1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of pyrethrins (as pyrethrins I & II) in sediment and soil. The test substance, Pyrethrum Concentrate (Stewardship Blend), consists of six different esters that are referred to, collectively, as pyrethrins I (PYR I) and pyrethrins II (PYR II). The method was validated (18 to 19 January 2017) to quantify the concentrations of Pyrethrum Concentrate (Stewardship Blend) present in recovery samples prepared in sediment and soil. The analytical method was validated with regards to accuracy and precision, linearity, specificity, limit of quantitation (LOQ), limit of detection (LOD), and method detection limit (MDL).

The method was validated by fortification of soil and sediment with the Pyrethrum Concentrate at concentrations of 8.00 (LOQ) and 80.0 (10X LOQ) µg/kg (as PYR I) and 6.45 (LOQ) and 64.5 (10X LOQ) µg/kg (as PYR II). Recovery samples were extracted with acetonitrile followed by dilution into the calibration standard range with 50:50 acetonitrile:purified reagent water (v:v). All samples were analyzed by liquid chromatography with tandem mass spectrometry detection (LC/MS/MS).

The study was initiated on 10 January 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 18 to 19 January 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 environmental chemistry method followed those described in the Smithers Viscient protocol entitled "Validation of an Environmental Chemistry Method for the Determination of Pyrethrins in Soil and Sediment" (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 (Stewardship Blend), is a naturally-occurring product which 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. Acetonitrile:	EMD, reagent grade
2. 0.1% Formic acid in reagent grade water:	Fisher, reagent grade
3. 0.1% Formic acid in acetonitrile:	Fisher, reagent grade
4. Purified reagent water:	Prepared from a Millipore Milli-Q® 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:	MDS Sciex 4000Q Trap® 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. Balances:	Mettler Toledo PG-2002-S, Mettler Toledo XSE205DU
3. Centrifuge:	Beckman Allegra X-12
4. Shaker table:	VWR 3500STD
5. Moisture balance:	Sartorius MA-45
6. Laboratory equipment:	Volumetric flasks, disposable glass and plastic pipets, positive displacement pipets, graduated cylinders, a vortex, autosampler vials, 50-mL Nalgene® centrifuge tubes, and amber glass bottles with Teflon®-lined caps

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

2.5 Test Matrices

Prior to use and characterization, the sediment was wet pressed through a 2-mm sieve to remove large particles and indigenous organisms. Characterization of soil and sediment was performed by Agvise Laboratories, Northwood, North Dakota.

Parameter	Natural Freshwater Sediment	Sandy Loam Soil
Smithers Viscient Batch No.:	102915-M-1	012616A
Collection location:	Glen Charlie Pond, Wareham, MA	Sunny Nook Farms, Rochester, MA
Percent organic carbon:	3.3%	0.70%[a]
USDA textural class:	sand	sand
Particle size distribution:	88% sand 10% silt 2% clay	94% sand 6% silt 0% clay
pH (1:1 matrix:water ratio):	5.4	6.9
Percent water holding capacity (at 1/3 Bar):	23.9%	3.3%

[a] Calculated as % organic matter/1.72 where % organic matter of soil was 1.2.

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 50:50 acetonitrile:purified reagent water (v:v) liquid reagent solution was prepared by combining 250 mL of acetonitrile with 250 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five 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.

A 10:90 acetonitrile:purified reagent water (v:v) autosampler needle wash solution was prepared by combining 200 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 per the table below:

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as 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 and 10X LOQ recovery samples

Sub-stock solutions were typically 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 (µg/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
		0.100	10.0		Stk 2	0.100/0.0818	LOQ 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 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 in the table below.

Test Substance Stock ID	Stock Concentration (mg/L), as (PYR I/PYR II)[a]	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L), as (PYR I/PYR II)[b]	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] PYR I = pyrethrins I and PYR II = pyrethrins II.

[b] PYR II concentration is 81.8% of the PYR I concentration of the stocks prepared from the raw material.

2.9 Sample Fortification and Preparation

A total of 14 recovery samples (5.00 g dry weight) were weighed into individual 50.0-mL Nalgene® centrifuge tubes for both soil and sediment. Samples were dosed with the appropriate test substance stock solution at concentrations of 8.00/6.54 (LOQ) and 80.0/65.4 (10X LOQ) µg/kg (dry weight) as PYR I/PYR II, respectively. Recovery samples for each matrix were prepared separately ("de novo") at these concentrations. Seven replicates were prepared for LOQ recovery samples and five replicates were prepared for the 10X LOQ recovery samples. In addition, two samples were left unfortified to serve as controls and were extracted in the same fashion as the LOQ recovery samples. In addition, one reagent blank was prepared and processed in the same manner as the control samples. The preparation procedure is outlined in the table below:

Sample ID	Stock Concentration (mg/L), as PYR I/PYR II	Fortification Volume (mL)	Dry Weight (g)	Sample Concentration (µg/kg), as PYR I/PYR II	Sample Concentration (µg/kg), as total pyrethrins
Reagent Blank	NA[a]	NA	0.00	0.00	0.00
Control A & B	NA	NA	5.00	0.00	0.00
LOQ A, B, C, D, E, F, & G	0.100/0.0818	0.400	5.00	8.00/6.54	14.5
10X LOQ A, B, C, D, & E	10.0/8.18	0.0400	5.00	80.0/65.4	145

[a] NA = Not Applicable.

2.10 Sediment and Soil Extraction

A 20.0-mL aliquot of acetonitrile was added to each sediment and soil recovery sample (5.00 g dry weight), which were then placed on a shaker table for 20 minutes at 150 rpm. The samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50.0-mL volumetric flasks. The extraction and centrifugation procedures were repeated as described above. The extracts were combined, brought to volume (50.0 mL) with acetonitrile and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 50:50 acetonitrile:purified reagent water (v:v). Secondary dilution volumes can be scaled up or down as necessary. The extraction and dilution procedures are detailed below.

Sample ID	Nominal Concentration (µg/kg), as PYR I/PYR II	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Sample Volume for Dilution (mL)	Diluted Final Volume[b] (mL)	Dilution Factor
Reagent Blank	0.00	NA[c]	20.0	50.0	1.25	10.0	80
Control A & B	0.00	5.00	20.0	50.0	1.25	10.0	80
LOQ A, B, C, D, E, F, & G	8.00 / 6.54	5.00	20.0	50.0	1.25	10.0	80
10X LOQ A, B, C, D, & E	80.0 / 65.4	5.00	20.0	50.0	0.375	10.0	267

[a] Extraction and dilution solvent: acetonitrile.
[b] Dilution solvent: 50:50 acetonitrile:purified reagent water (v:v).
[c] 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 purified reagent 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	2.0
0.50	0.600	98.0	2.0
2.00	0.600	30.0	70.0
5.00	0.600	2.0	98.0
6.00	0.600	2.0	98.0
6.10	0.600	98.0	2.0
7.00	0.600	98.0	2.0

Run time: 7.00 minutes
Autosampler needle wash: 30:30:40 acetonitrile:methanol:purified reagent water (v:v:v)
10:90 acetonitrile:purified reagent water (v:v)
Column temperature: 25 °C
Sample temperature: ambient
Injection volume: 100 µL
Retention Time: approximately 4.2 minutes (for pyrethrin I)
approximately 4.5 minutes (for jasmolin I)
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 4000 Q Trap 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 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 recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in as mean recovery ± the relative standard deviation for the test substance in the sample matrix. Recoveries of 70.0 to 120% (for the individual mean concentrations) are acceptable. The precision was reported as repeatability of recovery at each fortification level. The precision was calculated in terms of the standard deviation (SD) and relative standard deviation (RSD) for the retention times, peak-area based quantitation (i.e., µg/kg), and observed recovery values. RSD values $\leq$ 2.00% were considered acceptable for the retention times and RSD values $\leq$20% were considered acceptable for the quantitation values and percent recovery. Specificity of the method was determined by examination of the control samples for peaks at the same retention time as pyrethrin I, jasmolin I, cinerin I, pyrethrin II, jasmolin II, and cinerin II which might interfere with the quantitation of the esters. Linearity of the method was determined by the correlation coefficient (r), 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 proposed 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 Minimum Detectable Limit (MDL)

The Limit of Detection (LOD) was calculated using the standard deviation of the average recovery of the 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 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) $y = mx + b$

(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 mass)
A	=	analytical result (µg/kg), 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., 80)
MDL	=	Method detection limit reported (0.0500 µg/L × 80 = 4.00 µg/kg for PYR I and 0.0409 µg/L × 80 = 3.27 µg/kg for PYR II)