INTRODUCTION

On 10 September 2019, the legal name of Smithers Viscient Ltd was changed to Smithers ERS Limited. The legal entity did not change. Smithers ERS Limited is a business unit of The Smithers Group that is engaged in providing contract research services, however, both Smithers Viscient and Smithers ERS may appear in the protocol and the study report during the transition.

The objective of this study was to independently validate the analytical method in Study No. 14181.6108, for measuring residues of Triadimefon in ground and surface water, in accordance with EPA OCSPP 850.6100 (2012) and SANCO/3029/99 rev 4 (2000) guidelines.

The analytical method (Study No. 14181.6108) was provided by Smithers ERS, Wareham on behalf of the sponsor. The method was re-written in Smithers ERS, Harrogate format as draft method SMI 3202454-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMI 3202454-01V when validation was complete.

Control samples of ground and surface water were fortified with Triadimefon at 0.1 and 1 µg/L in quintuplicate and analysed. Samples were diluted with acetonitrile: water (20:80 v/v) to give a final composition of acetonitrile: test matrix: water (18:10:72 v/v). Samples were further diluted into the calibration range with acetonitrile: test matrix: water (18:10:72 v/v) for matrix matching.

To assess matrix effects, triplicate standards were prepared in acetonitrile: water (20:80 v/v) and in acetonitrile: test matrix: water (18:10:72 v/v).

Samples were analysed for Triadimefon 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 Triadimefon. One primary and one confirmatory LC-MS/MS transition were analysed for Triadimefon.

STUDY TIMETABLE

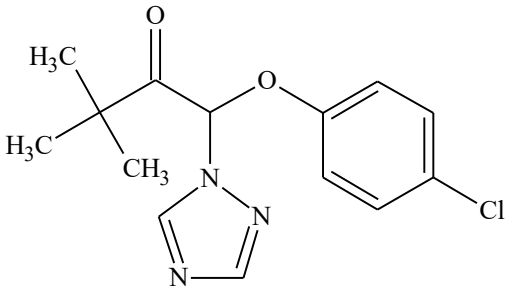
Study initiation:	17 March 2020 (date the protocol was signed by the Study Director).
Experimental start:	28 March 2020 (stock preparation).
Experimental completion:	26 April 2020 (LC-MS/MS analysis).
Study completion:	Date the final report was signed by the Study Director.

MATERIALS AND METHODS

Protocol Adherence

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

Test Substance

Test Substance Name:	**Triadimefon**
IUPAC Name:	1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone
CAS Number:	43121-43-3
Structure:	
Molecular Formula:	$C_{14}H_{16}ClN_3O_2$
Molecular Weight:	293.75
Batch Number:	BCBW0134
Purity:	99.1%
Storage Conditions:	Room Temperature (15-25°C)
Expiry Date:	31 October 2022

The Certificate of Analysis for the test substance is presented in Appendix 1.

Test Matrices

Control ground and surface water were sourced by Smithers ERS. The waters used were CS 13/18 Borehole groundwater and CS 01/20 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	CS 13/18	Ground	2	436	349	8.0	0.00
Fountains Abbey	CS 01/20	Surface	5	140	132	7.51	8.53

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

Reagents

• Acetonitrile	HPLC grade, 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

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 x 50 mm
- Analytical balance
- Positive displacement pipettes
- Glass jars
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

An environmental chemistry method, which had been validated for the analysis of Triadimefon in water by Smithers ERS, Wareham (Study No. 14181.6108), was supplied on behalf of the Sponsor. A summary of correspondence with the Sponsor is given in Appendix 5.

The method was re-written in Smithers ERS, Harrogate format as draft method SMI 3202454-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMI 3202454-01V when validation was complete. The complete analytical procedure is presented in Appendix 3.

Preparation of Reagents

Acetonitrile: water (20:80 v/v)

200 mL acetonitrile was mixed with 800 mL Milli-Q water.

Acetonitrile: test matrix: water (18:10:72 v/v)

18 mL acetonitrile was mixed with 10 mL control ground or surface water and 72 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 Triadimefon were prepared as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]	Stock Use
Stock 1	10.19	99.1	Acetonitrile	10.099	1000	Secondary stock solution
Stock 2	10.19			10.099	1000	

[1] Corrected for Purity.
Duplicate stocks were prepared for correlation purposes.

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

Secondary Stock Solutions

Secondary stock solutions of Triadimefon 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 stocks were stored refrigerated in amber glass bottles and given a nominal expiry of one month.

Sub-Stock Solutions

Sub-stock solutions of Triadimefon 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	1	1	Sub-stock solution
1	0.1		1	0.1[1]	Fortification at 10 × LOQ
0.1	0.1		1	0.01[2]	Fortification at LOQ
0.01	0.1		1	0.001[3]	Calibration standards

[1] Equivalent to 100 µg/L.
[2] Equivalent to 10 µg/L.
[3] Equivalent to 1 µg/L.
The final volume of sub-stock solutions were scaled as appropriate using appropriate volumes and concentrations of secondary stock solution to give the required concentration.

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 Triadimefon were prepared in acetonitrile: water (20:80 v/v) for comparison with matrix-matched standards.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.1	Acetonitrile: water (20:80 v/v)	10	0.01
1	0.1		10	0.01
1	0.1		10	0.01

Preparation of Matrix Matched Standards for Matrix Assessment
Matrix-matched standards of Triadimefon were prepared in acetonitrile: test matrix: water (18:10:72).

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.1	Acetonitrile: test matrix: water (18:10:72)	10	0.01
1	0.1		10	0.01
1	0.1		10	0.01

The three matrix-matched standards for each water were analysed alternately with the three non-matrix matched standards and their peak areas compared.

Preparation of Calibration Standards

Matrix-matched calibration standards of Triadimefon were prepared for the validation of groundwater and surface water as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
1	0.5	Acetonitrile: test matrix: water (18:10:72 v/v)	10	0.05
0.05	0.8		1	0.04
0.05	0.6		1	0.03
0.05	0.4		1	0.02
0.05	0.2		1	0.01
0.05	0.1		1	0.005

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

Sample Preparation and Fortification
5 mL of sample water was measured into a glass vial. Quintuplicate water samples were fortified at the LOQ (0.1 µg/L) and at 10 × LOQ (1 µg/L) with stock solutions of Triadimefon. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Borehole groundwater

Sample ID	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank A[1]	5	N/A	N/A	N/A
Control A-B	5	N/A	N/A	N/A
F0.1 A-E	5	0.01	0.05	0.1
F1 A-E	5	0.1	0.05	1

N/A = Not applicable.
[1]Milli-Q water was used as the reagent blank.

Fountains Abbey surface water

Sample ID	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank B[1]	5	N/A	N/A	N/A
Control C-D	5	N/A	N/A	N/A
F0.1 F-J	5	0.01	0.05	0.1
F1 F-J	5	0.1	0.05	1

N/A = Not applicable.
[1] Milli-Q water was used as the reagent blank.

Sample Dilution

5 mL of sample water was measured into a glass vial. 45 mL of acetonitrile: water (20:80 v/v) was added and mixed. Samples were further diluted into the calibration range using acetonitrile: test matrix: water (18:10:72 v/v) for matrix matching as described in the following tables.

Borehole groundwater

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank A	N/A	5	50	N/A	10
Control A-B	N/A	5	50	N/A	10
F0.1 A-E	0.1	5	50	N/A	10
F1 A-E	1	5	50	0.3-1	33.3

N/A = Not applicable.

Fountains Abbey surface water

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank B	N/A	5	50	N/A	10
Control C-D	N/A	5	50	N/A	10
F0.1 F-J	0.1	5	50	N/A	10
F1 F-J	1	5	50	0.3-1	33.3

N/A = Not applicable.

Instrument Conditions

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

LC Parameters:

Instrument:	Shimadzu Nexera series HPLC system		
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[1]:	0.5 mL/min		
Gradient[2]:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	80	20
	0.5	80	20
	3.0	0	100
	4.0	0	100
	4.1	80	20
	5.5	80	20
Run Time:	5.5 minutes		
Column Temperature:	40°C		
Autosampler Temperature:	4°C		
Injection Volume:	50 µL		
Retention Time:	Approx. 2.4 minutes		
Valco Valve Diverter:	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.0	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:	4500 V		
Collision Gas (CAD):	5		
Curtain Gas (CUR):	25		
Gas Flow 1 (GS1):	50		
Gas Flow 2 (GS2):	50		
Vaporiser Temperature (TEM):	550°C		
Interface Heater (ihe):	On		
Entrance Potential (EP):	10		
Declustering Potential (DP):	40		
Collision Exit Potential (CXP)	10		
Resolution Q1/Q3:	Unit/Unit		
Transition Name:	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Triadimefon (Primary):	294.1/197.6	22	200
Triadimefon (Confirmatory):	294.1/69.1	30	200

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

[1]The flow rate was increased from 0.4 to 0.5 mL/min to improve peak sensitivity.
[2]The ramp of the gradient was shortened from reaching 100 mobile phase B at 4 minutes to 3 minutes to reduce peak broadening observed with a longer ramp.

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:

$$x = \frac{(y - c)}{m} \times DF$$

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 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 (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 Triadimefon:

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 Triadimefon were found in the control samples at > 30% of the LOQ peak area response.

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ concentration (after dilution). The highest calibration standard concentration was ≥ 120% of the 10 × LOQ concentration (after dilution). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

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

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 0.005 µg/L and a dilution factor of 10).

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in acetonitrile: water (20:80 v/v) and in acetonitrile: test matrix: water (18:10:72). This was assessed for Triadimefon 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 significant.

If matrix effects were determined to be significant, matrix-matched calibration standards would be used for method validation.