or > 50% of the MDL peak height response at the retention time of CPU or CLA.

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 30% of the equivalent LOQ concentration (after dilution) and the highest calibration standard concentration was ≥ 120% of the 10 × LOQ concentration (after dilution). The correlation coefficient (r) was acceptable if it was ≥ 0.9975.

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

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 0.003 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 (50:50 v/v) and in control water final extract. This was assessed for CPU and CLA 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.

Solvent calibration lines were used for both the ground water and surface water validations.

The lowest calibration point was equivalent to an initial sample concentration of 0.03 µg/L (using a dilution factor of 10), which is 30% of the LOQ concentration after dilution.

The highest calibration point was equivalent to 1.25 µg/L (using a dilution factor of 25), which is ≥ 120% of the 10 × LOQ concentration after dilution.

Limit of Quantification (LOQ)
The LOQ based upon the lowest level validated was confirmed to be 0.1 µg/L for CPU and CLA in ground water and surface water.

Limit of Detection (LOD)
The LOD based upon the sample concentration equivalent to 3 × baseline noise was calculated in ground water and surface water for CPU and CLA (primary and confirmatory). The LOD values are presented in the summary tables at the beginning of the results section.

Method Detection Limit (MDL)
The MDL for CPU and CLA was calculated to be equivalent to an initial sample concentration of 0.03 µg/L (based upon the lowest standard concentration of 0.003 µg/L and a dilution factor of 10).

Matrix Effects
An assessment of matrix effects was made by comparison of peak areas from standards prepared using acetonitrile: water (50:50 v/v) and control water final extract. The difference from the mean non-matrix standard peak areas was calculated.

The initial matrix assessment for the CPU primary transition was inconclusive due to poor sensitivity resulting in unacceptable precision in ground and surface water. The matrix assessment was repeated after re-tuning the instrument to improve sensitivity and acceptable precision was obtained for both transitions of CPU and CLA.

Matrix effects were insignificant (< 20% difference from non-matrix standards) for CPU and CLA for both mass transitions in ground and surface water. Therefore, non-matrix matched calibration standards were used for the validation of ground and surface water, which is in agreement with the method validated in study 14125.6100.

Validation Attempts

Surface water

The first validation attempt for CPU and CLA in surface water passed the method validation acceptance criteria.

Ground water

The first validation attempt for CLA in ground water passed the method validation acceptance criteria. However, the lowest calibration point for CPU was excluded as an outlier. Therefore, the required MDL was not demonstrated. The same samples were re-injected the next day with freshly prepared calibration standards and the calibration line was acceptable. As the same vials of samples were injected, this was not considered to be a second validation attempt. Therefore, the first validation attempt for CLA in ground water was considered to have passed the method validation acceptance criteria.

PERFORMANCE CRITERIA

The method validation for CPU in Borehole ground water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	Confirmatory
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.9975.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (< 20% difference from non-matrix standards).	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.1 and 1 µg/L; 0.1 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analysed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 20% of the LOQ.	All blank sample values were < 20% of the LOQ (0.1 µg/L).	All blank sample values were < 20% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be estimated as the sample concentration equivalent to three times the baseline height in the control samples.	0.01944 µg/L	0.01511 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.03 µg/L	0.03 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 353.0/275.1 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 353.0/108.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for CLA in Borehole ground water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.9975.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (< 20% difference from non-matrix standards).	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.1 and 1 µg/L; 0.1 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analysed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 20% of the LOQ.	All blank sample values were < 20% of the LOQ (0.1 µg/L).	All blank sample values were < 20% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be estimated as the sample concentration equivalent to three times the baseline height in the control samples.	0.00302 µg/L	0.00870 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.03 µg/L	0.03 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 310.0/108.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 310.0/127.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for CPU in Fountains Abbey surface water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.9975.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (< 20% difference from non-matrix standards).	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.1 and 1 µg/L; 0.1 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ	10×LOQ, 1 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analysed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 20% of the LOQ.	All blank sample values were < 20% of the LOQ (0.1 µg/L).	All blank sample values were < 20% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be estimated as the sample concentration equivalent to three times the baseline height in the control samples.	0.02317 µg/L	0.01001 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.03 µg/L	0.03 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 353.0/275.1 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 353.0/108.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for CLA in Fountains Abbey surface water met the performance criteria as presented in the following table:

Criterion	Acceptable Limits	Study Performance	
		Primary	**Confirmatory**
Specificity	Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.	No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance.
Linearity: Correlation Coefficient	The data should have a correlation coefficient (r) of not less than 0.9975.		
Matrix Effects	Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the comparison of solvent-based and matrix-matched standards.	Matrix-matched and solvent-based standards were prepared and analysed. Matrix effects were insignificant (< 20% difference from non-matrix standards).	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Accuracy: Test Concentrations	The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing.	This portion of the study was performed at levels of 0.1 and 1 µg/L; 0.1 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable.	LOQ, 0.1 µg/L:	LOQ, 0.1 µg/L:
		10×LOQ, 1 µg/L:	10×LOQ, 1 µg/L:
Precision: Repeatability of Recovery	Five determinations will be made at each fortification level.	Five replicates were prepared and analysed for each of the two fortification levels.	
Limit Of Quantitation (LOQ)	Blank values (reagent blanks and untreated control samples) should not exceed 20% of the LOQ.	All blank sample values were < 20% of the LOQ (0.1 µg/L).	All blank sample values were < 20% of the LOQ (0.1 µg/L).
Limit Of Detection (LOD)	The LOD will be estimated as the sample concentration equivalent to three times the baseline height in the control samples.	0.00339 µg/L	0.00751 µg/L
Method Detection Limit (MDL)	The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.	0.03 µg/L	0.03 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 310.0/108.0 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 310.0/127.0 amu Meets all method and guideline specifications outlined in this table.

Appendix 3
Analytical Procedure

Analytical Procedure

Procedure Title	Determination of Novaluron Degradates CPU and CLA in Ground Water and Surface Water by LC-MS/MS
Procedure Code	SMV 3202770-01V
Issue Date	17 September 2020
Page Number	1 of 11

The methodology described in this procedure has been validated in Borehole Ground Water and Fountains Abbey Surface Water at 0.1 and 1 µg/L.

SMITHERS

REVISION HISTORY

SMV 3202770-01D	New method for independent laboratory validation based upon Smithers ERS, Wareham study 14125.6100.
SMV 3202770-01V	Method re-issued following validation

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining concentrations of Novaluron degradates CPU and CLA in ground water and surface water by LC-MS/MS. Water samples are diluted with acetonitrile. An aliquot is diluted into calibration range with acetonitrile: water (50:50 v/v) and quantified using LC-MS/MS.

Matrix effects for CPU and CLA in ground and surface water will be determined by comparing peak areas of calibration standards prepared in control water final extract and acetonitrile: water (50:50 v/v). Matrix effects are considered significant if the matrix matched standard area is ≥ 20% different from the non-matrix standard area. Provide that matrix effects are insignificant, non-matrix matched calibration standards should be used.

CPU and CLA may contaminate re-usable glassware, therefore disposable glassware should be used where possible, to avoid cross-contamination.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- 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

Equivalent equipment may be used if required

Materials

• Acetonitrile	HPLC grade, Honeywell
• 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

Equivalent materials may be used if required

Reagents

Acetonitrile: water (50:50 v/v)

Mix 250 mL HPLC grade acetonitrile with 250 mL Milli-Q water.

Reagent volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stock

Separately prepare duplicate stock solutions of CPU and CLA at 1000 µg/mL in acetonitrile. Accurately weigh ≥ 10 mg test substance, corrected for purity and transfer into a 10 mL volumetric flask. Adjust the volume to give exactly 1000 µg/mL. Transfer into an amber glass bottle. The primary stocks should be stored refrigerated and given a nominal expiry date of 3 months.

Standard Correlation

Dilute the duplicate primary stocks to the mid-point of the calibration line. Correlate the standard solutions by injecting each of the two calibration standards 5 times into the LC-MS/MS. Ensure that the two solutions are injected alternately in the run sequence. The results for the correlation should be ± 5% of the overall mean calculated by peak areas.

Review of Results

Review the data and document the correlation calculations. If the correlation is out of specification, either repeat the injections, re-dilute, or prepare two new stock standards and repeat the procedures in sections *<<Primary Standard Stock>>* to *<<Review of Results>>*.

If the acceptance criteria from section <<*Standard Correlation*>> have been met, then the calibration solutions are acceptable for use. If required, fortification solutions for method validation will be made from the same stock standard, or its dilutions, from which the calibration line has been prepared.

Secondary Standard Stocks

Prepare secondary stock solutions of CPU and CLA in acetonitrile. The following dilution scheme is suggested:

Test substance	Primary stock concentration (µg/mL)	Volume taken (mL)	Solvent	Final volume (mL)	Secondary Stock Concentration (µg/mL)
CPU	1000	0.1	Acetonitrile	10	10
CLA	1000	0.1		10	10

Transfer into amber glass bottles. The secondary stocks should be stored refrigerated and given a nominal expiry date of 1 month.

Sub-Stocks

Prepare sub-stock solutions of CPU and CLA in acetonitrile. The following dilution scheme is suggested:

Test Substance	Fortifying stock concentration (µg/mL)	Volume taken (mL)	Solvent	Final volume (mL)	Concentration (µg/mL)[1]
CPU	10	0.01	Acetonitrile	10	0.01
CLA	10	0.01			
Mixed	0.01	1		10	0.001[2]

[1] Mixed stock of CPU and CLA.
[2] Equivalent to 1 µg/L

Transfer into disposable glass vials. The sub-stock solutions should be prepared on the day of use.

Matrix Matched Standards for Matrix Assessment

Prepare ground water or surface water matrix matched standards of CPU and CLA in disposable glass vials as described in the following table:

Stock concentration (µg/L)	Volume taken (mL)	Solvent	Final volume (mL)	Concentration (µg/L)
1	0.05	Control water final extract	5	0.01
1	0.05		5	0.01
1	0.05		5	0.01

Non-Matrix Matched Standards for Matrix Assessment

Prepare non-matrix matched standards of CPU and CLA in acetonitrile: water (50:50 v/v). The following dilution scheme is suggested:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.25	Acetonitrile: water (50:50 v/v)	5	0.05
1	0.25		5	0.05
1	0.25		5	0.05

Calibration Standards

Prepare calibration standards of CPU and CLA in acetonitrile: water (50:50 v/v). The following dilution scheme is suggested:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.5	Acetonitrile: water (50:50 v/v)	10	0.05
0.05	0.8		1	0.04
0.05	0.6		1	0.03
0.05	0.4		1	0.02
0.05	0.2		1	0.01
0.05	0.15		1	0.0075
0.05	0.1		1	0.005
0.05	0.06		1	0.003

A single set of calibration standards should be prepared for each validation batch and injected twice, interspersed with and bracketing the samples.

PROCEDURES

All procedures will be carried out in compliance with departmental SOPs, following departmental safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]

Measure 5 mL of either ground water or surface water into a glass vial. Fortify samples using the sub-stocks of CPU and CLA in acetonitrile as shown in the following table:

Number of Replicates	Sample Type	Stock Concentration (µg/L)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
1	Reagent blank[1]	N/A	N/A	5	N/A
2	Control	N/A	N/A	5	N/A
5	LOQ	10	0.05	5	0.1
5	10 × LOQ	10	0.5	5	1

N/A = Not Applicable.

[1] Use Milli-Q water as the reagent blank.

Sample Dilution [1b, 4a]

1. Measure 5 mL of water into a glass jar.
2. Fortify the samples as shown above.
3. Add 5 mL of acetonitrile to each sample.
4. Mix well.
5. Dilute further into the calibration range using acetonitrile: water (50:50 v/v) or control water final extract if matrix matching.
6. Transfer into an HPLC vial for analysis.

Sample type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank	N/A	5	10	N/A	10
Control	N/A	5	10	0.2-1	10
LOQ	0.1	5	10	0.2-1	10
10 × LOQ	1	5	10	0.08-1	25

N/A = Not Applicable.

LC-MS/MS CONDITIONS

HPLC Parameters:

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	0.3 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	70	30
	0.5	70	30
	1.5	40	60
	4.0	0	100
	5.0	0	100
	5.1	70	30
	6.1	70	30
Run Time	6.1 minutes		
Column Temperature	40°C		
Autosampler Temperature	4°C		
Injection Volume	20 µL		
Retention Time	Approx. 2.9 minutes (CPU)		
	Approx. 3.2 minutes (CLA)		
Valco Valve Diverter	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.5	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	5000 V			
Collision Gas (CAD)	5			
Curtain Gas (CUR)	25			
Gas Flow 1 (GS1)	20			
Gas Flow 2 (GS2)	10			
Vaporiser Temperature (TEM)	500°C			
Interface Heater (ihe)	On			
Entrance Potential (EP)	10			
Collision Exit Potential (CXP)	13			
Compound Name	MRM Transition Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (ms)
CPU (Primary)	352.8/275.2	91.0	35.0	50
CPU (Confirmatory)	352.8/108.2	91.0	53.0	50
CLA (Primary)	309.7/108.0	86.0	45.0	50
CLA (Confirmatory)	309.7/127.0	86.0	50.0	50

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

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where 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 extract.

$$x = \frac{(y - c)}{m} \times DF$$

Where:-

x = concentration of test substance in sample (µg/L)
y = area of peak due to test substance
m = gradient
c = Y intercept on calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/L)
S = concentration added to fortified sample (µg/L)

METHOD CRITERIA

For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.9975 with a 1/x weighting.
- All sample extracts should be within the appropriate range of calibration standards.
- Mean recovery from fortified samples should be within the range of 70 to 110% at each concentration.
- Precision of fortified sample recoveries should be ≤ 20% RSD at each concentration.
- The control sample should not contain interference > 30% of the LOQ at the retention time of the test substance.

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main Division	CONTROL Name and Specification
1	GLOVES
a	Disposable latex
b	Disposable nitrile
c	Rubber gloves
d	Specific type for the job (see assessment giving details)
2	PROTECTIVE CLOTHING
a	Laboratory coat or equivalent
b	Disposable overalls
c	Oversleeves
d	Overshoes
e	Plastic apron
3	EYE/FACE PROTECTION
a	Safety glasses to BS 2092/2 C or better
b	Face shield to BS 2092/2 C or better
c	Safety goggles to BS 2092/2 C or better
4	ENGINEERING CONTROLS
a	Open bench in ventilated area
b	Fume cupboard to BS 7258
c	Laminar flow cabinet to BS 5295 Class 1
d	Re-circulating fume chamber
e	Radioisotope lab
f	Biohazard lab
g	Glove box
5	RESPIRATORY PROTECTIVE EQUIPMENT
a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
c	Respirator with specified canister (HSE approved)
6	SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7	ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8	REFER TO MATERIAL SAFETY DATA SHEET
9	KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10	POISON – ensure antidote is available and is within its expiry date (must specify details)