MRID 51561601

Study Title

Independent Laboratory Validation of the Analytical Method For Determination of Novaluron in Aqueous Matrices by LC-MS/MS

Test Guidelines

OCSPP 850.6100
OCSPP 860.1340

1.0 INTRODUCTION

This independent laboratory validation (ILV) study is required by the U.S. EPA under the Guideline for Environmental Chemistry Method and Associated Independent Laboratory Validations OCSPP No. 850.6100 (U.S. EPA, 2012), Residue Analytical Methods OCSPP No. 860.1340 (U.S. EPA, 1996), to confirm that the original analytical method, developed by one laboratory, can be independently validated by a second laboratory. This analytical method was validated by fortification of two water types with novaluron at the limit of quantification (LOQ, 0.0100 µg/L) and 10X LOQ (0.100 µg/L) concentration levels.

The study was initiated on 19 January 2021, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the ILV study was conducted on 22 to 23 January 2021 at Smithers, located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers' archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

The objective of this study is to confirm that the analytical method for novaluron in ground water and surface water, developed by one group, can be independently validated by a second group in the absence of major interaction between the two. This study was performed following the Smithers protocol entitled "Independent Laboratory Validation of the Analytical Method For Determination of Novaluron in Aqueous Matrices by LC-MS/MS" (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA, 2012), and the OSCPP Guideline 860.1340: Residue Analytical Method (U.S. EPA, 1996).

2.2 Test Substance

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

Name:	Novaluron
IUPAC name:	N-((3-chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]phenyl)carbamoyl)-2,6-difluoro-benzmide
Batch No.:	96869065
CAS No.:	116714-46-6
Purity:	100.00%
Re-test Date:	10 August 2022

Upon receipt at Smithers, the test substance (SMV No. 8690) was stored at room temperature in a dark, ventilated cabinet in the original container.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance, and archival of samples of the test substance are the responsibility of the Study Sponsor.

2.3 Reagents

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

2.4 Equipment

1.	Instrument:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACXR autoinjector Shimadzu DGU-20A5R vacuum degassers 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.	Balance:	Mettler Toledo XSE205DU
3.	Centrifuge:	Beckam Coulter Microfuge Centrifuge MFA13A009
4.	Laboratory equipment:	Volumetric flasks, graduated cylinders, disposable glass pipets, disposable glass vials, positive displacement pipets, stir bars, stir plates, sonicator, vortexer, micro centrifuge tubes, amber HPLC vials with crimp caps, and amber glass bottles with Teflon-lined caps

2.5 Test Systems

The matrices used during this method validation were ground water and surface water. The samples were stored refrigerated at a temperature set to maintain 2 to 8 °C.

Ground water information:

Ground water consists of unadulterated water from a 100-meter bedrock well prepared by filtering to remove any potential organic contaminants.

Prior to use, the ground water was characterized by Agvise Laboratories, Northwood, North Dakota:

Parameter	Results
Smithers Batch No.:	GROUND WATER 2019
pH:	7.6
Calcium:	24 ppm
Magnesium:	7.8 ppm
Sodium:	92 ppm
Hardness:	92 mg equivalent CaCO3/L
Conductivity:	0.70 mmhos/cm
Sodium adsorption ratio (SAR):	4.19
Total dissolved solids:	228 ppm
Turbidity:	0.15 NTU

Surface water information:

The surface water used for this method validation analysis was collected from the Weweantic River, West Wareham, Massachusetts, Lot No. 28Dec20WAT-A WEWEANTIC WATER. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water. Prior to use, the surface water was characterized by Agvise Laboratories, Northwood, North Dakota:

Parameter	Results
Smithers Batch No.:	28Dec20WAT-A WEWEANTIC WATER
pH:	6.6
Calcium:	3.0 ppm
Magnesium:	1.4 ppm
Hardness:	14 mg equivalent CaCO3/L
Conductivity:	0.10 mmhos/cm
Total dissolved solids:	52 ppm
Turbidity:	1.47 NTU
Biological oxygen demand:	0.8 ppm
Total organic carbon:	9.2 ppm
Dissolve organic carbon:	7.8 ppm
Nitrogen (total kjeldahl):	0.4 ppm
Nitrogen (nitrate):	0.1 ppm
Nitrogen (nitrile):	Below detection limit of 0.1 ppm
Nitrogen (ammoniacan distillation):	Below detection limit of 0.2 ppm
Total phosphorus (as PO_4^-):	0.1 ppm
Dissolved orthophosphate:	Below detection limit of 0.1 ppm

All documentation relating to the preparation, storage, and handling is maintained by Smithers.

2.6 Preparation of Liquid Reagent and Mobile Phase Solutions

The volumes listed in this section were those used during the independent laboratory 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 typically prepared by combining 200 mL of acetonitrile and 200 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 typically 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.

2.7 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 typically prepared as described in the table below.

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8690AT	0.0250	0.0250	Acetonitrile	25.0	1000	Secondary stock solution
8690AS	0.0200	0.0200	Acetonitrile	20.0	1000	Secondary stock solution

Secondary stock solutions were typically prepared as described in 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
8690AT	1000	0.250	25.0	Acetonitrile	8690AT-1	10.0	Sub-stock solution
8690AS	1000	0.250	25.0	Acetonitrile	8690AS-1	10.0	Sub-stock solution

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (µg/L)	Stock Use
8690AT-1	10.0	0.100	10.0	Acetonitrile	Tech Stk-1	100	Sub-stock solution
Tech Stk-1	0.100	1.00	10.0	Acetonitrile	Tech Stk-2	10.0	Sub-stock solution and 10X LOQ recovery samples
Tech Stk-2	0.0100	1.00	10.0	Acetonitrile	Tech Stk-3	1.00	LOQ-level recovery samples
8690AS-1	10.0	0.100	10.0	Acetonitrile	Ana Stk-1	100	Sub-stock solution
Ana Stk-1	0.100	1.00	10.0	Acetonitrile	Ana Stk-2	10.0	Sub stock solution and calibration standards
Ana Stk-2	0.0100	1.00	10.0	Acetonitrile	Ana Stk-3	1.00	Calibration standards and matrix effects investigation samples

All stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh daily and stored refrigerated for possible future use.

2.8 Preparation of Calibration Standards

2.8.1 Solvent-Based Calibration Standards

Standards were prepared in 50/50 acetonitrile/purified reagent water (v/v) using the 1.00 and 10.0 µg/L sub-stock solutions according to the table below. Following fortification, each solution was vortex-mixed for 15 seconds, and then standards were transferred to amber vials with crimp caps for analysis.

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk-3	1.00	0.0200	10.0	0.00200	Std 1
		0.0500	10.0	0.00500	Std 2
		0.100	10.0	0.0100	Std 3
Ana Stk-2	10.0	0.0200	10.0	0.0200	Std 4
		0.0500	10.0	0.0500	Std 5
		0.0750	10.0	0.0750	Std 6
		0.100	10.0	0.100	Std 7

2.8.2 Matrix Effects Standards

In an effort to observe any potential matrix effects, an aliquot of control sample final dilution for both matrices was fortified with the 1.00 µg/L sub-stock solution in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix-matched (solvent) standards fortified at the same concentration.

Matrix-Matched Standards (ground water)

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk-3	1.00	0.0700	10.0[a]	0.00700	GW-MM-Std A
		0.0700	10.0[a]	0.00700	GW-MM-Std B
		0.0700	10.0[a]	0.00700	GW-MM-Std C

[a] Diluted with Control Sample (14125-6133-02)

Matrix-Matched Standards (surface water)

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk-3	1.00	0.0700	10.0[a]	0.00700	SW-MM-Std A
		0.0700	10.0[a]	0.00700	SW-MM-Std B
		0.0700	10.0[a]	0.00700	SW-MM-Std C

[a] Diluted with Control Sample (14125-6133-15)

Non Matrix-Matched Standards

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk-3	1.00	0.0700	10.0[a]	0.00700	Sol-Std A
		0.0700	10.0[a]	0.00700	Sol -Std B
		0.0700	10.0[a]	0.00700	Sol -Std C

[a] Diluted with 50/50 acetonitrile/purified reagent water (v/v)

2.9 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (ground water and surface water) by fortification with the appropriate sub-stock solution of novaluron at concentrations of 0.0100 (LOQ) and 0.100 (10X LOQ) µg/L. Recovery samples for both matrices were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples of each matrix were left unfortified to serve as controls and were processed in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared for each sample set and processed in the same manner as the control samples. The dosing procedure is detailed in the following table.

Ground water

Sample ID: 14125-6133-	Sample Type	Stock ID	Fortifying Stock Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Nominal Concentration (µg/L)
01	Reagent Blank	NA[a]	NA	NA	7.00[b]	0.00
02 & 03	Control	NA	NA	NA	28.0[cd]	0.00
04, 05, 06, 07, & 08	LOQ	Tech Stk-3	1.00	0.0700	7.00[c]	0.0100
09, 10, 11, 12, & 13	10X LOQ	Tech Stk-2	10.0	0.0700	7.00[c]	0.100

[a] NA = Not Applicable
[b] Purified reagent water
[c] Ground water
[d] Control volumes were increased to ensure ample final volume was available for the matrix effects assessment.

Surface water

Sample ID: 14125-6133-	Sample Type	Stock ID	Fortifying Stock Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Nominal Concentration (µg/L)
14	Reagent Blank	NA[a]	NA	NA	7.00[b]	0.00
15 & 16	Control	NA	NA	NA	28.0[c, d]	0.00
17, 18, 19, 20, & 21	LOQ	Tech Stk-3	1.00	0.0700	7.00[c]	0.0100
22, 23, 24, 25, & 26	10X LOQ	Tech Stk-2	10.0	0.0700	7.00[c]	0.100

[a] NA = Not Applicable
[b] Purified reagent water
[c] Surface water
[d] Control volumes were increased to ensure ample final volume was available for the matrix effects assessment.

2.10 Dilution of Fortified Recovery 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 samples were diluted into the calibration range with acetonitrile by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 30/70 acetonitrile/test matrix (v/v). Following addition of acetonitrile, samples were mixed using a vortexer for 15 seconds, followed by centrifugation using micro centrifuge tubes at 14,000 rpm for 5 minutes to removed any undissolved materials that may be present prior to analysis. The dilution procedures are outlined in the tables below.

Ground water

Sample ID: 14125-6133-	Sample Type	Nominal Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
01	Reagent Blank	0.00	7.00	10.0	1.43
02 & 03	Control	0.00	28.0	40.0[b]	1.43
04, 05, 06, 07, & 08	LOQ	0.0100	7.00	10.0	1.43
09, 10, 11, 12, & 13	10X LOQ	0.100	7.00	10.0	1.43

[a] Diluted with acetonitrile
[b] Volume increased for use in matrix effects assessment

Surface water

Sample ID: 14125-6133-	Sample Type	Nominal Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
14	Reagent Blank	0.00	7.00	10.0	1.43
15 & 16	Control	0.00	28.0	40.0[b]	1.43
17, 18, 19, 20, & 21	LOQ	0.0100	7.00	10.0	1.43
22, 23, 24, 25, & 26	10X LOQ	0.100	7.00	10.0	1.43

[a] Diluted with acetonitrile
[b] Volume increased for use in matrix effects assessment

2.11 LC-MS/MS Instrumental Conditions

The LC-MS/MS analysis was conducted using 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 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
Injector Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 75.0 µL
Retention Times: approximately 4.1 minutes

MS Parameters:

Instrument: AB Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5000 V
Scan type: MRM
Dwell Time: 200 msec
Source Temperature: 500 °C
Curtain Gas: 25.0
Ion Source – Gas 1 / Gas 2: 20.0 / 10.0
Collision Gas: 5.00
Collision Cell Entrance Potential: 10.0
Collision Cell Exit Potential: 13.0
Declustering Potential: 81.0
Resolution Q1/Q3: Low/Low

Analyte	Transition	Q1/Q3 Mass (Da/Da)	Collision Energy
Novaluron	Primary	493.0/158.1	30.0
	Confirmatory	493.0/141.0	65.0

2.11.1 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 three to five injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11.2 Method Differences

The analytical method used for novaluron in this independent laboratory validation followed the procedures described in the original method validation (Study No. 3202771). The analytical method used for novaluron in this independent laboratory validation utilized the following minor modifications from the original method validation. These modifications were:

- Injection volume was modified from 50 µL to 75 µL in order to optimize instrument sensitivity.
- Autosampler temperature was increased from 4 °C to 10 °C, as 10 °C is our standard autosampler temperature setting.
- Q1 and Q3 resolution on the mass spectrometer was set to Low/Low (no setting was specified in the validation method) to optimize instrument sensitivity.
- The autosampler wash solution utilized during analysis was comprised of 30/30/40 acetonitrile/methanol/puritified reagent water (v/v/v), as this is our standard rinse solution used in analytical methods (no rinse solution was specified in the validation method).

2.12 Evaluation of Accuracy, Precision, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the LOQ and 10X LOQ recovery samples. Recoveries of 70.0 to 110% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the peak area quantitation and the percent recovery values of the LOQ and 10X LOQ recovery samples. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. The specificity of the method was determined by examination of the control samples for peaks at the same retention time as novaluron which might interfere with the quantitation of the analytes. Interferences with peak areas that are less than or equal to 20% of the LOQ and less than or equal to 50% of the MDL peak height response are not considered significant. The linearity of the method was determined by the correlation coefficient (r), y-intercept, and slope of the regression line. The calibration range covered from ≤30% of the LOQ concentration to ≥120% of the 10 × LOQ concentration. A 1/x weighted linear regression was used for the LC-MS/MS analysis. The calibration curves were evaluated based on the correlation coefficient and

the recoveries of the calibration standards. The signal response data should have an intercept close to zero and a correlation coefficient (r) not less than 0.9975 (or coefficient of determination, $r^2 \geq 0.995$). The precision of the method at the LOQ was reported in terms of the coefficient of variation of the observed recovery values being ≤20%.

2.13 Limit of Quantitation (LOQ)

The method was validated at the LOQ. This was defined as the lowest fortification level, with mean recoveries ranging between 70 and 110%, and a relative standard deviation not exceeding 20%. Blank values (reagent blanks and untreated control samples) did not exceed ≤20% of the LOQ and ≤50% of the MDL peak height response at the retention time of the test substance. These conditions were fulfilled for the 0.0100 µg/L fortification level.

2.14 Limit of Detection (LOD)

The LOD was defined as the mean sample concentration equivalent to three times the baseline height in the control samples for each matrix. Representative calculations for the LOD can be found in Section 3.0.

2.15 Method Detection Limit (MDL)

The MDL will be defined as the lowest calibration standard multiplied by the dilution factor used for samples fortified at the LOQ. Representative calculations for the MDL can be found in Section 3.0.

2.17 Time Required for Analysis

There were two water matrices investigated in this ILV. Each water matrix investigation included one set of samples used for LC-MS/MS analysis. Both matrices were processed on the same day, and are considered one set. One set of samples consisted of 20 fortified, four unfortified samples, two reagent blanks, nine matrix effects standards, and 7 calibration standards (42 samples total). A single analyst completed a set of 42 samples in one working day (eight hours) with LC-MS/MS analysis performed overnight (approximately 9 hours).

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 with 1/x weighting, 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.

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

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

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

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

NOTE: A 1/x weighting was used for calibration curves and sample quantitation using Analyst software, version 1.6.3.

The LOD was calculated using the following equation:

$$(4) \qquad LOD = ((3 \times (N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{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., 1.43)
LOD	=	limit of detection for the analysis

The MDL is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (equation 5) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(5) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.00200 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 1.43)
MDL	=	method detection limit reported for the analysis (0.00200 µg/L × 1.43= 0.00286 µg/L)

The 95% confidence interval for a mean was calculated using the following equation:

$$(6) \qquad 95\%\ CI = t_{df,95\%} \times \frac{s}{\sqrt{n}}$$

where:

$t_{df,\ 95\%}$	=	t value (at n-1 degrees of freedom) for 95% confidence = 2.776
s	=	standard deviation
n	=	number of replication

APPENDIX 1 - STUDY PROTOCOL

Independent Laboratory Validation of the Analytical Method (Study No. 3202771) for Determination of Novaluron in Aqueous Matrices by LC-MS/MS

1.0 INTRODUCTION

The purpose of this study is to confirm that an analytical method, developed by one group, can be independently validated by a second group. This study is required by EPA under guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174], and guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation, and must also satisfy SANCO/825/00 rev. 8.1: Guidance Document on pesticide residue analytical methods. Independent labs are allowed to analyze three sample sets in order to validate the method as written. A complete set of samples should consist of, at a minimum, a reagent blank, two un-spiked matrix control samples, five matrix control samples fortified at the limit of quantification (LOQ), and five matrix control samples fortified at 10X LOQ for each distinct matrix. A complete set may include more than thirteen samples depending on the number of reagents, un-fortified and fortified control matrix samples. It may be necessary, however, to divide a complete set into two subsets for efficient handling. Each subset should contain a reagent blank, two un-fortified matrix control samples, and five matrix control samples fortified at the LOQ or 10X LOQ.

A maximum of 3 validation attempts may be made. All communication with the Sponsor (e-mail or telephone), including any modifications to the methodology provided, will be presented in the raw data and summarised in the final report. A successful ILV trial will require adequate results on at least one complete set of samples on a given matrix.

The purpose of this protocol is to perform an ILV for the LC-MS/MS analytical method (Study No. 3202771) used to determine the test substance(s) in surface water and ground water. The analytical method will be validated for the test substance with regards to accuracy, precision, linearity, specificity, and limits of quantification.

2.0 OBJECTIVE

The objective of this study is to confirm that the analytical method for novaluron in surface water and ground water, developed by one group, can be independently validated by a second group in the absence of any interaction between the two.

3.0 JUSTIFICATION OF THE TEST SYSTEM

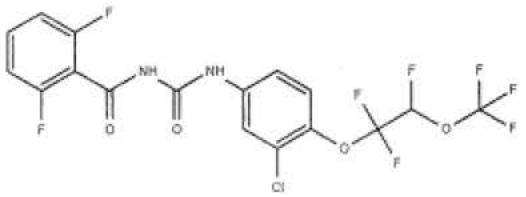
The method validation described in this protocol are designed to conform to EPA guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174], OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation, and SANCO/825/00 rev. 8.1: Guidance Document on pesticide residue analytical methods. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as accepted by the OECD principles on GLP.

4.0 MATERIALS

Upon arrival at Smithers, the test substance (also reference substance) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each test and reference substance will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, SDS, and safe handling procedures, and a verified expiration or reanalysis date.

4.1 Test Substance

Test Substance Name:	Novaluron
IUPAC Name:	*N*-({3-chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]phenyl}carbamoyl)-2,6-difluorobenzamide
CAS Number:	116714-46-6
Structure:	
Molecular Formula:	$C_{17}H_9ClF_8N_2O_4$
Molecular Weight:	492.7 g/mol
Lot Number:	96869065
Purity:	100.0%
Storage Conditions:	Room temperature (15-25 °C)
Retest Date:	10 August 2022

4.2 Validation Matrices

The water used for the independent laboratory validations will be two types of aqueous matrices (i.e. groundwater & surface water). The samples will be stored refrigerated at a temperature set to maintain 2 to 8°C. The following parameters of the ground water and surface water used in the validation will be experimentally determined:

- Suspended Solids (mg/L)
- Conductivity (µs/cm)
- Total Hardness as Calcium Carbonate (mg/L)
- pH
- Dissolved organic carbon content (mg/L)

All documentation relating to the preparation, storage and handling will be maintained by Smithers.

4.2.1 Ground Water

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

4.2.2 Surface Water

The surface water used for this method validation analysis will be collected from river water in Massachusetts. The water will be collected from an area of the river with approximately 30 to 60 cm of overlying water. All documentation relating to the preparation, storage and handling will be maintained by Smithers.

5.0 ANALYTICAL METHOD

The analytical method to be used during the ILV is, "Validation of the Analytical Method for the Determination of Novaluron in Aqueous Matrices by LC-MS/MS", Study No. 3202771, Sponsor Reference No. 000106386, 01 December 2020, Final report.

6.0 VALIDATION DESIGN

The test design will consist of surface water and ground water (identified in raw data and final report) fortified with the test substances at two concentrations with five replications for each fortification level. The control matrix for the validation will be untreated surface water and ground water. The validation study levels (approximate concentrations) for the test substance are:

• One reagent blank sample	0.0 µg/L
• Two control samples	0.0 µg/L
• Five samples fortified at the limit of quantitation (LOQ)	0.010 µg/L
• Five samples fortified at 10 x LOQ	0.10 µg/L

6.1 Validation Pass Criteria

The validation will be deemed acceptable if the following criteria are met for the primary and confirmatory transitions monitored:

Mean Recovery (Accuracy) and Precision – Each fortification level should have a mean recovery between 70 and 110% and a %RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity – Amounts found in blank samples should be ≤ 20% of the LOQ and ≤ 50% of the MDL peak height response at the retention time of the test substance.

Linearity – The calibration range should cover from ≤ 30% of the LOQ concentration to ≥ 120% of the 10 × LOQ concentration (after dilution if applicable). Solvent-based calibration standards will be used if matrix effects are not deemed significant (see Section 6.4). The correlation coefficient (r) should be ≥ 0.9975 (or coefficient of determination, $r^2 \geq 0.995$).

6.2. Limit of Detection (LOD) Assessment

The LOD will be estimated as the mean sample concentration equivalent to three times the baseline height in the control samples for each water.

6.3. Method Detection Limit (MDL) Assessment

The MDL will be defined as the lowest calibration standard multiplied by the dilution factor used for samples fortified at the LOQ.

6.4. Matrix Assessment

An assessment of matrix effects will be made by comparison of standards prepared using control matrix against non-matrix (solvent-based) standards. This applies to the primary and confirmatory transitions. Results will be presented as a % difference from the mean non-matrix standard value. A difference of < 20% will be considered acceptable when using a non-matrix matched calibration line.

6.5. Proposed Statistical Methods to be Used

In the event that outlying recoveries are suspected, Grubbs' test will be performed to check for significant outliers. If the outlier is significant, it will be excluded from calculation of the mean recovery and %RSD, but will still be reported. Up to one significant outlier may be removed from each set of five replicate fortified samples.

7.0 TEST SYSTEM IDENTIFICATION

The test system will be defined as the fortified recovery samples. The fortified recovery samples will be labeled as defined in Section 6.0 and each sample replicate will be assigned a unique identifier.

8.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples, replicate analysis, and maintenance of material balance.

9.0 SAMPLE DISPOSAL

All study specimens, and/or samples collected during the study, and test materials and reference standards, etc., provided by the Sponsor, client, or customer will either be returned to the originator, shipped to a third party archival facility on behalf of the Study Sponsor who will incur the costs of shipping and archival, or disposed of according to Smithers SOPs.

10.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

11.0 REPORTING

The raw data generated at Smithers will be peer-reviewed and the final report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The Quality Assurance Unit will inspect the final report to confirm that the methods, procedures, and observations are accurately and completely described, that the reported results accurately and completely reflect the raw data generated at Smithers and to confirm adherence with the study protocol. A single copy of the draft report will be submitted to the Sponsor for review. The report will be finalized according to Standard Operating Procedures and will meet the formatting requirements of EPA's PR Notice 2011-3. All reports will include, but will not be limited to, the following information:

- Protocol and all amendments.
- Name and address of study director and other contact person for ILV laboratory.
- Description of the analytical method.
- All recovery and control values for all matrices that were obtained during all ILV trials.
- Representative chromatograms/spectra for each analyte in each matrix.
- Description of the instruments used and operating parameters.
- Description of any problems encountered and a written description of any changes or modifications that were made during the ILV.
- Any steps considered critical, i.e. steps where little variation is allowable or directions must be followed precisely.
- The number of worker-hours required to complete one set of samples.

- The number of calendar days required for one set of samples.
- Any contact between the independent laboratory and the method developers or others familiar with the method, including the reasons for the contact, any changes in the method that resulted, and the time of this communication with respect to the progress of the confirmatory trial (i.e., after the first set, during the second set, etc.).
- The report and project numbers from Smithers and Sponsor study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program Coordinator (if applicable), Study Director and Principal Investigator.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, degree of purity of test substance (percent test chemical) (Sponsor-supplied, if available).
- The determined accuracy, precision, linearity, limit of detection, and method LOD.
- The mathematical equations and statistical methods used in generating and analyzing the data as well as calculations using these equations. Tabular and graphical representations (if appropriate) of the data.
- Description of any problems experienced and how they were resolved.
- Good Laboratory Practice (GLP) Compliance Statement signed by the Study Director.
- Statement of non-confidentiality and that the method contains no traded secrets or proprietary data.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the protocol, raw data and final report.

12.0 PROTOCOL CHANGES

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's Representative. Protocol amendments and deviations must include the reasons for the change and the impact of the change on the results of the study, if any.

13.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide, Fungicide and Rodenticide Act (40 CFR, Part 160) and as accepted by OECD Principles on Good Laboratory Practice.