1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of triadimefon in soil samples by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated to quantify the concentrations of triadimefon present in recovery samples prepared in sandy loam soil and loamy sand soil. The analytical method was validated with regards to accuracy, precision, specificity, linearity, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in sandy loam soil and loamy sand soil by fortification with triadimefon at concentrations of 0.0530 (LOQ) and 0.530 (10X LOQ) mg/kg. Samples were extracted twice with acetonitrile. The recovery samples were further diluted into the calibration range with 20/80 acetonitrile/purified reagent water (v/v). All samples were analyzed using LC-MS/MS.

The study was initiated on 22 April 2019, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted from 23 to 28 May 2019 at Smithers, located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers' archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of an Environmental Chemistry Method for the Determination of Triadimefon in Soil by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practice (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the

OECD principles on GLP (OECD, 1998), and followed the SANCO/3029/99 rev. 4 guidance document (EC, 2000) and OCSPP 850.6100 guideline (U.S. EPA, 2012).

2.2 Test and Reference Substances

2.2.1 Test Substance

The test substance, triadimefon TGAI, was received on 1 May 2019 from ChemStarr, LLC. The following information was provided:

Name:	triadimefon TGAI
Synonym:	triadimefon; 1-(4-chlorphenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone
Batch No.:	TRI2019032501
CAS No.:	43121-43-3
Purity:	98.4% (determined by Smithers Viscient; Certificate of Analysis, Appendix 2)
Recertification Date:	19 June 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 10047) was stored at room temperature in a dark, ventilated cabinet in amber glass jars. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples during testing.

2.2.2 Reference Substance

The reference substance, triadimefon analytical standard, was received on 8 April 2019 from Sigma Aldrich, Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	triadimefon analytical standard
Synonym:	1-(4-chlorphenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone
Lot No.:	BCBW0134
CAS No.:	43121-43-3
Purity:	98.7% (determined by Smithers Viscient; Certificate of Analysis, Appendix 2)
Recertification Date:	17 June 2020

Upon receipt at Smithers Viscient, the reference substance (SMV No. 9942) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the reference substance. This sample of reference substance was used to prepare calibration standards during testing.

Determination of stability and characterization, verification of the test and reference substance identities, maintenance of records on the test and reference substances, and archival of a sample of the test and reference substances are the responsibility of the Study Sponsor.

2.3 Reagents

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

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	AB MDS Sciex 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACHT autosampler Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD binary pumps Shimadzu CTO-20A column oven Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo Top Loader PG-2002-S; Mettler Toledo XSE205DU Sartorius Top Loader ENTRIS2202-1SUS
3.	Shaker table:	VWR Standard Analog 3500STD
4.	Centrifuge:	Thermo Scientific Sorvall Legend XFR
5.	Moisture balance:	Mettler Toledo HB43-S

6.	Laboratory equipment:	Positive displacement pipets, graduated cylinders, volumetric flasks, disposable glass pipets, stir bars, stir plate, vortex mixer, 50-mL centrifuge tubes, amber bottles, clear vials with snap caps, amber vials with crimp caps, autosampler vials, and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used in future testing but may require optimization to achieve the desired separation and sensitivity.

2.5 Test Matrices

The matrices used during this method validation were sandy loam soil and loamy sand soil.

Characterization of the sandy loam soil and loamy sand soil was performed by Agvise Laboratories, Northwood, North Dakota.

Parameter	Sandy Loam Soil	Loamy Sand Soil
Smithers Batch No.:	24Oct18Soil-A	041917B
Collection location:	Grand Forks, ND	Rochester, MA
Percent organic matter:	3.7%	13.5%
USDA textural class:	Sandy loam	Loamy sand
Particle size distribution:	64% sand 17% silt 19% clay	83% sand 16% silt 1% clay
pH (1/1 matrix/water ratio):	6.6	6.6
Percent water holding capacity (at 1/3 bar):	23.6%	31.1%
Bulk density (gm/cc):	1.05	0.96

2.6 Preparation of Liquid Reagent Solutions

The volumes listed in this section were those used during the validation. For future testing, the actual volumes used may be scaled up or down as necessary.

A 20/80 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by adding 100 mL of acetonitrile to 400 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water. The solution was mixed well before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

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

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
Test Substance						
10047AA	0.0539	0.0530	Acetonitrile	50.0	1060	Secondary stock solution
Reference Substance						
9942G	0.0537	0.0530	Acetonitrile	50.0	1060	Secondary stock solution

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
Test Substance							
10047AA	1060	0.500	50.0	Acetonitrile	10047AA-1	10.6	10X LOQ-level recovery samples and sub-stock solutions
Reference Substance							
9942G	1060	0.500	50.0	Acetonitrile	9942G-1	10.6	Sub-stock solutions

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
Test Substance							
10047AA-1	10.6	1.00	10.0	Acetonitrile	Tech-Mix-Stk 1 (25 May 2019)	1.06	LOQ-level recovery samples (sandy loam soil)
10047AA-1	10.6	1.00	10.0	Acetonitrile	Tech-Mix-Stk 1 (23 May 2019)	1.06	LOQ-level recovery samples (loamy sand soil)
Reference Substance							
9942G-1	10.6	0.0200	20.0	Acetonitrile	Ana-Stk 1 (25 May 2019)	0.0106	Calibration standards and sub-stocks (sandy loam soil)
Ana Stk 1	0.0106	1.00	10.0	Acetonitrile	Ana Stk 2 (25 May 2019)	0.00106	Calibration standards (sandy loam soil)
9942G-1	10.6	0.0200	20.0	Acetonitrile	Ana-Stk 1 (23 May 2019)	0.0106	Calibration standards and sub-stocks (loamy sand soil)
Ana Stk 1	0.0106	1.00	10.0	Acetonitrile	Ana Stk 2 (23 May 2019)	0.00106	Calibration standards (loamy sand soil)

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

Calibration standards were prepared in 20/80 acetonitrile/purified reagent water (v/v) by fortifying with the 0.00106 or 0.0106 mg/L sub-stock solution to yield concentrations of 0.00530, 0.0106, 0.0265, 0.0530, 0.106, and 0.265 µg/L. Calibration standards were prepared according to the table below. Following fortification, each solution was mixed using a vortex mixer for 15 seconds.

Fortifying Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Stock Concentration (µg/L)	Sample ID
Ana Stk 2	0.00106	0.0500	10.0	0.00530	Std 1
		0.100	10.0	0.0106	Std 2
		0.250	10.0	0.0265	Std 3
Ana Stk 1	0.0106	0.0500	10.0	0.0530	Std 4
		0.100	10.0	0.106	Std 5
		0.250	10.0	0.265	Std 6

2.9 Matrix Effect Investigation

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based calibration standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate in control matrix blank final dilution (see Section 2.11) and 20/80 acetonitrile/purified reagent water (v/v) by fortifying with the 10.6 µg/L sub-stock to yield a concentration of 0.0265 µg/L.

Sandy loam soil validation

Sample ID	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
L-MM-Std A, B, & C	Matrix-matched calibration standard	10.6	0.0250	10.0[a]	0.0265
L-Sol-Std A, B, & C	Solvent-based calibration standard	10.6	0.0250	10.0[b]	0.0265

[a] Diluted with the control matrix blank final dilution 14181-6107-16
[b] Diluted with 20/80 acetonitrile/purified reagent water (v/v)

Loamy sand soil validation

Sample ID	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
SL-MM-Std A, B, & C	Matrix-matched calibration standard	10.6	0.0250	10.0[a]	0.0265
SL-Sol-Std A, B, & C	Solvent-based calibration standard	10.6	0.0250	10.0[b]	0.0265

[a] Diluted with the control matrix blank final dilution 14181-6107-03
[b] Diluted with 20/80 acetonitrile/purified reagent water (v/v)

2.10 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (sandy loam soil and loamy sand soil) by fortification with stock solutions of triadimefon at concentrations of 0.0530 (LOQ) and 0.530 (10X LOQ) mg/kg. Recovery samples for both matrices were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples of each matrix were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared for each sample set and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Sandy loam soil recovery samples

Sample ID 14181-6107-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
14	Reagent Blank	NA[a]	NA	NA	NA	0.00
15 & 16	Control	NA	NA	6.12	5.00	0.00
17, 18, 19, 20, & 21	LOQ	1.00	0.250	6.12	5.00	0.0530
22, 23, 24, 25, & 26	10X LOQ	10.0	0.250	6.12	5.00	0.530

[a] NA = Not Applicable

Loamy sand soil recovery samples

Sample ID 14181-6107-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Weight (g)	Dry Weight (g)	Fortified Concentration (mg/kg)
01	Reagent Blank	NA[a]	NA	NA	NA	0.00
02 & 03	Control	NA	NA	7.03	5.00	0.00
04, 05, 06, 07, & 08	LOQ	1.00	0.250	7.03	5.00	0.0530
09, 10, 11, 12, & 13	10X LOQ	10.0	0.250	7.03	5.00	0.530

[a] NA = Not Applicable

2.11 Extraction of Samples

Samples were extracted twice with acetonitrile. A 20-mL aliquot of acetonitrile was added to each soil recovery sample (5.00 g dry weight), which were sonicated for 10 minutes and then placed on a shaker table for 30 minutes at 250 rpm. Samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50-mL volumetric flasks. The extraction and

centrifugation procedures were repeated one more time with an additional 20-mL aliquot of acetonitrile. The extracts were combined, taken to volume (50 mL) with acetonitrile and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v). The extraction and dilution procedures for each separate matrix is outlined in the tables below.

Sandy loam soil recovery samples

Sample ID 14181-6107-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
14	Reagent Blank	0.00	NA[c]	20.0	50.0	0.0500	10.0	2000
15	Control	0.00	5.00	20.0	50.0	0.0500	10.0	2000
16	Control	0.00	5.00	20.0	50.0	0.250	50.0[d]	2000
17, 18, 19, 20, & 21	LOQ	0.0530	5.00	20.0	50.0	0.0500	10	2000
22, 23, 24, 25, & 26	10X LOQ	0.530	5.00	20.0	50.0	0.0200	10	10,000

[a] Extraction solvent: acetonitrile
[b] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects.

Loamy sand soil recovery samples

Sample ID 14181-6107-	Sample Type	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
01	Reagent Blank	0.00	NA[c]	20.0	50.0	0.0500	10.0	2000
02	Control	0.00	5.00	20.0	50.0	0.0500	10.0	2000
03	Control	0.00	5.00	20.0	50.0	0.250	50.0[d]	2000
04, 05, 06, 07, & 08	LOQ	0.0530	5.00	20.0	50.0	0.0500	10	2000
09, 10, 11, 12, & 13	10X LOQ	0.530	5.00	20.0	50.0	0.0200	10	10,000

[a] Extraction solvent: acetonitrile
[b] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Volume increased to prepare matrix-matched calibration standards to assess matrix effects.

2.12 Analysis

2.12.1 Instrumental Conditions

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column:	Waters Xbridge BEH C18, 50 × 2.1 mm, 2.5 µm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.50	0.400	80.0	20.0
4.00	0.400	0.00	100.0
5.00	0.400	0.00	100.0
5.10	0.400	80.0	20.0
6.00	0.400	80.0	20.0

Run Time: 6.0 minutes
Autosampler Wash Solvent: 30/30/40 acetonitrile/methanol/reagent grade water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 15 °C
Injection Volume: 100.0 µL
Retention Time: approximately 3.6 minutes

MS parameters:

Instrument: AB MDS Sciex 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5000 V
Scan Type: MRM
Dwell Time: 200 milliseconds
Source Temperature: 650 °C
Curtain Gas: 20.0
Ion Source - Gas 1 / Gas 2: 50.0 / 50.0
Collision Gas: 7.00
Entrance Potential: 10.0
Declustering Potential: 65.0
Resolution Q1/Q3: Unit / Unit

Analyte	Analysis	Q1/Q3 Masses (amu)	Collision Energy	Collision Cell Exit Potential
Triadimefon	Primary	294.3/197.1	22.0	10.0
	Confirmatory	294.3/69.1	29.0	10.0

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.12.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, every five to seven injections.

Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.13 Evaluation of Accuracy, Precision, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 110% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples and retention times. RSD values less than 20% were considered acceptable for the recovery samples and RSD values less than 2% were considered acceptable for the retention times. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as triadimefon which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.14 Limit of Quantitation (LOQ)

The method was validated at the LOQ. This was defined as the lowest fortification level (0.0530 mg/kg). Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.15 Limit of Detection (LOD) and Method Detection Limit (MDL)

The LOD was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

The MDL was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Section 3.0.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (Equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

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

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

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

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (mg/kg) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (mg/kg), concentration in the original sample

The MDL is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (Equation 4) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(4) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.00530 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 2000 mL/g)
MDL	=	method detection limit reported for the analysis (0.00530 µg/L × 2000 mL/g = 0.0106 mg/kg)

The LOD was calculated using the following equation:

$$(5) \quad LOD = ((3\times(N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 2000 mL/g)
LOD	=	limit of detection for the analysis