1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of novaluron and its degradates, CPU and CLA, in aqueous solutions. The method was validated (26 to 27 October 2017) to quantify the concentrations of novaluron and its degradates, CPU and CLA, present in recovery samples prepared in ground water and surface water. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in ground water and surface water by fortification with novaluron and its degradates, CPU and CLA, at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. Recovery samples were diluted with acetonitrile and subsequently diluted into the calibration range with 50/50 acetonitrile/purified reagent water (v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of the Analytical Method for the Determination of Novaluron and its Degradates in Aqueous Matrices by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the guidance documents SANCO/825/00 rev. 8.1 (EC, 2010) and OCSPP 850.6100 (U.S. EPA, 2012).

2.1 Test Substances

The test substance, novaluron technical, was received on 9 January 2017 from ADAMA Makhteshim Ltd., Beer-Sheva, Israel. The following information was provided:

Name:	novaluron technical
Lot No.:	96869065
CAS No.:	116714-46-6
Purity:	98.8% (Certificate of Analysis, Appendix 2)
Recertification Date:	1 March 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 8690) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, CPU (novaluron degradate), was received on 7 April 2017 from ADAMA Makhteshim Ltd., Beer-Sheva, Israel. The following information was provided:

Name:	CPU (novaluron degradate)
Synonym:	1-[-3-chloro-4-(1,1,2-trifluoro-2 trifluoromethoxyethoxy)phenyl]urea
Lot No.:	554-187-04
CAS No.:	Not Listed
Purity:	86.9% (Certificate of Analysis, Appendix 2)
Recertification Date:	7 June 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 8853) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, CLA (novaluron degradate), was received on 9 January 2017 from ADAMA Makhteshim Ltd., Beer-Sheva, Israel. The following information was provided:

Name:	CLA (novaluron degradate)
Synonym:	3-chloro-4-(1,1,2-trifluoro-2-trifluoromethoxyethoxy)aniline
Batch No.:	554-136-01
CAS No.:	Not Listed
Purity:	98.9% (Certificate of Analysis, Appendix 2)
Recertification Date:	3 March 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8692) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identities, maintenance of records on the test substances, and archival of a sample of the test substances are the responsibility of the Study Sponsor.

2.2 Reagents

1.	0.1% Formic acid in purified reagent water:	Fisher Chemical, reagent grade
2.	0.1% Formic acid in acetonitrile:	Fisher Chemical, reagent grade
3.	Methanol:	EMD reagent grade
4.	Acetonitrile:	EMD, reagent grade
5.	Purified reagent water:	Prepared from a Millipore MilliQ Direct 8 water purification system (meets ASTM Type II requirements)

2.3 Instrumentation and Laboratory Equipment

1.	Instrument:	Sciex 6500+ QTRAP mass spectrometer equipped with an Sciex IonDrive Turbo V ion source Shimadzu SIL-20ACXR autosampler Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column compartment Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo XS205, Mettler Toledo AG285
3.	Centrifuge:	Beckman 367160
4.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, Teflon centrifuge tubes, graduated cylinders, Pasteur pipets, autosampler vials and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used in future testing but may require optimization to achieve the desired separation and sensitivity.

2.4 Test Matrices

The matrices used during this method validation were ground water and surface water.

Ground water information:

Ground water used in the study was filtered Town of Wareham, Massachusetts well water and was prepared by filtering to remove any potential organic contaminants. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

Surface water information:

The surface water used for this method validation analysis was collected from the Taunton River (SMV Lot No. 18Aug17Wat-B, collected on 18 August 2017) in Taunton, Massachusetts. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water and was determined to have a pH of 7.03 (YSI model pH100 pH meter), a dissolved oxygen concentration of 5.22 mg/L (YSI model Pro20 dissolved oxygen meter), and a total organic carbon concentration of 6.49 mg/L. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

2.5 Preparation of Liquid Reagent Solutions

The volumes listed in this section were those used during the validation. For future testing, the actual volumes used may be scaled up or down as necessary.

A 50/50 acetonitrile/purified reagent water (v/v) liquid reagent solution was prepared by combining 250 mL of acetonitrile and 250 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water. The solution was mixed well before use.

2.6 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

Primary stock solutions were prepared as described in the table below:

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8690S	0.05061	0.05000	Acetonitrile	50.0	1000	Secondary stock solution
8853AH	0.05763	0.05008	Acetonitrile	50.0	1000	Secondary stock solution
8692U	0.05063	0.05007	Acetonitrile	50.0	1000	Secondary stock solution

Secondary stock solutions were prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8690S	1000	0.500	50.0	Acetonitrile	8690S-1	10.0	Sub-stock solution
8853AH	1000	0.500	50.0	Acetonitrile	8853AH-1	10.0	Sub-stock solution
8692U	1000	0.500	50.0	Acetonitrile	8692U-1	10.0	Sub-stock solution

Sub-stock solutions were prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8690S-1	10.0	0.0500	50.0	Acetonitrile	Mix-Stk 1	0.0100	Calibration standards, LOQ-, and High-level recovery samples
8853AH-1		0.0500					
8692U-1		0.0500					
Mix-Stk 1	0.0100	1.00	10.0	Acetonitrile	Mix-Stk 2	0.00100	Calibration standards for matrix effect investigation

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.7 Preparation of Calibration Standards

Calibration standards were prepared in 50/50 acetonitrile/purified reagent water (v/v) by fortifying with the 0.0100 mg/L mixed test substance sub-stock solution to yield concentrations of 0.00500, 0.00750, 0.0100, 0.0200, 0.0300, 0.0400, and 0.0500 µg/L. This procedure is detailed in the table below.

Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Mix-Stk 1	0.0100	0.0200	40.0	0.00500	Std 1
		0.0300	40.0	0.00750	Std 2
		0.0200	20.0	0.0100	Std 3
		0.0200	10.0	0.0200	Std 4
		0.0300	10.0	0.0300	Std 5
		0.0400	10.0	0.0400	Std 6
		0.0500	10.0	0.0500	Std 7

Additional data to extend the low end of the calibration curve to 0.0025 ug/L is included in Appendix 4.

2.7.1 Matrix Effect Investigation

Calibration standards used to assess possible matrix effects were prepared as follows by fortifying with the 0.00100 mg/L mixed test substance sub-stock solution to yield a concentration of 0.0100 µg/L.

Matrix-matched standards

Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Mix-Stk 2	0.00100	0.0400	4.00[a]	0.0100	MM-Std A-g
		0.0400	4.00[a]	0.0100	MM-Std B-g
		0.0400	4.00[a]	0.0100	MM-Std C-g
		0.0400	4.00[b]	0.0100	MM-Std A-s
		0.0400	4.00[b]	0.0100	MM-Std B-s
		0.0400	4.00[b]	0.0100	MM-Std C-s

[a] Diluted with the final fraction of Control B-g in 50/50 acetonitrile/purified reagent water (v/v). See Section 2.9 for extract preparation and dilution procedures.

[b] Diluted with the final fraction of Control B-s in 50/50 acetonitrile/purified reagent water (v/v). See Section 2.9 for extract preparation and dilution procedures.

Non-matrix-matched standards

Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Mix-Stk 2	0.00100	0.0400	4.00	0.0100	Std A
		0.0400	4.00	0.0100	Std B
		0.0400	4.00	0.0100	Std C

[a] Samples were diluted with 50/50 acetonitrile/purified reagent water (v/v). See Section 2.5 for preparation procedures.

2.8 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (ground water and surface water) with novaluron and its degradates, CPU and CLA, at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. Recovery samples for all three matrices were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples per matrix were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water

Sample ID	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
Reagent BLK-1	Reagent Blank	NA[a]	NA	5.00	0.00
A-g & B-g	Control	NA	NA	5.00	0.00
A-g, B-g, C-g, D-g, & E-g	LOQ	0.0100	0.0500	5.00	0.100
A-g, B-g, C-g, D-g, & E-g	High	0.0100	0.500	5.00	1.00

[a] NA = Not Applicable

Surface water

Sample ID	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
Reagent BLK-1	Reagent Blank	NA[a]	NA	5.00	0.00
A-s & B-s	Control	NA	NA	5.00	0.00
A-s, B-s, C-s, D-s, & E-s	LOQ	0.0100	0.0500	5.00	0.100
A-s, B-s, C-s, D-s, & E-s	High	0.0100	0.500	5.00	1.00

[a] NA = Not Applicable

2.9 Dilution of Samples

To minimize the potential for losses of the test substance during processing, the aqueous test samples were not sub-sampled prior to dilution. The first dilution with 100% acetonitrile was performed by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified. The recovery samples were subsequently diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v) prior to analysis according to the tables below.

Ground water

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent BLK-1	Reagent Blank	0.00	5.00	10.0	1.00	5.00	10.0
A-g	Control	0.00	5.00	10.0	1.00	5.00	10.0
B-g	Control	0.00	5.00	10.0	5.00	25.0	10.0
A-g, B-g, C-g, D-g, & E-g	LOQ	0.100	5.00	10.0	1.00	5.00	10.0
A-g, B-g, C-g, D-g, & E-g	High	1.00	5.00	10.0	0.400	5.00	25.0

[a] Diluted with 100% acetonitrile.
[b] Diluted with 50/50 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

Surface water

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent BLK-1	Reagent Blank	0.00	5.00	10.0	1.00	5.00	10.0
A-s	Control	0.00	5.00	10.0	1.00	5.00	10.0
B-s	Control	0.00	5.00	10.0	5.00	25.0	10.0
A-s, B-s, C-s, D-s, & E-s	LOQ	0.100	5.00	10.0	1.00	5.00	10.0
A-s, B-s, C-s, D-s, & E-s	High	1.00	5.00	10.0	0.400	5.00	25.0

[a] Diluted with 100% acetonitrile.
[b] Diluted with 50/50 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable

2.10 Analysis

2.10.1 Instrumental Conditions

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column:	Waters XBridge BEH C18, 2.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% formic acid in reagent grade water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.300	70.0	30.0
0.50	0.300	70.0	30.0
1.50	0.300	40.0	60.0
4.00	0.300	0.00	100
5.00	0.300	0.00	100
5.10	0.300	70.0	30.0
6.10	0.300	70.0	30.0

Run Time: 6.10 minutes
Autosampler Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 20 µL
Retention Time: approximately 3.8 minutes (novaluron)
approximately 2.9 minutes (CPU)
approximately 3.2 minutes (CLA)

MS parameters:

Instrument: Sciex 6500+ QTRAP mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5000 V
Scan Type: MRM
Source Temperature: 500 °C
Curtain Gas: 25.0
Ion Source – Gas 1 / Gas 2: 20.0 / 10.0
Collision Gas: Medium
Collision Cell Entrance Potential: 10.0
Resolution Q1/Q3: Unit/Unit

Analyte	Analysis	Q1/Q3 Mass (amu/amu)	Dwell Time (milliseconds)	Declustering Potential	Collision Energy	Collision Cell Exit Potential
Novaluron	Primary	493.1/158.0	50.0	81.0	31.0	10.0
	Confirmatory	493.1/140.9	50.0	81.0	65.0	12.0
CPU	Primary	353.0/275.2	50.0	91.0	60.0	20.0
	Confirmatory	353.0/108.1	50.0	91.0	40.0	12.0
CLA	Primary	310.1/108.0	50.0	86.0	45.0	18.0
	Confirmatory	310.1/127.2	50.0	86.0	41.0	10.0

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.10.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; calibration standards were interspersed among analysis of the recovery samples, every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 120% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples. RSD values less than 20% were considered acceptable for the recovery samples (with less than 10% considered ideal). Specificity of the method was determined by examination of the control samples for peaks at the same retention times as novaluron and its degradates, CPU and CLA, which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.12 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level.

2.13 Limit of Detection (LOD) and Method Detection Limit (MDL)

The Limit of Detection (LOD) was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

The Method Detection Limit (MDL) was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Section 3.0.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = mx + b$$

$$(2) \qquad DC\,(x) = \frac{(y - b)}{m}$$

$$(3) \qquad A = DC \times DF$$

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The method detection limit (MDL) is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (Equation 4) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(4) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.00500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 10.0)
MDL	=	method detection limit reported for the analysis (0.00500 µg/L × 10.0 = 0.0500 µg/L)

The LOD was calculated using the following equation:

$$(5) \qquad LOD = ((3\times(N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

SN_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10)
LOD	=	limit of detection for the analysis

APPENDIX 3 - METHOD FLOW CHART

Dilute the aqueous samples with 100% acetonitrile

↓

Dilute the samples further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v)

↓

Place in autosampler vials

↓

Analyse by LC-MS/MS