INTRODUCTION

The objective of this study was to independently validate the analytical method 14125.6101, for measuring residues of Novaluron and its degradates CLA and CPU in two soils of differing USDA Textural Classification, in accordance with EPA 850.6100 (2012) and SANCO/825/00 rev.8.1 (2010) guidelines.

Analytical method 14125.6101 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 3201700-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was used for method validation, and re-issued as SMV 3201700-01V when validation was complete.

Control samples of Brierlow and Speyer 5M soil were fortified with Novaluron, CLA and CPU at 50 and 500 µg/kg in quintuplicate and analysed. Samples were extracted with methanol. 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 soil 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 23 April 2018 (stock dilution) and completed on 10 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 soil with differing USDA Textural Classification were sourced by Smithers Viscient (ESG). The soils used were CS 27/16 Speyer 5M (Sandy loam) and CS 30/16 Brierlow (Silt loam).

Soil characterisation data are listed in the following table:

Soil Name	Textural class[1]	% Sand, Silt, Clay[2]	CEC (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
Brierlow	Silt loam	26, 58, 16	20.0	2.5	6.4	5.6
Speyer 5M	Sandy loam	59, 30, 11	17.7	1.0	8.5	7.3

[1, 2] USDA classification.

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

The moisture content of the soil was determined to be 21.423% for Brierlow and 7.573% for Speyer 5M soil (expressed as a % of the dry soil weight).

Reagents

Acetonitrile	HPLC grade, Honeywell
Methanol	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 14125.6101 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 3201700-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was used for method validation, and re-issued as SMV 3201700-01V 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 (under study 3201701: Novaluron- Independent Laboratory Validation of Analytical Method 14125.6100 for the Determination of Novaluron and its Degradates in Water) 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 3	CLA	10.47	98.9		10.355	1000
Stock 5	CPU	11.62	86.9		10.0982	1000

[1] Corrected for Purity.

Duplicate stocks were prepared for correlation purposes, but only stocks used in this study have been presented. Stocks 1 and 2 failed correlation (> 5% from the mean), 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.

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

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	1000	0.1	Acetonitrile	10	10
CPU	1000	0.1			
CLA	1000	0.1			
Mixed	10	0.01		10	0.01

[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 table below:

Fortifying Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.2	Acetonitrile: water (50:50 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

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

Preparation of Matrix Matched Standards for Matrix Assessment

Matrix matched standards of Novaluron, CPU and CLA were prepared in control soil final extract.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.1	Speyer 5M soil final extract	10	0.1
10	0.1		10	0.1
10	0.1		10	0.1
10	0.1	Brierlow soil final extract	10	0.1
10	0.1		10	0.1
10	0.1		10	0.1

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)
10	0.1	Acetonitrile: water (50:50 v/v)	10	0.1
10	0.1		10	0.1
10	0.1		10	0.1

Sample Fortification

The moisture content of the soil was determined. The sample amount equivalent to 5 ±0.05 g dry weight (6.071 ± 0.061 g for Brierlow soil and 5.379 ± 0.054 g for Speyer 5M soil) was weighed into a Teflon tube. Quintuplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with a mixed stock solution of Novaluron, CPU and CLA. Duplicate control soil samples and a reagent blank (without soil) were also prepared, as described in the following tables:

CS 27/16 Speyer 5M soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
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
F50 A-E	5	10	0.025	50
F500 A-E	5	10	0.25	500

N/A = Not applicable.
Control A was used to prepare matrix matched standards for matrix assessment.
Control C was used to prepare matrix matched calibration standards and dilutions for Novaluron and CLA.

CS 30/16 Brierlow soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/kg)
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
F50 F-J	5	10	0.025	50
F500 F-J	5	10	0.25	500

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 Novaluron and CLA.

Sample Extraction

20 mL methanol was added to the soil, placed on a shaker for 30 minutes at 150 rpm and centrifuged for 10 minutes at 3000 rpm (2095 g). The supernatant was transferred into a glass jar and the extraction repeated with a second 20 mL of methanol. The combined extracts were made up to 50 mL with methanol. A portion of extract was diluted with acetonitrile: water (50:50 v/v). A second dilution was performed for the 500 µg/kg samples in acetonitrile: water (50:50 v/v) for CPU and in control soil extract for Novaluron and CLA. A portion of diluted extract was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm (16200 g) for 10 minutes, before transferring 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 27/16 Speyer 5M soil

Sample ID	Fortified Concentration (µg/kg)	Sample Weight (g)	Volume of Extract (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank A	N/A	N/A	50	0.2-10	500
Control A	N/A	5	50	0.02-1[1]	500
				0.2-10[2]	500
Control C-D	N/A	5	50	0.2-10[3]	500
F50 A-E	50	5	50	0.2-10	500
F500 A-E	500	5	50	0.2-10 then 0.1-1[4]	5000

N/A = Not applicable.
[1] One aliquot of Control A was analysed un-fortified with the matrix assessment.
[2] Three aliquots of Control A extract were used to prepare matrix matched standards for matrix assessment.
[3] Two portions of Control C extract were used to prepare matrix matched standards and dilutions for Novaluron and CLA.
[4] F500 A-E had the second dilution in acetonitrile: water (50:50 v/v) for CPU and in Control C final extract for Novaluron and CLA.

CS 30/16 Brierlow soil

Sample ID	Fortified Concentration (µg/kg)	Sample Weight (g)	Volume of Extract (mL)	Sample Dilution (mL to mL)	Dilution Factor
Reagent Blank B	N/A	N/A	50	0.2-10	500
Control B	N/A	5	50	0.02-1[1]	500
				0.2-10[2]	500
Control E-F	N/A	5	50	0.2-10[3]	500
F50 F-J	50	5	50	0.2-10	500
F500 F-J	500	5	50	0.2-10 then 0.1-1[4]	5000

N/A = Not applicable.
[1] One aliquot of Control B was analysed un-fortified with the matrix assessment.
[2] Three aliquots of Control B extract were used to prepare matrix matched standards for matrix assessment.
[3] Two portions of Control E extract were used to prepare matrix matched standards and dilutions for Novaluron and CLA.
[4] F500 F-J had the second dilution in acetonitrile: water (50:50 v/v) for CPU and in Control E final extract for Novaluron and CLA.

Matrix matched and non-matrix matched calibration standards and samples were analysed in the same LC-MS/MS sequence.

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	0.4 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	60	40
	1.0	60	40
	1.1	20	80
	3.0	0	100
	4.6	0	100
	4.7	60	40
	6.0	60	40
Run Time	6 minutes		
Column Temperature	40°C		
Autosampler Temperature	10°C		
Injection Volume	25 µL		
Retention Time	Approx. 1.9 minutes (Novaluron)		
	Approx. 1.6 minutes (CPU)		
	Approx. 1.8 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	65
Novaluron (Confirmatory)	493.1/141.1	81.0	65.0	65
CPU (Primary)	353.0/275.4	91.0	35.0	65
CPU (Confirmatory)	353.0/310.2	50.0	31.0	65
CLA (Primary)	310.1/108.0	86.0	45.0	65
CLA (Confirmatory)	310.1/127.1	86.0	50.0	65

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. Data was processed for Novaluron and CLA using the matrix matched calibration standards and dilutions, and separately processed for CPU using the non-matrix matched calibration standards and dilutions.

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

Dilution factor = Final extract volume (mL) / dry weight of soil in final extract (g)

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

Validation Pass Criteria
The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions or fragment ions monitored for each compound:

Mean Recovery and Precision
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 120% and a %RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if the amounts found in blank samples were ≤ 30% of the LOQ.

Linearity
Linearity was acceptable if the lowest calibration standard concentration was ≤ 30% of the equivalent LOQ final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract concentration (after dilution if applicable). If matrix effects were determined to be significant, matrix matched standards would be used. 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 for primary and confirmatory transitions for all compounds.

MDL (Method Detection Limit)
The MDL was calculated as the sample concentration equivalent to the lowest calibration standard (based upon a lowest standard concentration of 0.01 µg/L and a dilution factor of 500).

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in blank solvent and in each control matrix final extract. This was assessed for all compounds and for the primary and confirmatory transitions.

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

A difference of $\geq$ 20% was considered significant.

Novaluron and CPU were analysed using matrix matched calibration standards to match the primary method, even if matrix effects were not significant. CLA was only analysed using matrix matched calibration standards if matrix effects were significant.