1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of *d*-phenothrin in surface water. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in surface water by fortification with *d*-phenothrin at concentrations of 0.00500 (limit of quantitation, LOQ) and 0.0500 (10 x LOQ) µg/L (ppb). Recovery samples were extracted twice with hexane followed by extract concentration and solvent transfer by subsequent reconstitution with 100% acetonitrile and 100% purified reagent water for a final composition of 80:20 acetonitrile:purified reagent water (v:v). Samples were additionally diluted into the standard curve range with 80:20 acetonitrile:purified reagent water (v:v), as necessary.
All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 11 May 2017, 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 22 May to 23 June 2017 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol and the final report produced during this study are stored in Smithers Viscient's 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 the Analytical Method for the Determination of d-phenothrin in Aqueous Matrix by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practices (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 guidance documents SANCO/825/00 REV 8.1 (EC, 2010) and OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substance

The test substance, *d*-phenothrin, was received on 2 September 2016 from Sumitomo Chemical Company, Ltd., Tokyo, Japan. The following information was provided (Certificate of Analysis, Appendix 2):

Name:	*d*-phenothrin
Synonyms:	*d*-phenothrin TGAI, Sumithrin®
Lot No.:	141208
CAS No.:	26046-85-5
Purity:	96.5%
Expiration Date:	9 December 2017

Upon receipt at Smithers Viscient, the test substance (SMV No. 8499) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

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

2.3 Reagents

1.	Acetone:	EMD, reagent grade
2.	Hexane:	EMD, reagent grade
3.	Acetonitrile:	EMD, reagent grade
4.	Ammonium acetate:	Fisher, reagent grade
5.	Methanol:	EMD, reagent grade
6.	Nitric acid:	Reagent grade
7.	Purified reagent water:	Prepared from a Millipore MilliQ® Direct 8 water purification system (meets ASTM Type II requirements)

Reagents of similar grade and comparable purity may be substituted for the specific reagents above in future testing with this method as long as acceptable performance is demonstrated.

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	AB Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source Waters Acquity binary solvent manager Waters Acquity sample manager – FTN Waters Acquity column compartment Analyst version 1.6 software for data acquisition
2.	Balance:	Mettler Toledo AG285
3.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, graduated cylinders, Nitric acid (acid-washing), Pasteur pipets, 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 Matrix

The surface water used for this method validation analysis was collected from the Weweantic River (SMV Lot No. 15 May 17 WAT-A, collected on 15 May 2017) in Wareham, Massachusetts. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water at a temperature of 13.2 °C. Measured water characteristics are shown in the table below. All documentation relating to the preparation, storage and handling is maintained by Smithers Viscient.

pH[a]	Dissolved Oxygen (mg/L)[b]	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)	Conductivity (µS/cm)[c]	Total Organic Carbon (mg/L)[d]
5.81	8.48	12	6.0	83.6	9.535

[a] YSI Model pH100 meter.
[b] YSI Model Pro 20 meter.
[c] YSI Model 3100-115V meter.
[d] Shimadzu TOC-L/ASI-L/SSM meter.

2.6 Preparation of Liquid Reagent and Mobile Phase Solutions

The volumes and masses listed in this section are representative of the solutions prepared during testing, but may not reflect the exact quantities for each individual solution prepared. Volumes and masses may be changed; however, the proportions must remain the same.

A 20% nitric acid in purified reagent water liquid reagent solution was typically prepared by combining 400 mL of nitric acid and 1600 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

An 80:20 acetonitrile:purified reagent water (v:v) liquid reagent solution was prepared by combining 200 mL of acetonitrile and 50.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 10 mM ammonium acetate in purified reagent water mobile phase solution was prepared by adding 0.7710 g of ammonium acetate to 1000 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for ten minutes.

A 10:90 acetonitrile:purified reagent water (v:v) autosampler purge solution was prepared by combining 500 mL of acetonitrile and 4500 mL of purified reagent water. The solution was mixed well before use.

A 30:30:40 acetonitrile:methanol:purified reagent water (v:v:v) autosampler needle wash solution was 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 are representative of the stocks prepared during testing, but may not reflect the exact quantities for each individual stock solution prepared. Volumes and masses may be changed; however, the proportions must remain the same.

Primary stock solutions were 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
8499AB	0.0519	0.0501	Acetone	50.0	1000	Secondary stock solution
8499AC	0.5190	0.5008	Acetone	50.0	10,000	Secondary stock solution

Secondary stock solutions were prepared as per 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
8499AB	1000	0.500	50.0	Acetone	8499AB-1	10.0	Sub-stock solution
8499AC	10,000	0.0500	50.0	Acetone	8499AC-1	10.0	Sub-stock solution

Sub-stock solutions were prepared as per 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
8499AB-1	10.0	0.100	10.0	Acetonitrile	Ana Stk 1	0.100	Sub-stock solution
Ana Stk 1	0.100	1.00	10.0	Acetonitrile	Ana Stk 2	0.0100	Calibration standards
8499AC-1	10.0	0.100	10.0	Acetone	Tech Stk 1	0.100	LOQ- and High-level recovery samples

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

2.8.1 Calibration Standards – Recovery Samples

Calibration standards were prepared in 80:20 acetonitrile:purified reagent water (v:v) by fortifying with the 0.0100 mg/L sub-stock solution to yield concentrations of 0.00500, 0.0100, 0.0250, 0.0500, 0.0800, and 0.100 µg/L.

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 2	0.0100	0.0200	40.0	0.00500	Std 1
		0.0200	20.0	0.0100	Std 2
		0.0250	10.0	0.0250	Std 3
		0.0500	10.0	0.0500	Std 4
		0.0800	10.0	0.0800	Std 5
		0.100	10.0	0.100	Std 6

2.8.2 Matrix Effect Investigation

In an effort to observe any potential matrix effects, an aliquot of control sample final fraction was fortified in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix matched standards fortified at the same concentration (the LOQ). Calibration standards used to assess possible matrix effects were prepared as follows by fortifying with the 0.100 mg/L test substance sub-stock solution, which were then further diluted to yield test substance concentrations of 0.0625 µg/L.

2.8.2.1 Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Dilution Volume (mL)	Final Volume (mL)[b]	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.100	0.0250	2.00	0.250	5.00	0.0625	MM-Std A-2
		0.0250	2.00	0.250	5.00	0.0625	MM-Std B-2
		0.0250	2.00	0.250	5.00	0.0625	MM-Std C-2

[a] Samples were fortified in the final fraction of recovery sample Control C-E in 80:20 acetonitrile:purified reagent water (v:v).

[b] Samples were additionally diluted with 80:20 acetonitrile:purified reagent water (v:v) to yield the final standard concentration.

2.8.2.2 Non Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume[a] (mL)	Dilution Volume (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.100	0.0250	2.00	0.250	5.00	0.0625	Std A-2
		0.0250	2.00	0.250	5.00	0.0625	Std B-2
		0.0250	2.00	0.250	5.00	0.0625	Std C-2

[a] Samples were prepared in and additionally diluted with 80:20 acetonitrile:purified reagent water (v:v) to yield the final standard concentration.

2.9 Sample Fortification and Preparation

Prior to conduct of the validation, all non-disposable glassware was rinsed with a 20% nitric acid solution. This was done by either soaking all non-disposable glassware for 15 minutes or rinsing three times with equivalent amounts of 20% nitric acid solution. Following the acid wash, all glassware was rinsed three times with an equivalent volume of purified reagent water until no nitric acid remained. At this stage, glassware was rinsed an additional two times with purified reagent water, followed by three equivalent volumes of acetone. The finishing glassware rinse was done using the final dilution solvent.

The recovery samples were prepared by fortifying surface water (500 mL sample volume) within individual separatory funnels with *d*-phenothrin to obtain concentrations of 0.00500 (LOQ) and 0.0500 (High) µg/L. Recovery samples were prepared separately ("de novo") at these concentrations, with five replicates prepared for each concentration level. In addition, two samples were left unfortified to serve as controls and were processed in the same fashion as the LOQ-level recovery samples. One additional sample was extracted using purified reagent water as a reagent blank (no test substance or matrix) in order to assess interference from extraction solvents. The fortification procedure is outlined in the table below.

Sample ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
Reagent BLK-1-2	NA[a]	NA	500[b]	0.00
Control A-2 & B-2	NA	NA	500	0.00
LOQ A-2, B-2, C-2, D-2, & E-2	0.100	0.0250	500	0.00500
High A-2, B-2, C-2, D-2, & E-2	0.100	0.250	500	0.0500

[a] NA = Not Applicable.
[b] Dilution solvent: Purified reagent water.

2.10 Sample Extraction

The fortified recovery samples were extracted twice with 200 mL of hexane, with both solvent extracts combined in separate round-bottom flasks. After the extraction process was finished, the post-extraction water was drained from the separatory funnels and discarded. Each funnel was rinsed well with an additional 50.0 mL of extraction solvent. The rinsate was added to the appropriate round-bottom flasks. The extracts were taken to low volume (approximately 2 mL) by rotary evaporation using minimal heating (< 35 °C). A solvent transfer step was then performed to remove any residual water from the round-bottom flasks. An aliquot of acetone (100 mL) was added to each round-bottom flask and mixed well. The extracts were taken to low volume (approximately 5 mL) by rotary evaporation using minimal heating (< 35 °C). The extracts were then transferred to glass centrifuge tubes and taken to incipient dryness under a gentle stream of nitrogen at room temperature. This transfer was performed first with extraction solvent followed by acetone to better rinse the flasks, totaling the volume of the glass centrifuge tube (approximately 15 mL). An aliquot of acetonitrile was added to each sample, which was vortexed for 30 seconds and sonicated for five minutes to aid in dissolution of the concentrated extract. Next, an aliquot of purified reagent water was added to each sample, which was vortexed for 30 seconds and sonicated for five minutes to yield a solvent to water ratio of 80:20 acetonitrile:purified reagent water (v:v). High concentration level samples were additionally diluted into the standard curve range with 80:20 acetonitrile:purified reagent water (v:v) prior to analysis. All recovery samples were transferred to HPLC vials for analysis via LC-MS/MS.

Secondary dilution volumes can be scaled up or down as necessary. The dilution procedures are outlined in the tables below.

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Hexane Extraction Volume (mL)	Acetonitrile Volume[a] (mL)	Purified Reagent Water Volume[b] (mL)	Final Volume[c] (mL)	Sample Volume (mL)	Final Volume[d] (mL)	Sample Volume (mL)	Final Volume[d] (mL)	Dilution Factor
Reagent BLK-1-2	0.00	500	200	1.60	0.400	2.00	NA	NA	0.250	5.00	0.0800
Control A-2 & B-2	0.00	500	200	1.60	0.400	2.00	NA	NA	0.250	5.00	0.0800
LOQ A-2, B-2, C-2, D-2, & E-2	0.00500	500	200	1.60	0.400	2.00	NA	NA	0.250	5.00	0.0800
High A-2, B-2, C-2, D-2, & E-2	0.0500	500	200	1.60	0.400	2.00	1.00	10.0	0.250	5.00	0.800

[a] Reconstitution solvent: 100% acetonitrile.
[b] Reconstitution solvent: 100% purified reagent water.
[c] Final composition: 80:20 acetonitrile:purified reagent water (v:v).
[d] Dilution solvent: 80:20 acetonitrile:purified reagent water (v:v).

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC parameters:

Column:	Agilent Poroshell 120 EC-C8, 2.7 µm, 3.0 × 50 mm
Mobile Phase A:	10 mM ammonium acetate in purified reagent water
Mobile Phase B:	100% methanol

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
Initial	0.400	70.0	30.0
5.00	0.400	10.0	90.0
7.00	0.400	0.00	100
9.00	0.400	0.00	100
9.10	0.400	70.0	30.0
10.0	0.400	70.0	30.0

Run Time:	10.0 minutes
Autosampler Wash Solvent:	30:30:40 acetonitrile:methanol:reagent grade water (v:v:v)
Autosampler Purge:	10:90 acetonitrile:purified reagent water (v:v)
Column Temperature:	40 °C
Sample Temperature:	5 °C
Injection Volume:	100 µL
Retention Time:	approximately 5.8 minutes

MS parameters:

Instrument:	AB Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Q1/Q3 Mass:	351.4/183.2 amu (primary transition) 351.4/249.3 amu (confirmatory transition 1) 351.4/305.4 amu (confirmatory transition 2)
Dwell Time:	300 milliseconds
Resolution Q1/Q3:	Low/Low
Source Temperature:	500 °C
Curtain Gas:	25.0
Ion Source – Gas 1 / Gas 2:	60.0 / 60.0
Collision Gas:	8.0

	Primary Transition	Confirmatory Transition 1	Confirmatory Transition 2
Q1/Q3 Masses (amu):	351.4/183.2	351.4/249.3	351.4/305.4
Declustering Potential:	93.00	127.00	128.00
Collision Cell Entrance Potential:	10.00	7.00	6.80
Collision Energy:	29.60	25.20	17.40
Collision Cell Exit Potential:	18.60	18.60	21.00

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.

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 120% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples. RSD values less than 20% were considered acceptable for the recovery samples. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as *d*-phenothrin, which might interfere with the quantitation of the analyte. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.13 Limit of Quantitation (LOQ)

The method was validated at the LOQ. This was defined as the lowest fortification level (0.00500 µg/L). Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.14 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 lowest 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) $y = mx + b$

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

(3) $A = DC \times DF$

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 (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The LOD was calculated using the following equation:

$$(4) \quad LOD = (3x(SN_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

SN_{ctl}	=	mean signal to 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., 0.0800)
LOD	=	limit of detection for the analysis

The MDL was calculated using the following equation:

$$(5) \quad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	the lowest concentration calibration standard (i.e., 0.00500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 0.0800)
MDL	=	method detection limit reported (0.00500 µg/L × 0.0800 = 0.000400 µg/L)

Figure 1. Flow chart of the analytical method used for quantification of *d*-phenothrin from surface water recovery samples during the method validation.