Final Report

Study Title	Validation of the Analytical Method for the Determination of Novaluron in Aqueous matrices by LC-MS/MS
Study Guideline(s)	OCSPP 850.6100 OPPTS 860.1340

SMITHERS

INTRODUCTION

The objective of this study was to validate an analytical method for measuring residues of Novaluron in surface and ground water by LC-MS/MS, in accordance with EPA OCSPP 850.6100 (2012) and OPPTS 860.1340 (1996) guidelines.

An analytical method was developed based upon Smithers ERS (Wareham) Study No. 14125.6100, which was modified to cover a lower LOQ of 0.01 µg/mL for Novaluron. The validated method was issued as SMV 3202771-01V.

Control samples of ground and surface water were fortified with Novaluron at 0.01 and 0.1 µg/L in quintuplicate and analysed. Samples were diluted with acetonitrile to give a final ratio of acetonitrile: water (30:70 v/v).

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

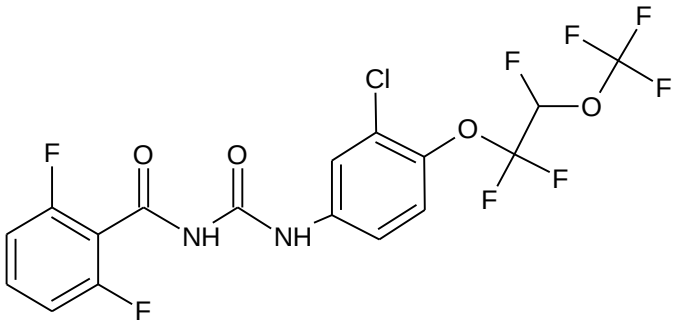
Samples were analysed for Novaluron using Liquid Chromatography with tandem Mass Spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy were calculated at each validation level in each water for Novaluron. One primary and one confirmatory LC-MS/MS transition were analysed for Novaluron.

MATERIALS AND METHODS

Protocol Adherence
The study was conducted in accordance with the protocol with no deviations.

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:	100.00%
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
- Acetonitrile — LC-MS grade, VWR
- Formic acid — LC-MS grade, Fisher
- 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 × 50 mm
- Analytical balance
- Micro centrifuge (Sorvall Legend Micro 21)
- Micro centrifuge tubes
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

An analytical method was developed based upon Smithers ERS (Wareham) Study No. 14125.6100, which was modified to cover a lower LOQ of 0.01 µg/mL for Novaluron. The validated method was issued as SMV 3202771-01V.

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 HPLC grade acetonitrile was mixed with 250 mL Milli-Q water.

0.1% formic acid in acetonitrile

Mix 1 mL LC-MS grade formic acid with 1000 mL LC-MS grade acetonitrile.

0.1% formic acid in water

Mix 1 mL LC-MS grade formic acid with 1000 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 Novaluron were prepared at 1000 µg/mL in acetonitrile as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]	Stock Use
Stock 1	10.07	100.00	Acetonitrile	10.07	1000	Secondary stock solution
Stock 2	10.06			10.06	1000	
Stock 3	10.55			10.55	1000	

[1] Corrected for Purity.

Three stocks were prepared for correlation purposes, as the correlation of Stocks 1 and 2 initially failed, however all three stocks were found to be acceptable following re-dilution.

Primary stock solutions were stored refrigerated in amber glass bottles for up to three months.

Secondary Stock Solutions

Secondary stock solution of Novaluron were prepared as described in the following table:

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

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

Sub-Stock Solutions

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

Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)	Stock Use
10	0.1	Acetonitrile	10	0.1	Sub-stock solution
0.1	1		10	0.01[1]	Fortification at 10 × LOQ
0.01	1		10	0.001[2]	Fortification at LOQ

[1] Equivalent to 10 µg/L.

[2] Equivalent to 1 µg/L.

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 Novaluron were prepared in acetonitrile: water (50:50 v/v) 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

Non-matrix matched standards were prepared on the day of use and were stored refrigerated until the analysis was complete.

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

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	0.1	Ground water final extract	10	0.1
10	0.1		10	0.1
10	0.1		10	0.1
10	0.1	Surface water final extract	10	0.1
10	0.1		10	0.1
10	0.1		10	0.1

Matrix matched standards were prepared on the day of use and were stored refrigerated until the analysis was complete.

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 Novaluron 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)
10	0.1	Acetonitrile: water (50:50 v/v)	10	0.1
0.1	0.75		1	0.075
0.1	0.5		1	0.05
0.1	0.2		1	0.02
0.1	0.1		1	0.01
0.05	0.1		1	0.005
0.02	0.1		1	0.002

A single set of calibration standards was prepared for each validation batch, which were injected twice during the batch, with at least one calibration standard every three sample injections.

Sample Preparation and Fortification

7 mL of water was measured into a glass vial. Quintuplicate water samples were fortified at the LOQ (0.01 µg/L) and at 10 × LOQ (0.1 µg/L) with stock solutions of Novaluron. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B	7	N/A	N/A	N/A
Control E+F	7	N/A	N/A	N/A
F0.01 F-J	7	1	0.07	0.01
F0.1 F-J	7	10	0.07	0.1

N/A = Not applicable.

Borehole ground water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/L)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank D	7	N/A	N/A	N/A
Control I+J	7	N/A	N/A	N/A
F0.01 P-T	7	1	0.07	0.01
F0.1 P-T	7	10	0.07	0.1

N/A = Not applicable.

Sample Dilution

3 mL of acetonitrile was added to the 7 mL of water and mixed. An aliquot of the sample was transferred into a micro centrifuge tube and centrifuged at 14,000 rpm for 5 minutes. The sample dilution is summarised in the following tables:

Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Reagent Blank B	N/A	7	10	1.43
Control E+F	N/A	7	10	1.43
F0.01 F-J	0.01	7	10	1.43
F0.1 F-J	0.1	7	10	1.43

N/A = Not applicable.

Borehole ground water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Reagent Blank D	N/A	7	10	1.43
Control I+J	N/A	7	10	1.43
F0.01 P-T	0.01	7	10	1.43
F0.1 P-T	0.1	7	10	1.43

N/A = Not applicable.

Samples were prepared on the day of use and were stored refrigerated until the analysis was complete.

Instrument Conditions

LC-MS/MS analysis was performed using the following instrument 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	50 µL		
Retention Time	Approx. 3.4 minutes		
Valco Valve Diverter	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.6	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 V		
Collision Exit Potential (CXP)	13 V		
Declustering Potential (DP)	81 V		
Compound Name	MRM Transition Ions Monitored	Dwell Time (ms)	Collision Energy (CE)
Novaluron (Primary)	493.0/158.1	200	30
Novaluron (Confirmatory)	493.0/141.0	200	65

The primary and confirmatory transitions can be monitored separately to improve sensitivity.

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

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 Novaluron:

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

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 30% of the equivalent LOQ concentration 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 (which is equivalent to a coefficient of determination (r^2) of ≥ 0.995).

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

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 0.002 µg/L and a dilution factor of 1.43).

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 Novaluron 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 acceptable when using a non-matrix matched calibration line.

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 0.00286 µg/L (using a dilution factor of 1.43), which is ≤ 30% of the LOQ concentration after dilution.

The highest calibration point was equivalent to 0.143 µg/L (using a dilution factor of 1.43), which is ≥ 120% of the 10 × LOQ concentration after dilution.

The correlation coefficients (r) for Novaluron were all ≥ 0.9975.

Limit of Quantification (LOQ)
The LOQ based upon the lowest level validated confirmed the LOQ to be 0.01 µg/L for Novaluron 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 Novaluron (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 Novaluron was calculated to be equivalent to an initial sample concentration of 0.00286 µg/L (based upon the lowest standard concentration of 0.002 µg/L and a dilution factor of 1.43).

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.

Matrix effects were insignificant (< 20% difference from non-matrix standards) for Novaluron 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.

Method Issues
The initial attempt at validating Novaluron in ground water was unsuccessful for the confirmatory transition, due to the background noise being > 50% of the lowest calibration standard (MDL). The high background noise was attributed to commercially prepared mobile phase. When freshly prepared mobile phase was used, the background noise decreased to < 50% of the lowest calibration standard (MDL).

The second attempt at validating Novaluron in ground water was unsuccessful due to poor precision at the LOQ. This was attributed to an insufficient number of data points across the Novaluron peak, which may have impacted the precision of the peak area measurement. When the primary and confirmatory transitions were collected in separate instrument runs, the scan rate was increased, which increased the number of

data points across the Novaluron peak, which improved instrument precision without reducing the instrument sensitivity.

PERFORMANCE CRITERIA

The method validation for Novaluron 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	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.		
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 0.01 and 0.1 µg/L; 0.01 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, 0.01µg/L:	LOQ, 0.01 µg/L:
		10 × LOQ, 0.1 µg/L:	10 × LOQ, 0.1 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 (0.01 µg/L).	All blank sample values were ≤ 20% of the LOQ (0.01 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.	0.00131 µg/L	0.00245 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.	0.00286 µg/L	0.00286 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 493.0/158.1 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 493.0/141.0 amu Meets all method and guideline specifications outlined in this table.

The method validation for Novaluron 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 (	
Accuracy: Mean Recoveries	Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable.	LOQ, 0.01 µg/L:	LOQ, 0.01 µg/L:
		10 × LOQ, 0.1 µg/L:	10 × LOQ, 0.1 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 0.01 and 0.1 µg/L; 0.01 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, 0.01 µg/L:	
		, 0.1 µg/L:	10 × LOQ, 0.1 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 of the LOQ (0.01 µg/L).	All blank sample values were the LOQ (0.01 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.	0.00108 µg/L	0.00431 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.	0.00286 µg/L	0.00286 µg/L
Confirmation of Analyte Identification	A chromatographic confirmatory method will be used to determine test solution concentrations during validation.	Primary ion: 493.0/158.1 amu Meets all method and guideline specifications outlined in this table.	Confirmatory ion: 493.0/141.0 amu Meets all method and guideline specifications outlined in this table.