INTRODUCTION

The objective of this study was to independently validate the analytical method 14125.6100, for measuring residues of Novaluron and its degradates CLA and CPU in surface and ground water, in accordance with EPA 850.6100 (2012) and SANCO/825/00 rev.8.1 (2010) guidelines.

Analytical method 14125.6100 was provided by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3201701-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was used for method validation, and re-issued as SMV 3201701-01V when validation was complete.

Control samples of Borehole ground water and Fountains Abbey surface water were fortified with Novaluron, CLA and CPU at 0.1 and 1 µg/L in quintuplicate and analysed. Samples were diluted with acetonitrile. An aliquot was diluted into calibration range with acetonitrile: water (1:1 v/v).

To assess matrix effects, calibration standards were prepared in control extract and in acetonitrile: water (1:1 v/v).

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

The study was initiated on 13 April 2018 (date the protocol was signed by the Study Director) and completed on the date the final report was signed by the Study Director. The practical phase of the study was conducted by Smithers Viscient (ESG) and was started on 18 April 2018 (stock preparation) and completed on 14 May 2018 (LC-MS/MS analysis).

MATERIALS AND METHODS

Test Substances

Test Substance Name:	**Novaluron Technical**
CAS Number:	116714-46-6
Molecular Formula:	$C_{17}H_9ClF_8N_2O_4$
Molecular Mass:	492.706 g/mol
Purity:	100.0 %
Batch Number:	96869065
Storage Conditions:	Room Temperature (15-30°C)
Expiry Date:	12 August 2021

Test Substance Name:	**CPU TGAI (Novaluron Degradate)**
Molecular Formula:	$C_{10}H_7ClF_6N_2O_3$
Molecular Mass:	352.62 g/mol
Purity:	86.9%
Lot Number:	554-187-04
Storage Conditions:	Room Temperature (15-30°C)
Retest Date:	07 June 2018

Test Substance Name:	**CLA TGAI (Novaluron Degradate)**
Molecular Formula:	$C_9H_6ClF_6NO_2$
Molecular Mass:	309.59 g/mol
Purity:	98.9%
Batch Number:	554-136-01
Storage Conditions:	Room Temperature (15-30°C)
Retest Date:	03 March 2019

Certificates of Analysis for the test substances are presented in Appendix 1.

Test System

Control samples of water were sourced by Smithers Viscient (ESG). The waters used were CS 14/18 Fountains Abbey surface water and CS 13/18 Borehole ground water.

Water characterisation data are listed in the following table:

Water Name	Water Type	Suspended Solids (mg/L)	Conductivity (µS/cm)	Hardness (mg/L $CaCO_3$)	pH	Dissolved Organic Carbon (mg/L)
Fountains Abbey	Surface	34	154	86	7.44	11.2
Borehole Water	Ground	2	436	349	8.0	0.00

The certificates of analysis for the water are presented in Appendix 2.

Reagents

Acetonitrile	HPLC grade, Honeywell
Water	Milli-Q with LCPAK polisher, In House
0.1% Formic acid in water	MS grade, Honeywell
0.1% Formic acid in acetonitrile	MS grade, Honeywell

Equivalent or better reagents may have been used.

Equipment

Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.

Analytical Method

Analytical method 14125.6100 was provided by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3201701-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was used for method validation, and re-issued as SMV 3201701-01V when validation was complete.

Preparation of Reagents

Acetonitrile: water (50:50 v/v) was prepared by mixing 500 mL HPLC grade acetonitrile with 500 mL water.

Preparation of Stock Solutions

Primary stock solutions of Novaluron, CPU and CLA were prepared as described in the following table:

Stock ID	Test Substance	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Stock 1	Novaluron	10.50	100.0	Acetonitrile	10.50	1000
Stock 2		10.94	100.0		10.94	1000
Stock 7		10.40	100.0		10.40	1000
Stock 8		10.62	100.0		10.62	1000
Stock 3	CLA	10.47	98.9		10.355	1000
Stock 4		10.36	98.9		10.246	1000
Stock 5	CPU	11.62	86.9		10.0982	1000
Stock 6		11.82	86.9		10.272	1000

[1] Corrected for Purity.

Duplicate stocks were prepared for correlation purposes.

Stocks 1 and 2 failed correlation, and were therefore re-prepared. Stock 1 was used for the Novaluron matrix assessment, which was analysed at the same time as the correlation. This matrix assessment was still reported, as the absolute concentrations were not considered to be critical. Additional Novaluron stocks 7and 8 were prepared, correlated and used for method validation.

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

Secondary stock solutions were prepared as described in the following table:

Test Substance	Fortifying Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Novaluron	1000	0.1	Acetonitrile	10	10
CPU	1000	0.1			
CLA	1000	0.1			

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

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

Test Substance	Fortifying Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Novaluron	10	0.01	Acetonitrile	10	10
CPU	10	0.01			
CLA	10	0.01			
Mixed	0.01	1		10	1[1]

[1] Mixed stock of Novaluron, CPU and CLA.

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

Preparation of Calibration Standards

Mixed calibration standards of Novaluron, CPU and CLA were prepared in as described in the following table:

Fortifying Stock Concentration (µg/L)	Volume Taken (mL)	Solvent[1]	Final Volume (mL)	Concentration (µg/L)
10	0.05	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

[1]Matrix matched standards were prepared for CLA in surface water using surface water control extract as the solvent.

Matrix matched calibration standards of Novaluron, CPU and CLA (for analysis of CLA in surface water) were prepared in as described in the following table:

Fortifying Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.05	Fountains Abbey surface water control final extract	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 once before the samples and once after the samples.

Preparation of Matrix Matched Standards for Matrix Assessment

Matrix matched standards of Novaluron, 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	0.05	Fountains Abbey surface water final extract	5	0.01
1	0.05		5	0.01
1	0.05		5	0.01
1	0.05	Borehole ground water final extract	5	0.01
1	0.05		5	0.01
1	0.05		5	0.01
10	0.025	Borehole ground water final extract	5	0.05
10	0.025		5	0.05
10	0.025		5	0.05

Additional matrix matched standards were prepared for Borehole ground water at a higher concentration because the precision of the peak areas was poor for CPU during the initial matrix assessment.

Preparation of Non-Matrix Matched Standards for Matrix Assessment

Non-matrix standards of Novaluron, CPU and CLA were prepared in blank solvent for comparison with matrix matched standards.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.05	Acetonitrile: water (50:50 v/v)	5	0.01
1	0.05		5	0.01
1	0.05		5	0.01
10	0.025	Acetonitrile: water (50:50 v/v)	5	0.05
10	0.025		5	0.05
10	0.025		5	0.05

Additional non-matrix matched standards were prepared at a higher concentration because the precision of the peak areas was poor for CPU in Borehole ground water during the initial matrix assessment.

Sample Fortification

5 mL water was measured into a disposable glass vial. Quintuplicate water samples were fortified at the LOQ (0.1 µg/L) and at 10 × LOQ (1 µg/L) with a mixed stock solution of Novaluron, CPU and CLA. Duplicate control water samples and a reagent blank (without water) were also prepared, as described in the following tables:

CS 13/18 Borehole ground water

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank A	N/A	N/A	N/A	N/A
Control A	5	N/A	N/A	N/A
Control C-D	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.
Control A was used to prepare matrix matched standards for matrix assessment.

CS 14/18 Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B	N/A	N/A	N/A	N/A
Control B	5	N/A	N/A	N/A
Control E-F	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.
Control B was used to prepare matrix matched standards for matrix assessment.
Control E was used to prepare matrix matched calibration standards and dilutions for CLA.

Sample Extraction

5 mL acetonitrile was added to the 5 mL of water and mixed well. A portion of extract was diluted with acetonitrile: water (50:50 v/v). A second dilution was performed for the 1 µg/L samples in acetonitrile: water (50:50 v/v) for Novaluron, CPU and CLA in Borehole ground water, and for Novaluron and CPU in Fountains Abbey surface water. The second dilution was performed in control surface water final extract for CLA in Fountains Abbey surface water (matrix matched). The final extract was transferred into an HPLC vial for analysis. Sample extracts were stored refrigerated in case further analysis was required. The extraction procedure is summarised in the following tables:

CS 13/18 Borehole ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Volume of Extract (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank A	N/A	N/A	10	0.2-1	10
Control A	N/A	5	10	4-20[1]	10
				4-20[2]	10
Control C-D	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	0.02-1	100

N/A = Not applicable.
[1] Three aliquots of Control A final extract were used to prepare matrix matched standards for matrix assessment.
[2] An additional dilution of Control A extract was used to prepare matrix matched standards to repeat the matrix assessment for CPU, due to poor precision of peak areas in the initial matrix assessment.

A single set of non-matrix matched calibration standards were prepared and analysed once before the samples and once after the samples in the LC-MS/MS sequence for Borehole ground water validation.

CS 14/18 Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Volume of Extract (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank B	N/A	N/A	10	0.2-1	10
Control B	N/A	5	10	4-20[1]	10
Control E-F	N/A	5	10	0.2-1	10
				4-20[2]	10
F0.1 F-J	0.1	5	10	0.2-1	10
F1 F-J	1	5	10	0.02-1	100
				0.02-0.1 then 0.1-1[3]	100

N/A = Not applicable.
[1] Three aliquots of Control B extract were used to prepare matrix matched standards for matrix assessment.
[2] A larger volume of Control E final extract was used to prepare matrix matched standards and dilutions for CLA.
[3] F1 F-J had a second dilution in Control E final extract for CLA (matrix matched).

A single set of matrix matched and non-matrix matched calibration standards were prepared and analysed once before the samples and once after the samples in the same LC-MS/MS sequence for Fountains Abbey surface water validation.

Instrument Conditions

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

LC Parameters:

Column#	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	10°C		
Injection Volume	20 µL		
Retention Time	Approx. 3.0 minutes (Novaluron)		
	Approx. 2.2 minutes (CPU)		
	Approx. 2.6 minutes (CLA)		
Valco Valve Diverter	Time (min)	Position	
	0	A (to waste)	
	1	B (to MS)	
	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)	40			
Gas Flow 2 (GS2)	40			
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)
Novaluron (Primary)	493.1/158.0	81.0	31.0	50
Novaluron (Confirmatory)	493.1/141.1	81.0	65.0	50
CPU (Primary)	353.0/275.4	91.0	35.0	50
CPU (Confirmatory)	353.0/108.3	91.0	60.0	50
CLA (Primary)	310.1/108.0	86.0	45.0	50
CLA (Confirmatory)	310.1/127.1	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.

LC-MS/MS data were collected using Analyst 1.6.2.

Calculation of Results

LC-MS/MS data were calculated using Analyst 1.6.2. The validation data for Fountains Abbey surface water was processed for Novaluron and CPU using the non-matrix matched calibration standards and dilutions, and separately processed for CLA using the matrix matched calibration standards and dilutions. The validation data for Borehole ground water validation contained non-matrix standards and dilutions only.

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 extract:

$x = (y - c) / m$

Where:

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

The concentration of test substance in the sample is calculated as follows:

Sample concentration (µg/L) = Extract concentration (µg/L) × Dilution factor

Dilution factor = Final extract volume (mL) / volume of water in final extract (mL)

Procedural recovery from fortified samples is calculated as follows:

Recovery (%) = Sample concentration / Fortified concentration × 100

95% confidence intervals were calculated for each validation level as follows:

95% confidence interval (±) = $t_{n-1}s/\sqrt{n}$

Where:

t_{n-1} = 2.78
s = standard deviation
n = number of samples (5)

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 = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (µg/mL) / height of calibration standard peak