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 Pyrethrins in Groundwater and Surface Water" (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 document OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substance

The test substance, Pyrethrum Concentrate (Stewardship Blend), was received on 24 June 2014 from EPL Archives Incorporated, Sterling Virginia. The following information was provided:

Name:	Pyrethrum Concentrate (Stewardship Blend)
Synonym:	BAS 383 HB I
Lot No.:	230-089
Purity:	29.46% Pyrethrin I
	24.02% Pyrethrin II
	53.48% Total Pyrethrins
Expiration Date:	27 September 2017

Upon receipt at Smithers Viscient, the test substance (SMV No. 7108) was stored in a refrigerator in the original container. Concentrations were adjusted for the purity of the test substance as PYR I and PYR II.

The test substance, Pyrethrum Concentrate, consists of six different esters that are referred to, collectively, as PYR I and PYR II. PYR I consists of three separate esters: pyrethrin I, jasmolin I and cinerin I; PYR II consists of three additional esters: pyrethrin II, jasmolin II and cinerin II. The purities indicated above of PYR I and PYR II were determined based on the sum of the

individual components: pyrethrin I, jasmolin I and cinerin I (for total PYR I) and pyrethrin II, jasmolin II and cinerin II (for total PYR II).

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

2.3 Reagents

1.	0.1% Formic acid in reagent grade water:	Fisher Chemical, reagent grade
2.	0.1% Formic acid in acetonitrile:	Fisher Chemical, reagent grade
3.	Methanol:	EMD reagent grade
4.	Acetonitrile:	EMD, reagent grade
5.	Dichloromethane:	EMD, reagent grade
6.	Purified reagent water:	Prepared from a Millipore MilliQ® Direct 8 water purification system (meets ASTM Type II requirements)

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	MDS Sciex 4000 QTRAP® mass spectrometer equipped with an ESI Turbo V source Agilent 1200SL/G1379B Vacuum Degasser Agilent 1200SL/G1312B Binary Pump Leap HTS PAL Autosampler Agilent 1200SL/G1316B Column Thermostat Analyst version 1.6.2 software for data acquisition
2.	Balance:	Mettler Toledo XSE205U
3.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, disposable plastic pipets, Teflon® centrifuge tubes, graduated cylinders, 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 Matrices

The matrices used during this method validation were ground water and surface water.

Ground water information:

Ground water used in the study consists of unadulterated water from a 100-meter bedrock well prepared by filtering to remove any potential organic contaminants. Prior to use, the ground water was characterized by Smithers Viscient and determined to have a total hardness and alkalinity (as $CaCO_3$) of 84 and 20 mg/L, respectively, a pH of 6.24 (YSI model pH100A pH meter) and a conductivity of 642 µS/cm (YSI model Pro 30 salinity and conductivity meter).

Surface water information:

The surface water used for this method validation analysis was collected from the Taunton River (SMV Lot No. 11NOV16 WAT-A) in Taunton, Massachusetts. The water was collected from an area of the river with approximately 60 cm of overlying water. Prior to use, the surface water was characterized by Agvise Laboratories, Northwood, North Dakota and was determined to have a total hardness (as $CaCO_3$) of 85 mg/L, a pH of 7.6 and a conductivity of 0.53 mmhos/cm. All documentation relating to the preparation, storage and handling is maintained by Smithers Viscient.

2.6 Preparation of Liquid Reagent and Mobile Phase 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 50:50 acetonitrile:purified reagent water (v:v) liquid reagent solution was prepared by combining 250 mL of acetonitrile and 250 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 0.1% formic acid in purified reagent water mobile phase solution was typically prepared by adding 1.00 mL of formic acid 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 0.1% formic acid in acetonitrile mobile phase solution was typically prepared by adding 1.00 mL of formic acid to 1000 mL of acetonitrile. 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 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.

A 10:90 acetonitrile: purified reagent water (v:v) autosampler needle wash solution was typically prepared by combining 2000 mL of acetonitrile and 1800 mL of purified reagent water.

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 separate validation. Volumes and masses may be changed; however, the proportions must remain the same.

A primary stock solution was prepared as described in the table below.

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as Active Ingredient (PYR I/PYR II)	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L), as PYR I/PYR II	Primary Stock Use
7108N	0.1702	0.0501/0.0409	Acetonitrile	50.0	1000/818	Secondary stock solution

A secondary stock solution was prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L), as PYR I/PYR II	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L), as PYR I/PYR II	Stock Use
7108N	1000/818	0.500	50.0	Acetonitrile	7108N-1	10.0/8.18	Sub-stock solutions

Sub-stock solutions were prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L), as PYR I/PYR II	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L), as PYR I/PYR II	Stock Use
7108N-1	10.0/8.18	0.0100	10.0	Acetonitrile	Stk 1	0.0100/0.00818	Calibration standards
7108N-1	10.0/8.18	0.100	10.0	Acetonitrile	Stk 2	0.100/0.0818	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

Calibration standards were prepared in 50:50 acetonitrile:purified reagent water (v:v) as described below:

Test Substance Stock ID	Stock Concentration (mg/L), as PYR I/PYR II	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L), as PYR I/PYR II[a]	Sample ID
Stk 1	0.0100/0.00818	0.0500	10.0	0.0500/0.0409	Std 1
		0.100	10.0	0.100/0.0818	Std 2
		0.200	10.0	0.200/0.164	Std 3
		0.300	10.0	0.300/0.245	Std 4
		0.400	10.0	0.400/0.327	Std 5
		0.500	10.0	0.500/0.409	Std 6

[a] Pyrethrins II concentration is 81.8% of the pyrethrins I concentration of the stocks prepared from the raw material.

2.9 Sample Fortification and Preparation

The recovery samples were prepared in each matrix (ground water and surface water) with Pyrethrum Concentrate at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L (as PYR I) and 0.0818 (LOQ) and 0.818 (10X LOQ) µg/L (as PYR II). Recovery samples for each matrix were prepared separately ("de novo") at these concentrations. Seven replicates were produced for the LOQ samples and five replicates were produced for the 10X LOQ. Two samples 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 of dichloromethane and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water recovery samples:

Sample ID	Stock Concentration (mg/L), as (PYR I/PYR II)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L), as (PYR I/PYR II)	Fortified Concentration (total pyrethrins) (µg/L)
Reagent Blank	NA[a]	NA	NA	0.00	0.00
Control A&B	NA	NA	40.0	0.00	0.00
LOQ A, B, C, D, E, F, & G	0.100/0.0818	0.0400	40.0	0.100/0.0818	0.182
10X LOQ A, B, C, D, & E	0.100/0.0818	0.400	40.0	1.00/0.818	1.82

[a] NA = Not Applicable.

Surface water recovery samples:

Sample ID	Stock Concentration (mg/L), as (PYR I/PYR II)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L), as (PYR I/PYR II)	Fortified Concentration (total pyrethrins) (µg/L)
Reagent Blank	NA[a]	NA	NA	0.00	0.00
Control A&B	NA	NA	40.0	0.00	0.00
LOQ A, B, C, D, E, F, & G	0.100/0.0818	0.0400	40.0	0.100/0.0818	0.182
10X LOQ A, B, C, D, & E	0.100/0.0818	0.400	40.0	1.00/0.818	1.82

[a] NA = Not Applicable.

2.10 Sample Extraction

The samples were extracted twice with 5.00-mL aliquots of dichloromethane using a 45-mL glass vials with PTFE lined caps. The extracts were then transferred using a transfer pipet from the bottom of the 45 mL glass vials into conical glass vials. The transfer of water was avoided as much as possible; however, small amounts of water will not interfere with analysis. The extracts were concentrated to low volume (approximately 100 µL) under a gentle stream of nitrogen at 40 °C. An aliquot of acetonitrile (7.50 mL) was added to the vials and the samples were vortex mixed for 30 seconds and sonicated for five minutes. An aliquot of purified reagent water (7.50 mL) was added in the same manner, bringing the final composition of the samples to 50:50 acetonitrile:purified reagent water (v:v). The 10X LOQ-level extracts were further diluted into the calibration standard range with 50:50 acetonitrile:purified reagent water. The following table summarizes the extraction procedure for each sample.

Sample ID	Fortified Concentration (total pyrethrins) (µg/L)	Sample Volume (mL)	Reconstituted Volume[a] (mL)	Sample Volume for Dilution (mL)	Diluted Final Volume[a] (mL)	Dilution Factor
Reagent Blank	0.00	NA[b]	15.0	NA	NA	0.375
Control A, B, C, D & E	0.00	40.0	15.0	NA	NA	0.375
LOQ A, B, C, D & E	0.182	40.0	15.0	NA	NA	0.375
High A, B, C, D & E	1.82	40.0	15.0	1.00	10.0	3.75

[a] Dilution solvent: 50:50 acetonitrile:purified reagent water (v:v).
[b] NA = Not Applicable.

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: 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 (%)
0.01	0.600	98.0	2.00
0.50	0.600	98.0	2.00
2.00	0.600	30.0	70.0
5.00	0.600	2.00	98.0
6.00	0.600	2.00	98.0
6.10	0.600	98.0	2.00
7.00	0.600	98.0	2.00

Run Time: 7.0 minutes
Autosampler Wash Solvent: 30:30:40 acetonitrile:methanol:reagent grade water (v:v:v)
10:90 acetontirile:purified reagent water (v:v)
Column Temperature: 25 °C
Sample Temperature: 5 °C
Injection Volume: 100 µL

Retention Times:	approximately 4.2 minutes (for pyrethrin I)
	approximately 4.5minutes (for jasmolin I)
(in ground water)	approximately 4.2 minutes (for cinerin I)
	approximately 3.6 minutes (for pyrethrin II)
	approximately 3.8 minutes (for jasmolin II)
	approximately 3.6 minutes (for cinerin II)
Retention Times:	approximately 4.2 minutes (for pyrethrin I)
	approximately 4.5 minutes (for jasmolin I)
(in surface water)	approximately 4.2 minutes (for cinerin I)
	approximately 3.6 minutes (for pyrethrin II)
	approximately 3.8 minutes (for jasmolin II)
	approximately 3.6 minutes (for cinerin II)

MS parameters:

Instrument:	MDS Sciex API 4000 QTrap mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Source Temperature:	550 °C
Curtain Gas:	20.00
Ion Source – Gas 1 / Gas 2:	70.00 / 50.00
Collision Gas:	Low

Instrument Parameters	Pyrethrin I	Jasmolin I	Cinerin I	Pyrethrin II	Jasmolin II	Cinerin II
Q1/Q3 Masses (amu)	329.30/161.30	331.40/163.20	317.40/149.30	373.40/161.10	375.30/163.20	361.30/149.00
Dwell Time (milliseconds)	50.00	50.00	50.00	50.00	150.00	50.00
Collision Energy	21.00	13.00	14.00	28.00	15.00	15.00
Collision Cell Entrance Potential	7.00	6.00	7.00	6.00	7.00	5.00
Collision Cell Exit Potential	8.00	22.00	22.00	22.00	10.00	10.00
Declustering Potential	46.00	42.00	42.00	40.00	47.00	40.00

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 for each matrix (for four sets in total) 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 standard deviation (SD) and the relative standard deviation (RSD or coefficient of variation (CV)) calculated for the retention times, peak area-based quantitation (i.e., µg/L), and the observed recovery values. RSD values less than or equal to 20% were considered acceptable for the recovery samples and peak area-based quantitation and RSD values less than or equal to 2.00% 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 Pyrethrum Concentrate which might interfere with the quantitation of the analytes. Linearity of the method was determined by the correlation coefficient (r^2), y-intercept, and slope of the regression line.

2.13 Limit of Quantitation

The method was validated at the proposed Limit of Quantitation (LOQ). This was defined as the lowest fortification level. 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 Limit of Detection (LOD) was calculated using the standard deviation of the average recovery in units of concentration of seven samples fortified at the proposed LOQ multiplied by

one-tailed t-statistic at the 99% confidence level for n-1 replicates. Representative calculations for the LOD can be found in Section 3.0.

The Minimum Detectable Limit (MDL) was defined as the lowest concentration in test solution samples which can be possibly 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.

2.15 Matrix Effects Determination

Matrix related enhancement or suppression was evaluated at the LOQ level in test solution samples. Mean recoveries were not impacted by greater than twenty percent.

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.

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

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

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

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 mass)
A	=	analytical result (µg/L), concentration in the original sample

The LOD was calculated using the following equation:

$$LOD = t_{0.99} \times SD$$

where:

$t_{0.99}$	=	One-tailed t-statistic at the 99% confidence level for n-1 replicates (i.e., 3.143 for seven replicates)
SD	=	Standard deviation of n samples spiked at the estimated LOQ

The MDL was calculated using the following equation:

$$MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	The lowest concentration calibration standard (i.e., 0.0500 µg/L for PYR I and 0.0409 µg/L for PYR II)
DF_{CNTL}	=	Dilution factor of the control samples (smallest dilution factor used, i.e., 0.375)
MDL	=	Minimum detectable limit reported (0.0500 µg/L × 0.375 = 0.0188 µg/L for PYR I and 0.0409 µg/L × 0.375 = 0.0153 µg/L for PYR II)