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TITLE

Independent Laboratory Validation of Analytical Method 12709.6509 for the Determination of V-10456, S-3100-CA, and S-3100-CA-UR in Ground and Surface Water

TEST GUIDELINE

850.6100

AUTHOR(S)

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STUDY COMPLETION DATE

11/9/2021

PERFORMING LABORATORY

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3203100

TOTAL PAGES

146

(Including Page 1i)

1. SUMMARY

The purpose of this study was to independently validate the Smithers (Wareham, USA) analytical method from report 12709.6509 (V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions) in ground and surface water in accordance with the EPA OCSPP 850.6100 (2012), EPA OPPTS 860.1340 (1996) and SANTE/2020/12830 rev.1 (2021) guidelines.

Five control samples each of ground and surface water were fortified with V-10456, S-3100-CA and S-3100-CA-UR at limit of quantification (LOQ), 10xLOQ and high-level then analysed. The fortified concentrations of V-10456 are 0.1 µ/L (LOQ), 1 µg/L (10xLOQ) and 1000 µg/L (high-level). The fortified concentrations of S-3100-CA and S-3100-CA-UR are 1 µg/L (LOQ), 10 µg/L (10xLOQ) and 1000 µg/L (high-level). Samples (5 mL) were made to 50 mL by adding 45 mL of 0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v) to give a final composition of 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate. Samples at 10xLOQ and high-level were further diluted into the calibration range using either 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate.

Two reagent blank samples and five control samples were also analysed with each set of fortified samples.

Samples were analysed for V-10456, S-3100-CA and S-3100-CA-UR using Liquid Chromatography with tandem Mass Spectrometry detection (LC-MS/MS).

Accuracy and precision, linearity, specificity, limit of detection (LOD), LOQ and matrix effects of the method were determined. Precision and accuracy were calculated at each validation level in each water for V-10456, S-3100-CA and S-3100-CA-UR. One primary and one confirmatory LC-MS/MS transition were analysed for V-10456, S-3100-CA and S-3100-CA-UR.

Mean recoveries met the protocol criteria of 70 to 120% in reference to SANTE/2020/12830 and the Analytical Method 12709.6509 acceptance criteria of 70 to 110% at each concentration for V-10456, S-3100-CA and S-3100-CA-UR for the primary and confirmatory transitions for the two waters tested.

Precision was acceptable (≤ 20%) for the primary and confirmatory transitions for the two waters tested.

Any interferences at the retention time of V-10456, S-3100-CA and S-3100-CA-UR found in ground or surface water were ≤ 30% of the LOQ peak area response.

The suitability of the calibration lines were demonstrated by random distribution of the regression residuals in the residual plots.

The correlation coefficients (r) for V-10456, S-3100-CA and S-3100-CA-UR were all ≥ 0.995.

The observed matrix enhancement or suppression of the analyte peak was not greater than 20% of V-10456, S-3100-CA and S-3100-CA-UR and, in-line with the environmental chemistry method 12709.6509, matrix-matched calibration standards were used for the validation of both water types.

The method for the analysis of V-10456, S-3100-CA and S-3100-CA-UR was successfully validated in the two waters tested.

2. INTRODUCTION

The purpose of this study was to independently validate the Smithers (Wareham, USA) analytical method from report 12709.6509 (V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions) in ground and surface water in accordance with the EPA OCSPP 850.6100 (2012), EPA OPPTS 860.1340 (1996) and SANTE/2020/12830 rev.1 (2021) guidelines.

The analytical method 12709.6509 was provided by the Sponsor. The method was re-written in Smithers ERS Limited, Harrogate format to include details of the equivalent equipment and reagents used. The independently validated method was then issued as SM 3203100-01V.

Five control samples each of ground and surface water were fortified with V-10456, S-3100-CA and S-3100-CA-UR at limit of quantification (LOQ), 10xLOQ and high-level then analysed. The fortified concentrations of V-10456 were 0.1 µ/L (LOQ), 1 µg/L (10xLOQ) and 1000 µg/L (high-level). The fortified concentrations of S-3100-CA and S-3100-CA-UR were 1 µg/L (LOQ), 10 µg/L (10xLOQ) and 1000 µg/L (high-level). Samples (5 mL) were made to 50 mL by adding 45 mL of 0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v) to give a final composition of 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate. Samples at 10xLOQ and high-level were further diluted into the calibration range using either 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate.

Two reagent blank samples and five control samples were also analysed with each set of fortified samples.

Samples were analysed for V-10456, S-3100-CA and S-3100-CA-UR using Liquid Chromatography with tandem Mass Spectrometry detection (LC-MS/MS).

Accuracy and precision, linearity, specificity, limit of detection (LOD), LOQ and matrix effects of the method were determined. Precision and accuracy were calculated at each validation level in each water for V-10456, S-3100-CA and S-3100-CA-UR. One primary and one confirmatory LC-MS/MS transition were analysed for V-10456, S-3100-CA and S-3100-CA-UR.

To assess matrix effects of both water types, standards were prepared using both final matrix extracts and non-matrix standards and the peak areas were compared.

2.1. Study Timetable

Study initiation:	01 July 2021 (date the protocol was signed by the Study Director).
Experimental start:	15 July 2021 (the date of the first recording of study specific experimental raw data).
Experimental completion:	29 September 2021 (the date of the last recording of study specific experimental raw data).
Study completion:	Date the final report was signed by the Study Director.

3. MATERIALS AND METHODS

3.1. Protocol Adherence

The study was conducted in accordance with the protocol with four amendments and one protocol deviation.

3.1.1. Protocol Amendment 1 Details

1. The number of reagent blank and control sample replicates analysed was updated in order to accurately reflect the original analytical method 12709.6509 (V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions).

3.1.2. Protocol Amendment 2 Details

1. The instrumentation and data acquisition system intended for use on this study was added.

2. The water type listed in the Water Characterisation section was corrected.

3.1.3. Protocol Amendment 3 Details

1. The address for Valent USA LLC was updated.

2. The archiving address was updated due to a change in legal name of Covance Laboratories Limited to Labcorp Early Development Laboratories Limited.

3. The Final Extract Refrigerated Storage Stability Assessment section was removed as not required under the scope of the ILV.

3.1.4. Protocol Amendment 4 Details

1. The requirement for stock solution stability was added in line with SANTE/2020/12830 rev.1 (2021).

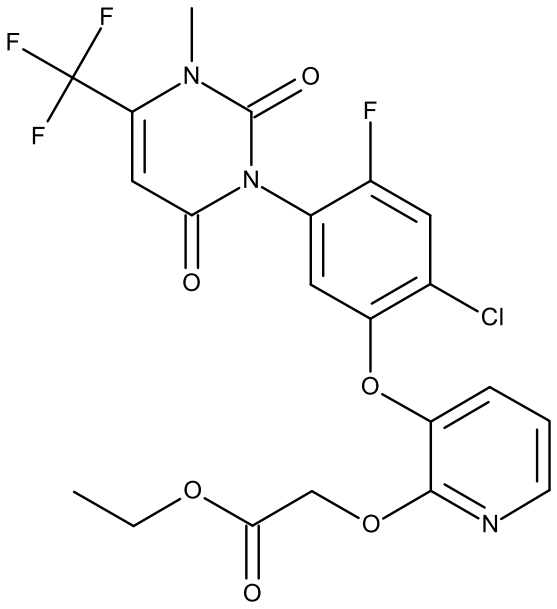
3.1.5. Protocol Deviation Details

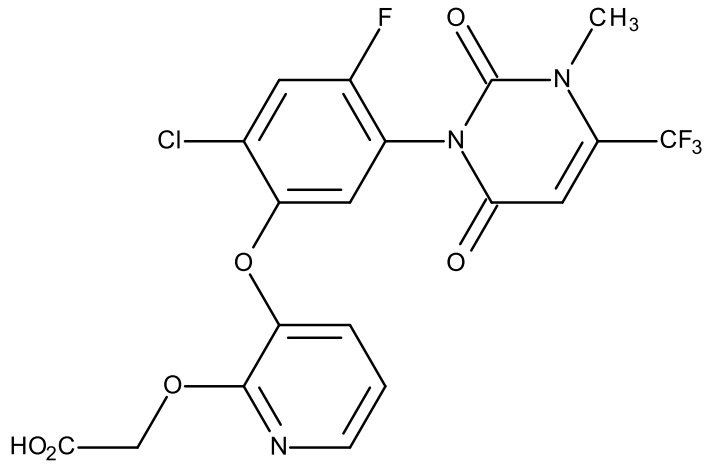
1. The protocol (and amendments) state that the calibration range should cover from ≤ 30% of the LOQ final extract concentration to ≥ 120% of the 10000 × LOQ (V-10456 in both water types) or 1000 × LOQ (S 3100 CA and S-3100-CA-UR in both water types) (after dilution if applicable). In the case of V-10456, the concentration of the lowest calibration point was 50% of the LOQ final extract concentration. This is in deviation to the protocol but there

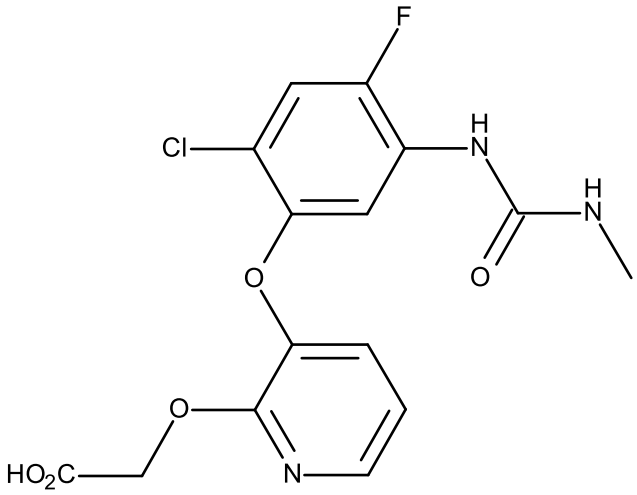
is no impact to the quality or integrity of the study because the calibration line for V-10456 was prepared in accordance with the procedures in the Smithers (Wareham, USA) analytical method 12709.6509.

3.2. Test Substances

The information below is presented as detailed in the test substance information supplied by the Sponsor:

Test Substance Name:	**V-10456**
Chemical Name:	ethyl 2-[[3-[2-chloro-4-fluoro-5-[3-methyl-2,6-dioxo-4-(trifluoromethyl)pyrimidin-1-yl]phenoxy]-2-pyridyl]oxy]acetate
Synonym:	S-3100
Molecular Formula:	$C_{21}H_{16}ClF_4N_3O_6$
Molecular Structure:	
Molecular Weight:	517.81 g/mol
Sponsor Lot Number:	SG-1610G
Purity:	99.4%
Appearance:	White solid
Storage Conditions:	Frozen (nominally -20°C)
Expiry Date:	09 April 2023

Test Substance Name:	**S-3100-CA**
Chemical Name:	2-[[3-[2-chloro-4-fluoro-5-[3-methyl-2,6-dioxo-4-(trifluoromethyl)pyrimidin-1-yl]phenoxy]-2-pyridyl]oxy]acetic acid
Molecular Formula:	$C_{19}H_{12}ClF_4N_3O_6$
Molecular Structure:	
Molecular Weight:	489.76 g/mol
Sponsor Lot Number:	19SC8673272
Purity:	98.6%
Appearance:	White powder
Storage Conditions:	Refrigerated (2-8°C)
Expiry Date:	30 November 2022

Test Substance Name:	**S-3100-CA-UR**
Chemical Name:	2-[[3-[2-chloro-4-fluoro-5-(methylcarbamoylamino)phenoxy]-2-pyridyl]oxy]acetic acid
Molecular Formula:	$C_{15}H_{13}ClFN_3O_5$
Molecular Structure:	
Molecular Weight:	369.73 g/mol
Sponsor Lot Number:	19SC8673273
Purity:	99.5%
Appearance:	White powder
Storage Conditions:	Refrigerated (2-8°C)
Expiry Date:	31 January 2023

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

3.3. Test Matrices

Control samples of ground water and surface water were sourced by Smithers ERS Limited. The unique identification number of the ground water used was CS61/21 and that of the surface water was CS60/21.

Water characterisation data are listed in the following table:

Water Type	**Ground Water**	**Surface Water**
Water Name	Harrogate Spa	Fountains Abbey
Unique ID	CS61/21	CS60/21
Suspended Solids (mg/L)	1	3
Electrical Conductivity (µS/cm)	404	296
Total Hardness (mg/L $CaCO_3$)	202	169
pH	7.02	9.53
Dissolved Organic Carbon (DOC, ppm)	1.40	10.1

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

3.4. Reagents

- Acetonitrile — HPLC grade, Honeywell
- Methanol — HPLC grade, Honeywell
- Formic acid — ≥ 98%, Honeywell
- 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

3.5. Equipment

- Shimadzu Nexera series HPLC system with Sciex API 5000 MS/MS detector
- Phenomenex Kinetex Phenyl-hexyl 2.6 µm, 3 × 50 mm
- Analytical balance
- Polypropylene centrifuge tubes (PPCT)
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- HPLC vials
- Various disposable laboratory glassware

3.6. Analytical Method

3.6.1. Preparation of Reagents

3.6.1.1. 0.1% Formic Acid in Acetonitrile:Milli-Q Water (50:50 v/v)
Mix 500 mL HPLC grade acetonitrile, 500 mL Milli-Q water and 1 mL formic acid.

3.6.1.2. 0.1% Formic Acid in Acetonitrile:Milli-Q Water (56:44 v/v)
Mix 560 mL HPLC grade acetonitrile, 440 mL Milli-Q water and 1 mL formic acid.

3.6.1.3. 0.1% Formic Acid in Acetonitrile:Ground Water:Milli-Q Water (50:10:40 v/v/v)
Mix 500 mL HPLC grade acetonitrile, 100 mL of ground water, 400 mL Milli-Q water and 1 mL formic acid.

3.6.1.4. 0.1% Formic Acid in Acetonitrile:Surface Water:Milli-Q Water (50:10:40 v/v/v)
Mix 500 mL HPLC grade acetonitrile, 100 mL of surface water, 400 mL Milli-Q water and 1 mL formic acid.

3.6.1.5. Acetonitrile:Milli-Q Water (50:50 v/v)
Mix 150 mL HPLC grade acetonitrile with 150 mL Milli-Q water.

3.6.1.6. Acetonitrile:Methanol:Water (30:30:40 v/v/v)
Mix 300 mL LC/MS grade acetonitrile, 300 mL LC/MS grade methanol and 400 mL Milli-Q water.

Reagents were scaled as appropriate and stored at room temperature (15-25°C) for up to one month.

3.6.2. Standard Solution Preparation

3.6.2.1. Primary Standard Stocks
Primary stock solutions of V-10456, S-3100-CA and S-3100-CA-UR were prepared as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)	Stock Use
V-10456 Stock A	10.17	99.4	MeCN	10.109	1000	Secondary solution (for fortification purposes)
V-10456 Stock B	10.13			10.070		Secondary solution (for calibration standards)
S-3100-CA Stock A	10.51	98.6	MeCN:H_2O (1:1 v/v)	10.363	1000	Secondary solution (for fortification purposes)
S-3100-CA Stock B	10.49			10.343		Secondary solution (for calibration standards)
S-3100-CA-UR Stock A	10.19	99.5		10.139	1000	Secondary solution (for fortification purposes)
S-3100-CA-UR Stock B	10.17			10.119		Secondary solution (for calibration standards)

Primary stock solutions were stored refrigerated in amber glass bottles with PTFE-lined caps and given a nominal expiry of one month.

3.6.2.2. Secondary Standard Stocks

Fortification solutions of V-10456, S-3100-CA and S-3100-CA-UR were prepared as described in the following table:

Test Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
V-10456	1000	0.5	Acetonitrile	50	10
S-3100-CA	1000	0.5	Acetonitrile:Milli-Q water (50:50 v/v)	50	10
S-3100-CA-UR	1000	0.5	Acetonitrile:Milli-Q water (50:50 v/v)	50	10

Secondary stock solutions were stored refrigerated in amber glass bottles with PTFE-lined caps and given a nominal expiry of one month.

3.6.2.3. Calibration Sub-Stocks and Fortification Standards

Calibration sub-stock standards and fortification standards were prepared from primary and secondary stocks as described in the following table:

Test Substance	Standard Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Mixed Standard Concentration (µg/mL)
V-10456	10	0.02	0.1% formic acid in Acetonitrile:Milli-Q water (50:50 v/v)	20	0.01 (V-10456) 0.1 (S-3100-CA and S-3100-CA-UR[1]
S-3100-CA	10	0.2			
S-3100-CA-UR	10	0.2			
V-10456	10	0.2		20	0.1 (V-10456) 1 (S-3100-CA and S-3100-CA-UR[2]
S-3100-CA	10	2			
S-3100-CA-UR	10	2			
V-10456	1000	0.2		20	10[3]
S-3100-CA	1000	0.2			
S-3100-CA-UR	1000	0.2			
V-10456	10	0.01		20	0.005 (V-10456) 0.025 (S-3100-CA and S-3100-CA-UR[4]
S-3100-CA	10	0.05			
S-3100-CA-UR	10	0.05			

[1]From mixed standard prepared from Stock A secondary stock, for fortification of LOQ recovery samples.
[2]From mixed standard prepared from Stock A secondary stock, for fortification of 10 x LOQ recovery samples.
[3]Prepared from Stock A for fortification of high-level recovery samples.
[4]From mixed standard prepared from Stock B secondary stock, used for calibration standards and matrix effects assessment.

Fortification and calibration sub-stock standards were prepared fresh for each assay.

3.6.2.4. Calibration Standards

Calibration solutions of V-10456, S-3100-CA and S-3100-CA-UR were prepared in 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v), as appropriate, as described in the following table:

Sub-Stock Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)	Volume Taken (mL)	Volume of Solvent (mL)	Final Volume (mL)	Calibration Standard ID	Calibration Standard Concentration V-10456/ S-3100-CA / S-3100-CA-UR (ng/mL)
5/25/25	0.1	9.9	10	Cal 1	0.05/0.25/0.25
5/25/25	0.08	9.92	10	Cal 2	0.04/0.2/0.2
5/25/25	0.06	9.94	10	Cal 3	0.03/0.15/0.15
5/25/25	0.04	9.96	10	Cal 4	0.02/0.1/0.1
5/25/25	0.02	9.98	10	Cal 5	0.01/0.05/0.05
5/25/25	0.01	9.99	10	Cal 6	0.005/0.025/0.025

Calibration standards were prepared fresh for each assay and transferred to 1.5 mL glass HPLC vials for injection.

3.6.2.5. Matrix-Matched Standards for Matrix Assessment

Matrix-matched standards and non-matrix matched standards of V-10456, S-3100-CA and S-3100-CA-UR were prepared in triplicate in volumetric flasks for the matrix assessment as described in the following table:

Mixed Standard Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)	Volume Taken (mL)	Dilution Solvent	Final Volume (mL)	Calibration Standard Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)
5/25/25	0.05	0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v)	50	0.005/0.025/0.025
5/25/25	0.05		50	0.005/0.025/0.025
5/25/25	0.05		50	0.005/0.025/0.025
5/25/25	0.05	0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)	50	0.005/0.025/0.025
5/25/25	0.05		50	0.005/0.025/0.025
5/25/25	0.05		50	0.005/0.025/0.025

The peak areas of the matrix-matched and non-matrix matched standards were compared and used to calculate matrix effects.

3.6.3. Sample Preparation and Fortification

5 mL of each water type was measured into 50 mL polypropylene centrifuge tubes (PPCT). Five control samples each of ground and surface water were fortified with V-10456, S-3100-CA and S-3100-CA-UR at the appropriate concentration levels.

Two reagent blank and five control water samples were also prepared per water type, as described in the following table:

Number of Replicates	Sample Type	Stock Concentration (ng/mL)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
2	Reagent Blank	N/A	N/A	5	N/A
5	Control	N/A	N/A	5	N/A
5	LOQ	10/100/100*[#]	0.05	5	0.1/1/1[#]
5	10xLOQ	100/1000/1000*[#]	0.05	5	1/10/10[#]
5	High-Level	10000	0.5	5	1000

N/A = Not Applicable.
[#]For V-10456, S-3100-CA and S-3100-CA-UR, respectively.
*10 ng/mL = 0.01 µg/mL, 100 ng/mL = 0.1 µg/mL, 1000 ng/mL = 1 µg/mL, 10000 ng/mL = 10 µg/mL.

3.6.4. Sample Extraction

Samples were made to 50 mL by adding 45 mL of 0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v) to give a final composition of 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate. Samples at 10xLOQ and high-level were further diluted into the calibration range using either 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate.

Full details of sample extraction are presented in the analytical method SM 3203100-01V in Appendix 3.

The method is summarised in the following table:

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Extract Volume (mL)	Dilution Factor
Reagent Blank A+B	N/A	5	50	10
Control A-E	N/A	5	50	10
LOQ A-E	0.1/1/1[#]	5	50	10
10LOQ A-E	1/10/10[#]	5	50	100
High-level	1000	5	50	5556 (S-3100-CA and S-3100-CA-UR) 27780 (V-10456)

N/A = Not applicable.
[#]For V-10456, S-3100-CA and S-3100-CA-UR, respectively.

It was possible to perform full experimental analysis of one batch of validation samples within one working day, or eight hours.

3.6.5. Instrument Conditions

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

3.6.5.1. LC Parameters

Instrument:	Shimadzu Nexera series HPLC system		
Column#:	Phenomenex Kinetex Phenyl-hexyl 2.6 µm, 3 × 50 mm		
Mobile Phase A#:	0.1% formic acid in water		
Mobile Phase B#:	0.1% formic acid in acetonitrile		
Flow Rate:	0.5 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.01	90	10
	0.5	90	10
	4.0	0	100
	4.5	0	100
	4.6	90	10
	6.0	90	10
Run Time:	6 minutes		
Autosampler Wash Solvent:	Acetonitrile:methanol:water (30:30:40 v/v/v)		
Column Temperature:	40°C		
Autosampler Temperature:	10°C		
Injection Volume:	35 µL		
Retention Time:	V-10456 – approximately 3.50 minutes S-3100-CA – approximately 3.05 minutes S-3100-CA-UR – approximately 2.50 minutes		

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

3.6.5.2. MS/MS Parameters

Instrument:	Sciex API 5000 mass spectrometer
Ionisation Type#:	Electrospray (ESI)
Polarity#:	Positive
Ion Spray Voltage:	4500 V
Scan Type#:	Multiple reaction monitoring (MRM)
Source Temperature:	650°C
Curtain Gas:	20.0
Ion Source – Gas 1/Gas 2:	50.0/50.0
Collision Gas:	8.0
Resolution (Q1/Q3):	Unit/Low
Dwell Time (milliseconds):	100

Test Substance	**V-10456**		**S-3100-CA**		**S-3100-CA-UR**	
Instrument Parameter	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da)	518.1/472.1	518.1/444.1	490.2/249.9	490.2/292.9	370.2/312.9	370.2/351.7
Collision Energy	27.0	30.0	40.0	40.0	21.0	20.0
Collision Cell Entrance Potential	10.0	10.0	10.0	10.0	7.0	7.0
Collision Cell Exit Potential	20.0	20.0	20.0	26.0	22.0	29.0
Declustering Potential	120	120	180	180	75.0	75.0

Note: Instrument parameters are provisional and may be subject to change after optimisation.

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

3.6.6. Calculation of Results

When the calibration fit is linear as in this study, Analyst 1.6.2 uses the following formula to calculate the concentration of test substance present in the sample extract:

$$\text{Amount } (\mu g/L) = \left[\frac{y - c}{m}\right] \times DF$$

Where:
y = Peak area
c = Y intercept on calibration graph
m = Slope of the linear calibration graph
DF = Software dilution factor

The dilution factor in this case was 10 (prior to any further dilutions), 100 for 10xLOQ samples, 5556 for S-3100-CA and S-3100-CA-UR high-level samples and 27780 for V-10456 high-level samples.

Procedural recovery data from fortified samples were calculated via the following equation:

$$\text{Recovery}\,(\%) = \frac{A}{S} \times 100$$

Where:
A = concentration found in fortified sample (µg/L)
S = concentration added to fortified sample (µg/L)

The 95% confidence intervals were calculated as follows:

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

Where:
t_{n-1} = 2.78 (for n=5)
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:

$$\text{LOD}\ (\mu g/L) = \frac{3 \times BN \times DF \times SC}{H}$$

Where:
BN = height of control baseline noise
DF = control sample dilution factor
SC = lowest calibration standard concentration (ng/mL)
H = 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:

$$\text{MDL}\ (\mu g/L) = SC \times DF$$

Where:
SC = lowest calibration standard concentration (ng/mL)
DF = control sample dilution factor

Matrix effects (%) were calculated as follows:

$$\text{Matrix effects}\ (\%) = \left(\frac{\text{Matrix PA} - \text{Solvent PA}}{\text{Solvent PA}}\right) \times 100$$

Where:
Matrix PA = mean peak area with matrix
Solvent PA = mean peak area with solvent

3.6.7. Validation Pass Criteria

The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored for V-10456, S-3100-CA and S-3100-CA-UR:

3.6.7.1. 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%.

3.6.7.2. Specificity/Selectivity

Specificity was acceptable if interferences at the retention time of V-10456, S-3100-CA and S-3100-CA-UR were found in the reagent blank and control samples at ≤ 30% of the LOQ peak area response.

3.6.7.3. Linearity

The linear range was acceptable if the lowest calibration standard concentration was ≤ 30% of the equivalent LOQ final extract concentration and if the highest calibration standard concentration was ≥ 120% of the 10×LOQ final extract concentration.

The suitability of the calibration line was demonstrated by a residual plot of the regression residuals (d_i):

$$d_i = y_i - y_{yi}$$

Where:
y_i = the measured value i.
y_{yi} = the estimated value which corresponds to yi and is derived from the calibration function.

The residual plot was visually inspected to decide if the regression residuals were randomly distributed, indicating linear calibration. If a trend was observed in the residuals, the calibration was not acceptable.

The correlation coefficient (r) for the calibration lines was acceptable at ≥ 0.995.

3.6.7.4. LOD (Limit of Detection) Assessment

An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions for V-10456, S-3100-CA and S-3100-CA-UR.

The MDL was calculated as the initial sample concentration equivalent to the lowest calibration standard (based upon the lowest standard concentration and a dilution factor of 10). The lowest standard concentration was 0.005 ng/mL for V-10456 and 0.025 ng/mL for S-3100-CA and S-3100-CA-UR.

Note that in SANTE/2020/12830 rev.1 (2021), the LOD, rather than the MDL (as in this report), is expressed as the lowest calibration standard.

3.6.7.5. Matrix Assessment

An assessment of matrix effects was made by comparison of the peak area response of standards prepared using final matrix extracts against non-matrix standards.
This applied to the primary and confirmatory transitions.

Results were presented as a % difference from the mean non-matrix standard value and a difference of $\geq$ 20% was considered significant.

3.6.7.6. Stock Solution Stability Assessment

The stability of primary stock solutions (V 10456, S 3100 CA and S 3100 CA UR) was investigated by comparing freshly prepared standard solutions diluted from freshly prepared stock solutions with freshly prepared standard solutions diluted from stocks previously prepared (>1 week old) stored in an appropriate solvent.
Each solution was injected in quintuplet and only the primary transitions were reported.

The stability of primary stock solutions was considered to be suitably demonstrated if the difference between the means from the five replicate measurements of standards diluted from stored and freshly prepared stock solutions were $\leq$ 10%.

4.1.11. Stock Solution Stability Assessment

The stability of primary stock solutions (V-10456, S-3100-CA and S-3100-CA-UR) was investigated by comparing freshly prepared standard solutions diluted from freshly prepared stock solutions with freshly prepared standard solutions diluted from stocks previously prepared (>1 week old) stored in an appropriate solvent.
Stock solution stability results are presented in Table 21 to Table 23.

V-10456 and S-3100-CA were found to be stable over the period of 8 days, which encompassed the time between stock preparation and validation batch extraction over the course of this study.

S-3100-CA-UR was not found to be stable over this period because the difference between the old and new stock solutions was approximately -33%. This does not affect the integrity of the study data because the fortification stock and the calibration stock were prepared on the same day as each other and both were diluted for use as fortification standards and calibration standards on the same day as batch extraction, so any degradation would have happened to both solutions, essentially negating any instability.

6. CONCLUSIONS

The analytical method in the Smithers (Wareham, USA) analytical method 12709.6509 (V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions) was successfully independently validated in ground and surface water in accordance with the EPA OCSPP 850.6100 (2012), EPA OPPTS 860.1340 (1996) and SANTE/2020/12830 rev.1 (2021) guidelines.

The independently validated method was issued as SM 3203100-01V.

Five control samples each of ground and surface water were fortified with V-10456, S-3100-CA and S-3100-CA-UR at limit of quantification (LOQ), 10xLOQ and high-level and analysed. The fortified concentrations of V-10456 were 0.1 µ/L (LOQ), 1 µg/L (10xLOQ) and 1000 µg/L (high-level). The fortified concentrations of S-3100-CA and S-3100-CA-UR were 1 µg/L (LOQ), 10 µg/L (10xLOQ) and 1000 µg/L (high-level). Recoveries of V-10456, S-3100-CA and S-3100-CA-UR (primary and confirmatory) were calculated and the validity of the analytical method was assessed.

Mean recoveries met the protocol criteria of 70 to 120% in reference to SANTE/2020/12830 and the Analytical Method 12709.6509 acceptance criteria of 70 to 110% at each concentration for V-10456, S-3100-CA and S-3100-CA-UR for the primary and confirmatory transitions for the two waters tested.

Precision was acceptable (≤ 20%) for the primary and confirmatory transitions for the two waters tested.

Any interferences at the retention time of V-10456, S-3100-CA and S-3100-CA-UR found in ground or surface water were ≤ 30% of the LOQ peak area response.

The suitability of the calibration lines were demonstrated by random distribution of the regression residuals in the residual plots.

The correlation coefficients (r) for V-10456, S-3100-CA and S-3100-CA-UR were all ≥ 0.995.

The observed matrix enhancement or suppression of the analyte peak was not greater than 20% of V-10456, S-3100-CA and S-3100-CA-UR and, in-line with the environmental chemistry method 12709.6509, matrix-matched calibration standards were used for the validation of both water types.

The method for the analysis of V-10456, S-3100-CA and S-3100-CA-UR was successfully validated in the two waters tested.

7. REFERENCES

- EPA OCSPP 850.6100 (2012): Environmental Chemistry Methods and Associated Independent Laboratory Validation.
- EPA OPPTS 860.1340 (1996): Residue Analytical Method.
- SANTE/2020/12830 rev.1 (2021): Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.
- Miller, James N. and Jane C.(2010). "Statistics and Chemometrics for Analytical Chemistry", Sixth Edition.

Appendix 3
Analytical Procedure and Flowchart

SM 3203100-01V

Analytical Procedure

Procedure Title	Determination of V-10456, S-3100-CA and S-3100-CA-UR in Ground Water and Surface Water by LC-MS/MS
Procedure Code	SM 3203100-01V
Issue Date	22 October 2021
Page Number	1 of 14

REVISION HISTORY

SMV 3203100-01V	Updated after validation.
SMV 3203100-02D	Updates to calibration standards and matrix assessment standards sections, instrument conditions, retention times and typographical corrections.
SMV 3203100-01D	New method for independent laboratory validation based upon the Smithers (Wareham, USA) analytical method in study 12709.6509 (V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions).

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining concentrations of V-10456, S-3100-CA and S-3100-CA-UR in ground water and surface water by LC-MS/MS.

Samples are fortified with V-10456, S-3100-CA and S-3100-CA-UR before being subjected to the prescribed method and quantified by LC-MS/MS.

Matrix effects for V-10456, S-3100-CA and S-3100-CA-UR in ground water and surface water will be determined by comparing peak areas of calibration standards prepared in control final extract and in the relevant final extract solvent. Matrix effects are considered significant if the matrix-matched standard area is $\geq 20\%$ different from the non-matrix standard area. If matrix effects are significant then matrix-matched calibration standards should be used.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- Shimadzu Nexera series HPLC system with Sciex API 5000 MS/MS detector
- Phenomenex Kinetex Phenyl-hexyl 2.6 µm, 3 × 50 mm
- Analytical balance
- Polypropylene centrifuge tubes (PPCT)
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- HPLC vials
- Various disposable laboratory glassware

With the exception of the HPLC column, equivalent equipment may be used if required.

Materials

• Acetonitrile	HPLC grade, Honeywell
• Methanol	HPLC grade, Honeywell
• Formic acid	≥ 98%, Honeywell
• 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

Equivalent or better grade materials may be used if required.

Reagents

0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)
Mix 500 mL HPLC grade acetonitrile, 500 mL Milli-Q water and 1 mL formic acid.

0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v)
Mix 560 mL HPLC grade acetonitrile, 440 mL Milli-Q water and 1 mL formic acid.

0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v)
Mix 500 mL HPLC grade acetonitrile, 100 mL of ground water, 400 mL Milli-Q water and 1 mL formic acid.

0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v)
Mix 500 mL HPLC grade acetonitrile, 100 mL of surface water, 400 mL Milli-Q water and 1 mL formic acid.

Acetonitrile:Milli-Q water (50:50 v/v)
Mix 150 mL HPLC grade acetonitrile with 150 mL Milli-Q water.

Acetonitrile:methanol:water (30:30:40 v/v/v)
Mix 300 mL LC/MS grade acetonitrile, 300 mL LC/MS grade methanol and 400 mL Milli-Q water.

Reagents may be stored at room temperature (15-25°C) for up to one month and volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stocks

Prepare duplicate stock solutions (stocks A and B) of V-10456, S-3100-CA and S-3100-CA-UR, separately. Accurately weigh ≥ the required amount of each test substance, corrected for purity and transfer into an appropriately sized volumetric flask. Adjust the volume to give the exact concentration required. The following dilution scheme is suggested:

Test Substance	Target Weight (mg)	Solvent	Final Volume (mL)	Primary Stock Concentration (µg/mL)
V-10456	10	Acetonitrile	10	1000
S-3100-CA	10	Acetonitrile:Milli-Q water (50:50 v/v)	10	1000
S-3100-CA-UR	10	Acetonitrile:Milli-Q water (50:50 v/v)	10	1000

Transfer into amber glass bottles with PTFE-lined caps. One stock is for preparing calibration standards and the other is for preparing quality control (QC) standards. Should further primary stocks be required, these should also be prepared in duplicate (stocks C and D) where one is for preparing calibration standards and the other is for preparing quality control (QC) standards.

The primary stocks of V-10456, S-3100-CA and S-3100-CA-UR should be stored refrigerated (2-8°C) and given a nominal expiry date of 1 month.

Secondary Standard Stocks

Prepare secondary stock solutions from the duplicate primary stocks of V-10456, S-3100-CA and S-3100-CA-UR, separately, at 10 µg/mL. The following dilution scheme is suggested:

Test Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
V-10456	1000	0.5	Acetonitrile	50	10
S-3100-CA	1000	0.5	Acetonitrile:Milli-Q water (50:50 v/v)	50	10
S-3100-CA-UR	1000	0.5	Acetonitrile:Milli-Q water (50:50 v/v)	50	10

Transfer into amber glass bottles with PTFE-lined caps. The secondary stocks should be stored refrigerated (2-8°C) and given a nominal expiry date of 1 month.

Calibration Sub-Stocks and Fortification Standards

Using the secondary standards prepared from the "A" stocks of V-10456, S-3100-CA and S-3100-CA-UR, prepare one set of mixed standard solutions to be used for sample fortification purposes. Using the secondary standards prepared from the "B" stocks of V-10456, S-3100-CA and S-3100-CA-UR, prepare one set of mixed standard solutions to be used for the preparation of the calibration lines and for matrix assessment.

The following dilution scheme is suggested for the preparation of calibration sub-stock standards and fortification standards from primary and secondary stocks:

Test Substance	Standard Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Mixed Standard Concentration (µg/mL)
V-10456	10	0.02	0.1% formic acid in Acetonitrile:Milli-Q water (50:50 v/v)	20	0.01 (V-10456) 0.1 (S-3100-CA and S-3100-CA-UR[1]
S-3100-CA	10	0.2			
S-3100-CA-UR	10	0.2			
V-10456	10	0.2		20	0.1 (V-10456) 1 (S-3100-CA and S-3100-CA-UR[2]
S-3100-CA	10	2			
S-3100-CA-UR	10	2			
V-10456	1000	0.2		20	10[3]
S-3100-CA	1000	0.2			
S-3100-CA-UR	1000	0.2			
V-10456	10	0.01		20	0.005 (V-10456) 0.025 (S-3100-CA and S-3100-CA-UR[4]
S-3100-CA	10	0.05			
S-3100-CA-UR	10	0.05			

[1]From mixed standard prepared from Stock A secondary stock, for fortification of LOQ recovery samples.
[2]From mixed standard prepared from Stock A secondary stock, for fortification of 10 x LOQ recovery samples.
[3]Prepare from Stock A for fortification of high-level recovery samples.
[4]From mixed standard prepared from Stock B secondary stock, use for calibration standards and matrix effects assessment.

Fortification and calibration sub-stock standards should be prepared fresh for each assay.

Calibration Standards

Prepare calibration solutions of V-10456, S-3100-CA and S-3100-CA-UR in 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v), as appropriate.
The following dilution scheme is suggested:

Sub-Stock Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)	Volume Taken (mL)	Volume of Solvent (mL)	Final Volume (mL)	Calibration Standard ID	Calibration Standard Concentration V-10456/ S-3100-CA / S-3100-CA-UR (ng/mL)
5/25/25	0.1	9.9	10	Cal 1	0.05/0.25/0.25
5/25/25	0.08	9.92	10	Cal 2	0.04/0.2/0.2
5/25/25	0.06	9.94	10	Cal 3	0.03/0.15/0.15
5/25/25	0.04	9.96	10	Cal 4	0.02/0.1/0.1
5/25/25	0.02	9.98	10	Cal 5	0.01/0.05/0.05
5/25/25	0.01	9.99	10	Cal 6	0.005/0.025/0.025

Calibration standards should be prepared fresh for each assay.

Matrix-Matched Standards for Matrix Assessment

Prepare triplicate matrix-matched standards and non-matrix matched standards of V-10456, S-3100-CA and S-3100-CA-UR in volumetric flasks for the matrix assessment. The following dilution scheme is suggested:

Mixed Standard Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)	Volume Taken (mL)	Dilution Solvent	Final Volume (mL)	Calibration Standard Concentration V-10456/ S-3100-CA/ S-3100-CA-UR (ng/mL)
5/25/25	0.1	0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v)	50	0.01/0.05/0.05
5/25/25	0.1		50	0.01/0.05/0.05
5/25/25	0.1		50	0.01/0.05/0.05
5/25/25	0.1	0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)	50	0.01/0.05/0.05
5/25/25	0.1		50	0.01/0.05/0.05
5/25/25	0.1		50	0.01/0.05/0.05

The peak areas of the matrix-matched and non-matrix matched standards will be compared and used to calculate matrix effects.

PROCEDURES

All procedures will be carried out in compliance with local SOPs, following local safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]
Measure 5 mL of control ground water or surface water into 50 mL polypropylene centrifuge tubes (PPCT). Fortify samples with the appropriate V-10456, S-3100-CA and S-3100-CA-UR standards. The following fortification scheme is suggested:

Number of Replicates	Sample Type	Stock Concentration (ng/mL)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
2	Reagent Blank	N/A	N/A	5	N/A
5	Control	N/A	N/A	5	N/A
5	LOQ	10/100/100*#	0.05	5	0.1/1/1#
5	10xLOQ	100/1000/1000*#	0.05	5	1/10/10#
5	High-Level	10000	0.5	5	1000

N/A = Not Applicable.
#For V-10456, S-3100-CA and S-3100-CA-UR, respectively.
*10 ng/mL = 0.01 µg/mL, 100 ng/mL = 0.1 µg/mL, 1000 ng/mL = 1 µg/mL, 10000 ng/mL = 10 µg/mL.

Sample Extraction [1b, 4a]

1. Measure 5 mL of control ground water or surface water into 50 mL polypropylene centrifuge tubes (PPCT).
2. Fortify samples as described in the table above.
3. Add 45 mL of 0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v) to give a final composition of 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v)., as appropriate.
4. Transfer the reagent blank, control and LOQ samples to HPLC vials for LC-MS/MS analysis.
5. Dilute the 10xLOQ samples 1 mL up to 10 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate and transfer into HPLC vials for LC-MS/MS analysis.
6. Dilute the high-level samples 0.09 mL up to 50 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate and transfer into HPLC vials for LC-MS/MS analysis of S-3100-CA and S-3100-CA-UR.
7. Further dilute the high-level samples 1 mL up to 5 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate and transfer into HPLC vials for LC-MS/MS analysis of V-10456.

Dilution Factor Calculation Summary

The following calculations are required to calculate the software dilution factor for reagent blank, control, LOQ and 10xLOQ samples:

$$Software\ Dilution\ Factor\ = \frac{Final\ Extract\ Volume\ (mL) \times Sample\ Dilution}{Sample\ Amount\ (g\ or\ mL)} \times Unit\ Conversions$$

Where (for samples which have no further dilutions performed):

Sample amount = 5 mL
Final extract volume = 50 mL
Sample dilution = 1 (no further dilution)
Unit conversion = 1 (no unit conversion)

Software dilution factor = 50/5 = 10.

Where (for 10xLOQ samples):

Sample amount = 5 mL
Final extract volume = 50 mL
Sample dilution = 10
Unit conversion = 1 (no unit conversion)

Software dilution factor = (50x10)/5 = 100.

The following calculations are required to calculate the software dilution factor for high-level samples:

$$Adjusted\ Sample\ Amount\ (g\ or\ mL) = \frac{Initial\ Sample\ Amount\ (g\ or\ mL)}{Initial\ Extract\ Volume\ (mL)} \times Aliquot\ Volume\ (mL)$$

$$Software\ Dilution\ Factor\ = \frac{Final\ Extract\ Volume\ (mL) \times Sample\ Dilution}{Adjusted\ Sample\ Amount\ (g\ or\ mL)} \times Unit\ Conversions$$

Where (for high-level S-3100-CA and S-3100-CA-UR samples only):

Initial sample amount = 5 mL
Initial extract volume = 50 mL
Aliquot volume = 0.09 mL
Adjusted sample amount = 0.009 mL
Final extract volume = 50 mL
Sample dilution = 1
Unit conversion = 1 (no unit conversion)

Software dilution factor = 50/((5/50)x0.09) = 5556.

Additional dilution (for high-level V-10456 samples only):

Aliquot volume = 1 mL
Final extract volume = 5 mL
Sample dilution = 5556
Unit conversion = 1 (no unit conversion)

Software dilution factor = (5/1)x5556 = 27780.

LC-MS/MS CONDITIONS

HPLC Parameters

Instrument:	Shimadzu Nexera series HPLC system
Column#:	Phenomenex Kinetex Phenyl-hexyl 2.6 µm, 3 × 50 mm
Mobile Phase A#:	0.1% formic acid in water
Mobile Phase B#:	0.1% formic acid in acetonitrile
Flow Rate:	0.5 mL/min

Gradient:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.01	90	10
0.5	90	10
4.0	0	100
4.5	0	100
4.6	90	10
6.0	90	10

Run Time:	6 minutes
Autosampler Wash Solvent:	Acetonitrile:methanol:water (30:30:40 v/v/v)
Column Temperature:	40°C
Autosampler Temperature:	10°C
Injection Volume:	35 µL
Retention Time:	V-10456 – approximately 3.50 minutes S-3100-CA – approximately 3.05 minutes S-3100-CA-UR – approximately 2.50 minutes

MS/MS Parameters

Instrument:	Sciex API 5000 mass spectrometer
Ionisation Type#:	Electrospray (ESI)
Polarity#:	Positive
Ion Spray Voltage:	4500 V
Scan Type#:	Multiple reaction monitoring (MRM)
Source Temperature:	650°C
Curtain Gas:	20.0
Ion Source – Gas 1/Gas 2:	50.0/50.0
Collision Gas:	8.0
Resolution (Q1/Q3):	Unit/Low
Dwell Time (milliseconds):	100

Test Substance	**V-10456**		**S-3100-CA**		**S-3100-CA-UR**	
Instrument Parameter	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da)	518.1/472.1	518.1/444.1	490.2/249.9	490.2/292.9	370.2/312.9	370.2/351.7
Collision Energy	27.0	30.0	40.0	40.0	21.0	20.0
Collision Cell Entrance Potential	10.0	10.0	10.0	10.0	7.0	7.0
Collision Cell Exit Potential	20.0	20.0	20.0	26.0	22.0	29.0
Declustering Potential	120	120	180	180	75.0	75.0

Note: Instrument parameters are provisional and may be subject to change after optimisation.

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

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where the calibration fit is linear as in this study, Analyst 1.6.2 uses the following formula to calculate the concentration of test substance present in the sample extract.

$$\text{Amount } (\mu g/L) = \left[\frac{y - c}{m}\right] \times DF$$

Where:
y = Peak area
c = Y intercept on calibration graph
m = Slope of the linear calibration graph
DF = Software dilution factor (see Dilution Factor Calculations Summary)

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/L)
S = concentration added to fortified sample (µg/L)

METHOD CRITERIA

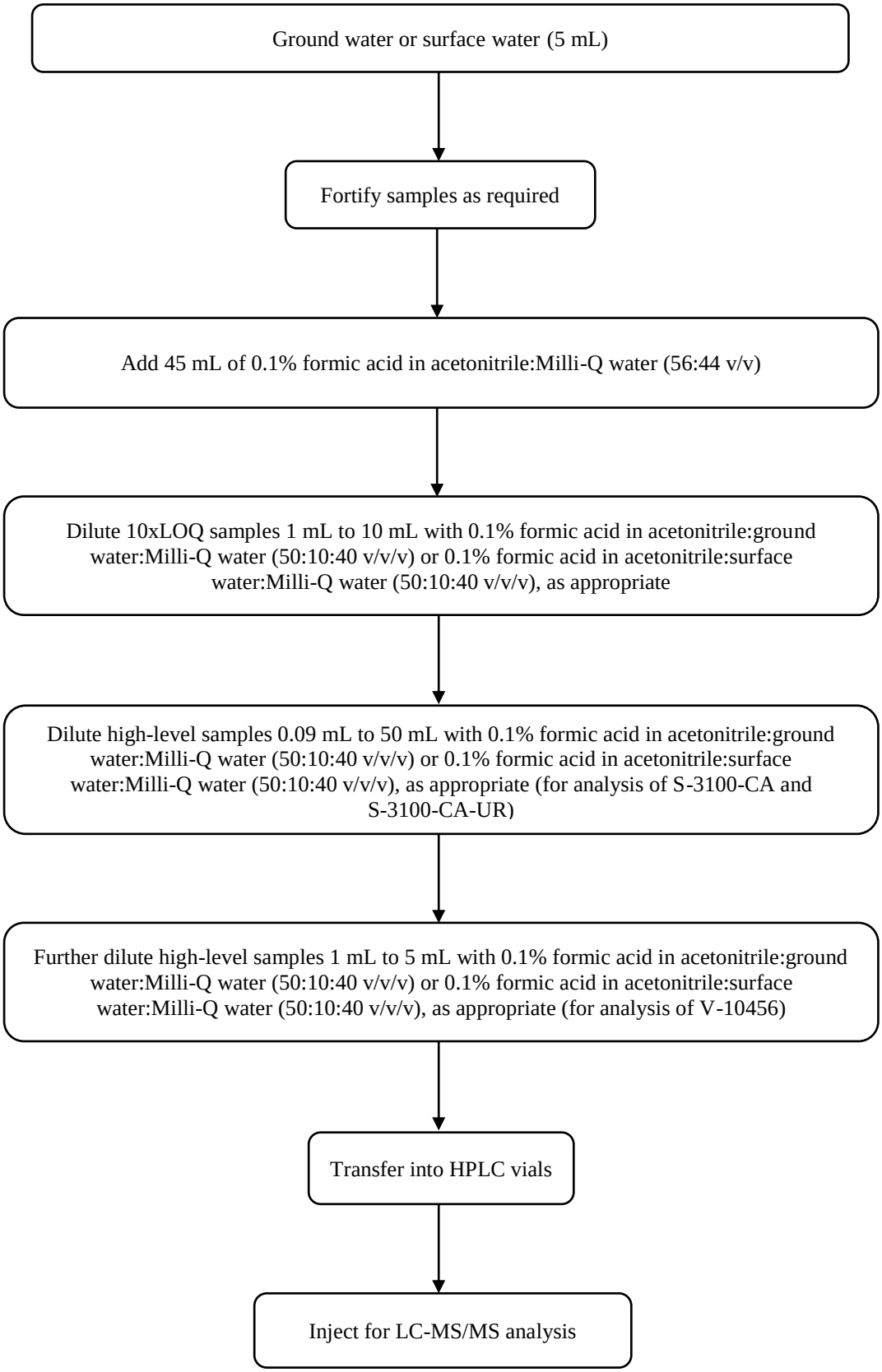
For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.995 with a 1/x weighting.
- The residual plot will have a visually random distribution of regression residuals.
- All sample extracts should be within the appropriate range of calibration standards.
- Mean recovery from fortified samples should be within the range of 70 to 120% at each concentration.
- Precision of fortified sample recoveries should be ≤ 20% RSD at each concentration.
- The control sample should not contain interference > 30% of the LOQ at the retention time of the test substance.

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main Division	CONTROL Name and Specification
1	GLOVES
a	Disposable latex
b	Disposable nitrile
c	Rubber gloves
d	Specific type for the job (see assessment giving details)
2	PROTECTIVE CLOTHING
a	Laboratory coat or equivalent
b	Disposable overalls
c	Oversleeves
d	Overshoes
e	Plastic apron
3	EYE/FACE PROTECTION
a	Safety glasses to BS 2092/2 C or better
b	Face shield to BS 2092/2 C or better
c	Safety goggles to BS 2092/2 C or better
4	ENGINEERING CONTROLS
a	Open bench in ventilated area
b	Fume cupboard to BS 7258
c	Laminar flow cabinet to BS 5295 Class 1
d	Re-circulating fume chamber
e	Radioisotope lab
f	Biohazard lab
g	Glove box
5	RESPIRATORY PROTECTIVE EQUIPMENT
a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
c	Respirator with specified canister (HSE approved)
6	SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7	ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8	REFER TO MATERIAL SAFETY DATA SHEET
9	KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10	POISON – ensure antidote is available and is within its expiry date (must specify details)

Flowchart


Ground water or surface water (5 mL)
Fortify samples as required
Add 45 mL of 0.1% formic acid in acetonitrile:Milli-Q water (56:44 v/v)
Dilute 10xLOQ samples 1 mL to 10 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate
Dilute high-level samples 0.09 mL to 50 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate (for analysis of S-3100-CA and S-3100-CA-UR)
Further dilute high-level samples 1 mL to 5 mL with 0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v/v) or 0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v/v), as appropriate (for analysis of V-10456)
Transfer into HPLC vials
Inject for LC-MS/MS analysis