## 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. 14181.6107, for measuring residues of Triadimefon in soil, in accordance with EPA OCSPP 850.6100 (2012) and SANCO/3029/99 rev 4 (2000) guidelines.

The analytical method (Study No. 14181.6107) 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 3202453-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMI 3202453-01V when validation was complete.

Control samples of sandy loam and silt loam soil were fortified with Triadimefon at 50 and 500 µg/kg in quintuplicate and analysed. Samples were extracted twice with acetonitrile. An aliquot was diluted into calibration range with acetonitrile: water (20:80 v/v).

To assess matrix effects, triplicate standards were prepared in control soil final extract and in acetonitrile: water (20:80 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 soil for Triadimefon.
One primary and one confirmatory LC-MS/MS transition were analysed for Triadimefon.

## STUDY TIMETABLE

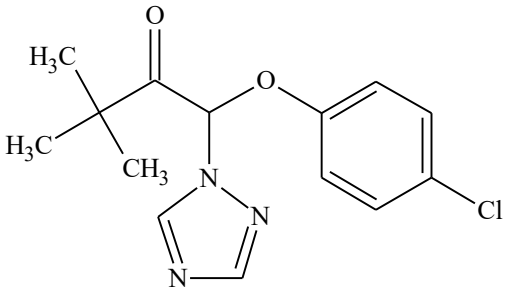
| | |
|---|---|
| Study initiation: | 17 March 2020 (date the protocol was signed by the Study Director). |
| Experimental start: | 20 April 2020 (soil moisture). |
| Experimental completion: | 10 May 2020 (LC-MS/MS analysis). |
| Study completion: | Date the final report was signed by the Study Director. |

## 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 sandy loam and silt loam soil were sourced by Smithers ERS. The soils used were CS 30/18 (sandy loam) and CS 17/18 (silt loam).

Soil characterisation data are listed in the following table:

| Soil Name | Unique ID | Textural class[1] | % Sand, Silt, Clay[2] | CEC (meq/100 g) | % Organic Carbon | pH in $H_2O$ | pH in 0.01M $CaCl_2$ |
|---|---|---|---|---|---|---|---|
| RefeSol 01-A | CS 30/18 | sandy loam[3] | 74, 20, 6[3] | 5.3 | 0.9[3] | 6.4 | 5.3[3] |
| Newhaven | CS 17/18 | silt loam | 25, 51, 24 | 17.4 | 3.2 | 6.0 | 5.4 |

[1, 2] USDA classification.
[3] Soil characterisation data provided by Fraunhofer.

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

The moisture contents of the soils were determined to be 4.77 % and 26.08 % of the dry soil weight for RefeSol 01-A and Newhaven soil respectively.

**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
- Centrifuge: Allegra X-15R
- Centrifuge tubes
- Glass jars
- Orbital shaker: Edmund Buhler SM 30 A
- Positive displacement pipettes
- 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 soil by Smithers ERS, Wareham (Study No. 14181.6107), 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 3202453-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMI 3202453-01V 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 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 at 1000 µg/mL in acetonitrile under Smithers ERS GLP Study No. 3202454 (Independent Laboratory Validation of Analytical Method 14181.6108 for the Determination of Triadimefon in Water). Primary stock solutions were stored refrigerated for up to 3 months.

*Secondary stock solutions*
Secondary stock solutions of Triadimefon were prepared at 10 µg/mL in acetonitrile under Smithers ERS GLP Study No. 3202454 for matrix assessment and validation of sandy loam soil.

Secondary stock solutions of Triadimefon were prepared for the validation of silt loam soil 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 | Fortification at 10 × LOQ |

Secondary stock solutions were stored refrigerated for up to 1 month.

***Sub-Stocks***
Sub-stock solutions of Triadimefon in acetonitrile 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[2] | 1 | Fortification at LOQ |
| 1 | 0.1 | | 1 | 0.1 | Sub-stock solution |
| 0.1 | 0.1 | | 1 | 0.01[1] | Intermediate calibration standard |

[1] Equivalent to 10 µg/L.
[2] The final volume of sub-stock solution was scaled as appropriate using the required volume and concentration of secondary stock solution.

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 as described in the following table:

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.03 | Acetonitrile: water (20:80 v/v) | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |

***Preparation of Matrix-Matched Standards for Matrix Assessment***
Matrix-matched standards of Triadimefon were prepared in control soil final extract as shown in the table below:

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.03 | Sandy loam soil final extract | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |
| 10 | 0.03 | Silt loam soil final extract | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |
| 10 | 0.03 | | 10 | 0.03 |

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

***Preparation of Intermediate Calibration Standards***
An intermediate calibration standard of Triadimefon was prepared as described in the following table:

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) | Stock Use |
|---|---|---|---|---|---|
| 10 | 0.1 | Acetonitrile: water (20:80 v/v) | 1 | 1 | Calibration standards |

***Preparation of Calibration Standards***
Non-matrix matched calibration standards of Triadimefon were prepared for the validation of sandy loam and silt loam soil as described in the following table:

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 1 | 0.3 | Acetonitrile: water (20:80 v/v) | 1 | 0.3 |
| 1 | 0.1 | | 1 | 0.1 |
| 1 | 0.05 | | 1 | 0.05 |
| 0.3 | 0.1 | | 1 | 0.03 |
| 0.1 | 0.1 | | 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±0.05 g dry weight equivalent of soil was weighed into a Polypropylene centrifuge tube. Quintuplicate soil samples were fortified at the LOQ (50 µg/kg) and at 10 × LOQ (500 µg/kg) with a stock solution of Triadimefon. Duplicate control soil samples and a reagent blank (no soil) were also prepared, as described in the following tables:

Sandy loam soil

| Sample ID | Sample Weight (g) | Stock Concentration (µg/mL) | Volume Added (mL) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent Blank A[1] | N/A | N/A | N/A | N/A |
| Control A[2] | 5 | N/A | N/A | N/A |
| Control C-D | 5 | N/A | N/A | N/A |
| F0.05 A-E | 5 | 1 | 0.25 | 50 |
| F0.5 A-E | 5 | 10 | 0.25 | 500 |

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control A was used for matrix assessment.

Silt loam soil

| Sample ID | Sample Weight (g) | Stock Concentration (µg/mL) | Volume Added (mL) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent Blank B[1] | N/A | N/A | N/A | N/A |
| Control B[2] | 5 | N/A | N/A | N/A |
| Control E-F | 5 | N/A | N/A | N/A |
| F0.05 F-J | 5 | 1 | 0.25 | 50 |
| F0.5 F-J | 5 | 10 | 0.25 | 500 |

N/A = Not applicable.
[1] No soil was used for the reagent blank.
[2] Control B was used for matrix assessment.

***Sample Extraction***

The samples were extracted twice with 20 mL acetonitrile by sonicating for 10 minutes, shaking at 200 rpm for 30 minutes and centrifuging at 3000 rpm for 10 minutes. The supernatant was transferred into a glass jar and made to 50 mL with acetonitrile in a volumetric flask. The sample extract was diluted into calibration range with acetonitrile: water (20:80 v/v). This was transferred into an HPLC vial for analysis. The extraction and dilution procedure is summarised in the following tables:

Sandy loam soil

| Sample ID | Fortified Concentration (µg/kg) | Sample Weight (g) | Extract Volume (mL) | Dilution (mL-mL) | Dilution Factor |
|---|---|---|---|---|---|
| Reagent blank A | N/A | N/A | 50 | 0.05-10 | 2000 |
| Control A | N/A | 5 | 50 | 0.05-10 | 2000 |
| Control C-D | N/A | 5 | 50 | 0.05-10 | 2000 |
| F0.05 A-E | 50 | 5 | 50 | 0.05-10 | 2000 |
| F0.5 A-E | 500 | 5 | 50 | 0.01-10 | 10000 |

N/A = Not applicable.

Silt loam soil

| Sample ID | Fortified Concentration (µg/kg) | Sample Weight (g) | Extract Volume (mL) | Dilution (mL-mL) | Dilution Factor |
|---|---|---|---|---|---|
| Reagent blank B | N/A | N/A | 50 | 0.05-10 | 2000 |
| Control B | N/A | 5 | 50 | 0.05-10 | 2000 |
| Control E-F | N/A | 5 | 50 | 0.05-10 | 2000 |
| F0.05 F-J | 50 | 5 | 50 | 0.05-10 | 2000 |
| F0.5 F-J | 500 | 5 | 50 | 0.01-10 | 10000 |

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: | 0.4 mL/min | | |
| Gradient: | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
| | 0.0 | 80 | 20 |
| | 0.5 | 80 | 20 |
| | 4.0 | 0 | 100 |
| | 5.0 | 0 | 100 |
| | 5.1 | 80 | 20 |
| | 6.0 | 80 | 20 |
| Run Time: | 6.0 minutes | | |
| Column Temperature: | 40°C | | |
| Autosampler Temperature: | 4°C | | |
| Injection Volume: | 50 µL | | |
| Retention Time: | Approx. 3.0 minutes | | |
| Valco Valve Diverter: | Time (min) | Position | |
| | 0 | A (to waste) | |
| | 0.5 | B (to MS) | |
| | 5.5 | 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): | 500°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.

***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/kg)
*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/kg)
*S* = concentration added to fortified sample (µg/kg)

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/kg) = 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/kg) = 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 final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract 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 2000).

*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 each control soil final extract. This was assessed for the primary and confirmatory transitions of Triadimefon.

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.