Study Title

Validation of the Analytical Method for the Determination of Veratridine and its Major Metabolite in Soil and Sediment by LC-MS/MS

Test Guidelines

OCSPP 850.6100 (2012)
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1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of veratridine and its major metabolite, veratric acid, in soil and sediment, as well as establish storage stability of the test substances in soil and sediment under frozen conditions. The analytical portion of the study was conducted (19 April to 3 June 2018), with a method validation portion to quantify concentrations of veratridine and veratric acid present in recovery samples prepared in soil and sediment with regards to linearity, accuracy, precision, a determination of the limit of quantitation (LOQ), and limit of detection (LOD), and confirmation of analyte identification (specificity). In addition, a frozen storage stability assessment was made for the analytes in soil and sediment.

The method was validated in soil and sediment by fortification with veratridine and veratric acid at concentrations of approximately 50.0 (LOQ) and 500 µg/kg (10X LOQ). All recovery samples were extracted twice with 5/1 acetonitrile/0.1 N hydrochloric acid (v/v). The soil and sediment recovery sample extracts were further diluted into the calibration standard range with 10/90 acetonitrile/purified reagent water (v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The storage stability testing was performed in both soil and sediment by fortification with veratridine and veratric acid at concentrations of 499 and 500 µg/kg, respectively. Duplicate samples of each matrix were extracted and analyzed at 0, 7, 14, and 28 days after freezer storage (i.e., ≤0 °C).

The study was initiated on 11 April 2018, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The method validation and storage stability portions of this study were conducted 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 Veratridine and its Major Metabolite in Soil and Sediment 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 OCSPP 850.6100 (U.S. EPA, 2012) and California Department Pesticide Regulation SOP# QAQC012.00 (CA DPR, 2017).

2.2 Test Substances

2.2.1 Veratridine

The test substance, veratridine, was received on 22 November 2016 from Sigma Aldrich, Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	veratridine
Synonym:	3-veratroylveracevine
Batch No.:	SLBF5557V
CAS No.:	71-62-5
Purity:	94.1% (Certificate of Analysis, Appendix 2)
Recertification Date:	12 April 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 8612) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

2.2.2 Veratric Acid

The test substance, 3,4-dimethoxybenzoic acid, was received on 15 January 2018 from Sigma-Aldrich, Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	3,4-dimethoxybenzoic acid
Synonym:	veratric acid
Batch No.:	BCBV0062
CAS No.:	93-07-2
Purity:	99.6 % (Certificate of Analysis, Appendix 2)
Retest Date:	15 January 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 9258) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

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

2.3 Reagents

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

Equivalent reagents/chemicals may be used and it is recommended to evaluate the reagents/chemical substitutes for interferences by taking a reagent blank through the entire analytical procedure.

2.4 Instrumentation and Laboratory Equipment

1.	Instruments:	MDS Sciex API 5000 and API 6500+ Qtrap mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACHT autoinjector Shimadzu DGU-20A3V vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo XS205; Mettler Toledo AB204-S; and Mettler Toledo PG-2002-S
3.	Centrifuges:	Thermo Scientific Sorvall Legend XFR and Allegra X-12
4.	Moisture balance:	Mettler Toledo HB43-S
5.	Shaker table:	VWR Shaker Table 3500
6.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, 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

Soil and sediment were the test matrices used during this method validation. Both soil and sediment matrices were stored refrigerated when not in use. Characterization of soil and sediment was performed by Agvise Laboratories, Northwood, North Dakota.

Parameter	Soil	Sediment
Smithers Viscient Batch No.:	R03-14-18-09	R03-06-18-08
Collection location:	Research For Hire, Porterville, California	Turner Ag Research, Inc. Yuba City, California
Percent sand/silt/clay:	73/20/7	25/34/41
USDA textural class:	Sandy Loam	Clay
Bulk density (disturbed, gm/cc)	1.18	1.09
Cation exchange capacity (meq/100 g)	9.1	23.4
Percent organic matter:	0.63	2.1
pH (1/1 matrix/water ratio, v/v):	8.6	7.4
Percent water holding capacity (at 1/3 bar):	11.9	30.9

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 0.1 N hydrochloric acid liquid reagent solution was typically prepared by diluting 2.46 mL of concentrated hydrochloric acid with 200 mL of purified reagent water and then adjusting to a final volume of 300 mL with purified reagent water. The solution was mixed for five minutes using a stir bar and stir plate.

A 5/1 acetonitrile/0.1 N hydrochloric acid (v/v) liquid reagent solution was typically prepared by diluting 300 mL of the 0.1 N hydrochloric acid solution with 1500 mL of acetonitrile. The solution was mixed for five minutes using a stir bar and stir plate.

A 10/90 acetonitrile/purified reagent water (v/v) solution was typically prepared by diluting 100 mL of acetonitrile with 900 mL of purified reagent water. The solution was mixed for five minutes using a stir bar and stir plate.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler 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 were those used during each separate validation. For the storage stability and any future testing, the actual volumes and masses used may be scaled up or down as necessary. Volumes and masses may be changed; however, the proportions must remain the same.

Primary stock solutions were typically 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	Stock Solvent	Final Volume (mL)	Primary Stock Concentration[a] (mg/L)	Primary Stock Use
8612-1F	0.0099	0.0093	Acetonitrile	10.0	932	Secondary stock and sub stock solutions
9258A	0.0252	0.0251		25.0	1000	Secondary stock and sub stock solutions

Secondary stock solutions were typically 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
8612-1F	932	0.250	25.0	Acetonitrile	8612-1F-1	9.32	Sub-stock solution
9258A	1000	0.500	50.0		9258A-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
8612-1F	932	0.107	10.0	Acetonitrile	Tech Mix-Stk1	9.97/10.0[a]	10X LOQ recovery samples and sub-stock solution
9258A	1000	0.100					
Tech Mix-Stk1	9.97/10.0[a]	1.00	10.0	Acetonitrile	Tech Mix-Stk2	0.997/1.00[a]	LOQ recovery samples
8612-1F-1	9.32	0.0540	50.0	Acetonitrile	Ana Mix-Stk1	0.0101/0.100[a]	Calibration standards
9258A-1	10.0	0.500					

[a] Concentrations are presented as veratridine/veratric acid.

All primary and secondary veratridine stock solutions were stored in a freezer (approximately <0 °C) in amber glass bottles fitted with Teflon-lined caps. All primary and secondary veratric acid 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 stored refrigerated for possible future use.

2.8 Preparation of Calibration Standards

Calibration standards were typically prepared in 10/90 acetonitrile/purified reagent water (v/v) by dosing with the 10.1/100 µg/L (veratridine/veratric acid) sub-stock solution to yield veratridine concentrations of 0.0253, 0.0505, 0.0758, 0.101, 0.202, 0.303, 0.404, and 0.505 µg/L and veratric acid concentrations of 0.250, 0.500, 0.750, 1.00, 2.00, 3.00, 4.00, and 5.00 µg/L.

2.9 Matrix Effect Investigation

In an effort to observe any potential matrix effects, prepared matrix blanks were fortified in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix matched standards fortified in triplicate at the same concentration. Standard solutions used to assess possible matrix effects were prepared as described in the following tables.

2.9.1 Matrix-Matched Standards

Veratridine:

Test Substance Stock ID	Stock Concentration (µg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Mix-Stk 1	10.1	0.0500	5.00	0.101	V-MM Std A
		0.0500	5.00	0.101	V-MM Std B
		0.0500	5.00	0.101	V-MM Std C

[a] Diluted with the final dilution of the control matrix blank.

Veratric Acid:

Test Substance Stock ID	Stock Concentration (µg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Mix-Stk 1	100	0.0500	5.00	1.00	VA-MM Std A
		0.0500	5.00	1.00	VA-MM Std B
		0.0500	5.00	1.00	VA-MM Std C

[a] Diluted with the final dilution of the control matrix blank.

2.9.2 Non-Matrix-Matched Standards

Veratridine:

Test Substance Stock ID	Stock Concentration (µg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Mix-Stk 1	10.1	0.0500	5.00	0.101	V-Std A
		0.0500	5.00	0.101	V-Std B
		0.0500	5.00	0.101	V-Std C

[a] Diluted with 10/90 acetonitrile/purified reagent water (v/v).

Veratric Acid:

Test Substance Stock ID	Stock Concentration (µg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Mix-Stk 1	100	0.0500	5.00	1.00	VA-Std A
		0.0500	5.00	1.00	VA-Std B
		0.0500	5.00	1.00	VA-Std C

[a] Diluted with 10/90 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 clay sediment) with veratridine and veratric acid at concentrations of approximately 50.0 (LOQ) and 500 (10X LOQ) µg/kg. Recovery samples for the soil and sediment matrices were prepared separately ("de novo") at these concentrations. Seven replicates were prepared for the LOQ concentration and five replicates were prepared for the 10X LOQ concentration. Two samples per 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 and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Soil recovery samples:

Sample ID 14012-6139-	Sample Type	Stock Concentration (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
1	Reagent Blank	NA[a]	NA	NA	0.00
2 & 3	Control	NA	NA	5.00	0.00
4, 5, 6, 7, 8, 9, & 10	LOQ	0.997/1.00[b]	0.250	5.00	49.9/50.0[b]
11, 12, 13, 14, & 15	10X LOQ	9.97/10.0[b]	0.250	5.00	499/500[b]

[a] NA = Not Applicable
[b] Concentration expressed as veratridine/veratric acid.

Sediment recovery samples:

Sample ID 14012-6139-	Sample Type	Stock Concentration (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
16	Reagent Blank	NA[a]	NA	NA	0.00
17 & 18	Control	NA	NA	5.00	0.00
19, 20, 21, 22, 23, 24, & 25	LOQ	0.997/1.00[b]	0.250	5.00	49.9/50.0[b]
26, 27, 28, 29, & 30	10X LOQ	9.97/10.0[b]	0.250	5.00	499/500[b]

[a] NA = Not Applicable
[b] Concentration expressed as veratridine/veratric acid.

2.11 Soil and Sediment Extraction Procedure

A 20.0-mL aliquot of 5/1 acetonitrile/0.1 N hydrochloric acid (v/v) was added to each soil and sediment recovery sample (5.00 g dry weight) and then placed on a shaker table for 30 minutes at 200 rpm. The samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50.0-mL centrifuge tubes. The extraction and centrifugation procedures were repeated with an additional 20.0-mL aliquot of 5/1 acetonitrile/0.1 N hydrochloric acid (v/v). The extracts were combined, taken to volume (50.0 mL) with 5/1 acetonitrile/0.1 N hydrochloric acid (v/v) and mixed well. The soil and sediment recovery sample extracts were further diluted into the calibration standard range with 10/90 acetonitrile/purified reagent water (v/v), with different dilution schemes utilized for veratridine and veratric acid as outlined the in tables below. These secondary dilution volumes can be scaled up or down as necessary.

Prior to analysis the soil recovery samples were centrifuged at 13,000 rpm for five minutes using low-binding centrifuge tubes in order to remove any particles in the samples (sediment samples were not centrifuged). All recovery samples were transferred to HPLC vials for analysis. The extraction and dilution procedures are detailed below.

Soil recovery samples - Veratridine

Sample ID 14012-6139-	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
1-V	0.00	NA[c]	20.0	50.0	0.200	10.0	500
2-V & 3-V	0.00	5.00	20.0	50.0	1.00[d]/0.200	50.0[d]/10.0	500
4-V, 5-V, 6-V, 7-V, 8-V, 9-V, & 10-V	0.0499	5.00	20.0	50.0	0.200	10.0	500
11, 12, 13, 14, & 15	0.499	5.00	20.0	50.0	0.0200	10.0	5000

[a] Extraction solvent: 5/1 acetonitrile/0.1 N hydrochloric acid (v/v)
[b] Dilution solvent: 10/90 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Increased volume for 14012-6139-2-V final dilution for use in matrix effects assessment.

Soil recovery samples – Veratric acid

Sample ID 14012-6139-	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
1-VA	0.00	NA[c]	20.0	50.0	2.00	10.0	50.0
2-VA & 3-VA	0.00	5.00	20.0	50.0	10.0[d]/2.00	50.0[d]/10.0	50.0
4-VA, 5-VA, 6-VA, 7-VA, 8-VA, 9-VA, & 10-VA	0.0500	5.00	20.0	50.0	2.00	10.0	50.0
11-VA, 12-VA, 13-VA, 14-VA, & 15-VA	0.500	5.00	20.0	50.0	0.200	10.0	500

[a] Extraction solvent: 5/1 acetonitrile/0.1 N hydrochloric acid (v/v)
[b] Dilution solvent: 10/90 acetonitrile/purified reagent water (v/v)
[c] NA = Not Applicable
[d] Increased volume for 14012-6139-2-VA final dilution for use in matrix effects assessment.

Sediment recovery samples - Veratridine

Sample ID 14012-6139-	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
16-V[c]	0.00	NA[d]	20.0	50.0	2.00	10.0	500
17-V & 18-V	0.00	5.00	20.0	50.0	1.00[e]/0.200	50.0[e]/10.0	500
19-V, 20-V, 21-V, 22-V, 23-V, 24-V, & 25-V	0.0499	5.00	20.0	50.0	0.200	10.0	500
26-V, 27-V, 28-V, 29-V, & 30-V	0.499	5.00	20.0	50.0	0.0200	10.0	5000

[a] Extraction solvent: 5/1 acetonitrile/0.1 N hydrochloric acid (v/v)
[b] Dilution solvent: 10/90 acetonitrile/purified reagent water (v/v)
[c] V in the suffix refers to veratridine.
[d] NA = Not Applicable
[e] Increased volume for 14012-6139-17-V final dilution for use in matrix effects assessment.

Sediment recovery samples – Veratric acid

Sample ID 14012-6139-	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
16-VA[c]	0.00	NA[d]	20.0	50.0	2.00	10.0	50.0
17-VA & 18-VA	0.00	5.00	20.0	50.0	10.0[e]/2.00	50.0[e]/10.0	50.0
19-VA, 20-VA, 21-VA, 22-VA, 23-VA, 24-VA, & 25-VA	0.0500	5.00	20.0	50.0	2.00	10.0	50.0
26-VA, 27-VA, 28-VA, 29-VA, & 30-VA	0.500	5.00	20.0	50.0	0.200	10.0	500

[a] Extraction solvent: 5/1 acetonitrile/0.1 N hydrochloric acid (v/v)
[b] Dilution solvent: 10/90 acetonitrile/purified reagent water (v/v)
[c] VA in the suffix refers to veratric acid.
[d] NA = Not Applicable
[e] Increased volume for 14012-6139-17-VA final dilution for use in matrix effects assessment.

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, 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

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.50	0.350	90.0	10.0
3.00	0.350	0.0	100.0
4.00	0.350	0.0	100.0
4.10	0.350	90.0	10.0
6.00	0.350	90.0	10.0

Run Time:	6.00 minutes
Autosampler Wash Solvent:	30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature:	40 °C
Sample Temperature:	10 °C
Injection Volume:	5.0-20.0 µL
Retention Times:	approximately 2.9 minutes (veratridine) approximately 2.7 minutes (veratric acid)

MS parameters:

Instruments:	MDS Sciex API 5000 and 6500+ QTrap mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Dwell Time:	150 milliseconds
Source Temperature:	600 °C
Curtain Gas:	20.0
Ion Source – Gas 1/Gas 2:	60.0/60.0
Collision Gas:	10.0
Collision Cell Entrance Potential:	10.0
Declustering Potential:	166 (Veratridine) and 75.0 (Veratric Acid)
Resolution Q1/Q3:	Low/Low

Veratridine:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	674.3/456.4	674.3/165.2
Collision Energy:	69.0	93.0
Collision Cell Exit Potential:	12.0	8.0

Veratric Acid:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	183.1/139.1	183.1/124.1
Collision Energy:	17.0	25.0
Collision Cell Exit Potential:	17.0	16.0

Other instrumentation parameters 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 recovery and storage stability samples, every three to seven injections. Injection of recovery and storage stability samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

2.13 Evaluation of Precision, Accuracy, Specificity and Linearity

During the method validation portion of the study, the accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70 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 and retention times. RSD values less than 20% were considered acceptable for the recovery 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 veratridine and veratric acid 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. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.15 Limit of Detection (LOD)

The LOD was calculated using the standard deviation of the average recovery in units of concentration of seven aliquots of the sample fortified at the LOQ, multiplied by the one-tailed t-statistic at the 99% confidence level for n-1 replicates (CA DPR, 2017). Representative calculations for the LOD can be found in Section 3.0.

2.16 Storage Stability

2.16.1 Sample Fortification and Preparation

The storage stability samples were prepared at Smithers Viscient to yield concentrations of 499 and 500 µg/kg veratridine and veratric acid, respectively, in both the soil and sediment. Duplicate samples of each matrix were analyzed after 0, 7, 14, and 28 days of freezer storage (i.e., ≤0 °C).

Eight samples for each matrix (soil and sediment) were fortified with the 9.97/10.0 mg/L veratridine/veratric acid mixed sub-stock solution in individual 50-mL centrifuge tubes to obtain concentrations of 499/500 µg/kg veratridine/veratric acid as presented in the tables below. In addition, three sets of contingency samples, in duplicate, were prepared in each matrix and were stored for possible future use.

Soil stability samples:

Sample ID C05-18-	Storage Interval	Stock Concentration[a] (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Fortified Sample Concentration[a] (µg/kg)
01	Day 0	9.97/10.0	0.250	5.00	499/500
02		9.97/10.0	0.250	5.00	499/500
03	Day 7	9.97/10.0	0.250	5.00	499/500
04		9.97/10.0	0.250	5.00	499/500
05	Day 14	9.97/10.0	0.250	5.00	499/500
06		9.97/10.0	0.250	5.00	499/500
07	Day 28	9.97/10.0	0.250	5.00	499/500
08		9.97/10.0	0.250	5.00	499/500
09, 10, 11, 12, 13, & 14	Contingency Samples	9.97/10.0	0.250	5.00	499/500

[a] Concentrations are presented as veratridine/veratric acid.

Sediment stability samples:

Sample ID C05-18-	Storage Interval	Stock Concentration[a] (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Nominal Concentration[a] (µg/kg)
15	Day 0	9.97/10.0	0.250	5.00	499/500
16		9.97/10.0	0.250	5.00	499/500
17	Day 7	9.97/10.0	0.250	5.00	499/500
18		9.97/10.0	0.250	5.00	499/500
19	Day 14	9.97/10.0	0.250	5.00	499/500
20		9.97/10.0	0.250	5.00	499/500
21	Day 28	9.97/10.0	0.250	5.00	499/500
22		9.97/10.0	0.250	5.00	499/500
23, 24, 25, 26, 27, & 28	Contingency Samples	9.97/10.0	0.250	5.00	499/500

[a] Concentrations are presented as veratridine/veratric acid.

Samples were stored in a freezer until the appropriate sampling interval, with the exception of day 0 samples, which were processed and analyzed on the same day of preparation.

2.16.2 Quality Control (QC) Sample Preparation

Four QC samples were prepared at each sampling interval in each matrix (5.00 g dry weight). QC samples were fortified individually with the 9.97/10.0 mg/L (veratridine/veratric acid) mixed sub-stock solution at concentrations of 249, 375, 499, and 499 µg/kg as veratridine and at concentrations of 250, 376, 500, and 500 µg/kg as veratric acid. A typical fortification scheme is presented in the table below:

Sample ID	Stock Concentration[b] (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration[a] (µg/kg)
QC #1 & #5	9.97/10.0	0.125	5.00	249/250
QC #2 & #6		0.188	5.00	375/376
QC #3 & #7		0.250	5.00	499/500
QC #4 & #8		0.250	5.00	499/500

[a] Concentrations are presented as veratridine/veratric acid.

In addition, one reagent blank (free of matrix) and one control in each matrix was prepared at each storage sampling interval and processed in the same manner as the LOQ samples during the validation portion of this study as presented in the table below:

Sample ID	Storage Interval	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
Reagent Blank	All Intervals	NA[a]	NA	NA	0.00
Control Blank	All Intervals	NA	NA	5.00	0.00

[a] NA = Not Applicable

2.16.3 Sample Processing

The storage stability samples were prepared and processed as detailed in Section 2.11. Duplicate storage stability samples for each matrix were processed at each sampling interval. The procedures are outlined in the table below.

Sample ID	Nominal Concentration (µg/kg)	Dry Weight (g)	Extraction Volume (mL)[a]	Final Volume (mL)[a]	Dilution (mL)	Final Volume (mL)[b]	Dilution Factor
Dilution Scheme for Veratridine							
Reagent Blank	0.00	NA	20.0	50.0	0.0200	10.0	5000
Control Blank	0.00	5.00	20.0	50.0	0.0200	10.0	5000
Storage Stability	499	5.00	20.0	50.0	0.0200	10.0	5000
Storage Stability	499	5.00	20.0	50.0	0.0200	10.0	5000
QC#1	249	5.00	20.0	50.0	0.0200	10.0	5000
QC#2	375	5.00	20.0	50.0	0.0200	10.0	5000
QC#3	499	5.00	20.0	50.0	0.0200	10.0	5000
QC#4	499	5.00	20.0	50.0	0.0200	10.0	5000
Dilution Scheme for Veratric Acid							
Reagent Blank	0.00	NA	20.0	50.0	0.200	10.0	500
Control Blank	0.00	5.00	20.0	50.0	0.200	10.0	500
Storage Stability	500	5.00	20.0	50.0	0.200	10.0	500
Storage Stability	500	5.00	20.0	50.0	0.200	10.0	500
QC#1	250	5.00	20.0	50.0	0.200	10.0	500
QC#2	376	5.00	20.0	50.0	0.200	10.0	500
QC#3	500	5.00	20.0	50.0	0.200	10.0	500
QC#4	500	5.00	20.0	50.0	0.200	10.0	500

[a] Extraction and Dilution solvent: 5/1 acetonitrile/0.1N hydrochloric acid (v/v)
[b] Dilution solvent: 10/90 acetonitrile/purified reagent water (v/v)

The storage stability samples were analyzed using the calibration standards detailed in Section 2.8 and the instrumental conditions in Section 2.13.1.

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. A 1/x-weighted linear regression was used to quantify the recovery samples. The equation for the line including the slope and intercept was generated using standard software (e.g., Microsoft Excel). 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/kg), concentration in the original sample

The LOD (aka MDL) was calculated using the following equation (CA DPR, 2017):

$$(4) \quad LOD = t_{0.99} \times SD$$

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 the detected concentrations of n samples spiked at the estimated LOQ
LOD	=	Limit of detection for the analysis

SMITHERS VISCIENT

TEST PROTOCOL

Title: Validation of the Analytical Method for the Determination of Veratridine and its Major Metabolite in Soil and Sediment by LC-MS/MS

Data Requirement(s): OCSPP 850.6100 and California Department of Pesticide Regulation SOP#QAQC0.12.00

Test Substances:
Name: Veratridine
Purity: 90.6%
Batch or Lot #: SLBF5557V

Name: 3,4-Dimethoxybenzoic acid (Veratric Acid)
Purity: 99.6%
Batch or Lot #: BCBV0062

Study Sponsor: Steve Klaysmat
Address: McLaughlin Gromley & King, MGK®
8810 Tenth Avenue North
Minneapolis, MN 55427

Study Monitor: Mark Lenz, Ph.D.
Email / Phone Number: mlenz@exponent.com Tel: (913) 213-9519

Sponsor Protocol/Project No. (when applicable): NA

Testing Facility: Smithers Viscient,
790 Main Street
Wareham, Massachusetts 02571

Study Director: Johnson I. Jutson, Ph.D.

Smithers Viscient Study No.: 14012.6139

Test Concentrations: 0.050 and 0.50 mg/kg

Proposed Experimental Dates
Start: April 12, 2018
Termination: May 24, 2018

Sponsor Approval	10 April 2018 **Date**
Study Director Signature	11 April 2018 **Study Initiation Date**

Validation of the Analytical Method for the Determination of Veratridine and its Major Metabolite Veratric Acid in Soil and Sediment by LC-MS/MS

1.0 INTRODUCTION

The purpose of this study is to validate an analytical method used to determine the content of veratridine and its major metabolite in a single soil matrix and a single sediment matrix by LC-MS/MS. The analytical method will be validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of identification.

2.0 JUSTIFICATION OF THE TEST SYSTEM

This study is conducted to support the registration of the test substance(s).

The method validations described in this protocol are designed to conform to EPA guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as compatible with OECD principles on GLP.

3.0 TEST SUBSTANCE

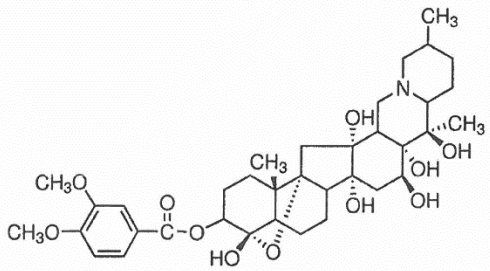
3.1 Test Substance

Upon arrival at Smithers Viscient, the test substance (also the reference substance) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

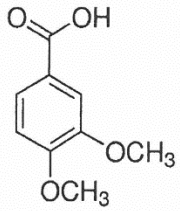
Each test and reference substance will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

3.2 Reference Standards

Residues of veratridine and veratric acid will be determined in soil and sediment. Current available information on the analytes is summarized below.



Name:	Veratridine
Synonym:	3-veratroylveracevine
Batch/Lot No.:	SLBF5557V
CAS No.:	71-62-5
Purity:	To be added by amendment
Recertification Date:	To be added by amendment



Name:	3,4-dimethyloxybenzoic acid
Synonym:	Veratric acid
Batch/Lot No.:	BCBV0062
CAS No.:	93-07-2
Purity:	99.6%
Expiration Date:	01/15/2019

3.3 Test Matrices

The soil and sediment used for the method validation will be one type of soil and one type of sediment matrix. Prior to testing, soil and sediment moisture content will be determined using a moisture analyzer. Soil and sediment characterization data such as % sand, silt clay, bulk density and % organic matter will be determined. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.

3.4 Reagents

Highly pure reagents will be used throughout the study. The actual reagent grade will be depending on the manufacturer's designation. Generally, these reagents will have grades, such

as high purity solvent, ACS grade, or Select. The reagents used are recorded along with test chemical information at the time of preparation.

4.0 VALIDATION DESIGN

The test design will consist of a single soil matrix and a single sediment matrix fortified with each test substance at two concentrations with seven replications at the target LOQ and five replicates at 10x LOQ level for each matrix. The procedural blank will be reagent blank without matrix. The control matrix for the validation will be untreated matrix representing soil and sediment. The validation study levels (approximate concentrations) for test substance are:

1.	Procedural blank-reagent blank	0.0 mg/kg
2.	Matrix blank-control matrix	0.0 mg/kg
3.	Control matrix fortified at LOQ	0.050 mg/kg
4.	Control matrix fortified at 10 x LOQ	0.50 mg/kg

4.1 Accuracy and Precision

The accuracy of the analytical method will be determined by applying the method to seven samples at the LOQ and five samples at 10X LOQ for each test substance. Accuracy will be reported as the mean recovery at each fortification level. Mean recoveries in the range 70 – 120% of nominal concentrations of the target analyte in the fortified samples will be considered acceptable.

The precision will be calculated for the fortified samples in terms of the standard deviation (SD) and relative standard deviation (RSD or coefficient of variation (CV)) calculated for the retention time, peak area based quantitation (i.e., mg/kg), and the observed recovery values. The retention time should have a RSD of less than or equal to 2%. The RSD of the peak area based quantitation (i.e., mg/kg) should be less than or equal to 20% per level. The RSD of the recovery values should be less than or equal to 20% per level as well.

4.2 Specificity

The specificity of the method will be determined by applying the method to the appropriate number of reagent blank and control matrix samples (n=2). Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte(s) peak of interest. Peaks attributable to the test substance(s) should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. Blank values (including procedural blanks and untreated samples) should not exceed 30% of the LOQ. If this is exceeded, detailed justification is required.

4.3 Regression Analysis

Quantitative analysis will be achieved with the aid of a calibration curve. The calibration curve will be constructed using a minimum of five analytical standards and will extend over a range appropriate to the lowest and highest nominal concentrations of the target analyte in relevant analytical solutions ± at least 20%.

The calibration data will be subjected to regression analysis; a plot of analyte concentration versus detector response will be included in the report along with the correlation coefficient (r) and the

equation describing the curve. The linearity of the detector response will be assessed according to the strength of the correlation coefficient: this should be ≥ 0.995 (or coefficient of determination, $r^2 \geq 0.990$). If non-linear calibration is used an explanation will be provided.

4.4 Limits of Quantitation (LOQ)

The method will be validated at the limit of quantitation (LOQ). This will be defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. If this is exceeded, it will be discussed with the Sponsor and detailed justification provided prior to processing.

4.5 Limits of Detection (LOD)

The Limits of Detection (LOD) will be calculated using the standard deviation of the average recovery in units of concentration of seven aliquots of the sample fortified at the LOQ multiplied by one-tailed t-statistic at the 99% confidence level for n-1 replicates (CA DPR, 2017).

4.6 Matrix Effects Determination

Determination of LC-MS/MS matrix effects will be evaluated through the assessment of solvent-based and matrix-matched calibration standards for both primary and confirmatory transitions. Matrix effects should be evaluated at the LOQ level for each test substance. Only if experiments clearly demonstrate that matrix effects are not significant (i.e. <20%), calibration with standards in solvent may be used.

4.7 Confirmatory Analyses

Unequivocal identification of the target analyte will be achieved by LC/MS-MS using a primary quantitation ion and secondary quantitation/ confirmatory ion. All of the required elements need to be met for this confirmatory method with full method validation results generated for both transitions. For triple-quad MS method, the confirmation method would be where confirmatory (secondary) product ion will be used for quantification. The confirmatory method analysis will also adhere to the aforementioned method specifications (Sections 4.1-4.6 above).

4.8 Storage Stability

A minimum of 28-day storage stability will be established in both soil and sediment matrices. Sediment and soil matrices will be fortified at 10X LOQ level and stored in the freezer below 0 °C for the duration of the test. Two replicate samples will be analyzed at four sampling intervals (For example, Day 0, Day7, Day 14 and Day 28) to validate residue's rate of decomposition in respective matrices. Analysis will be conducted with validated analytical method with acceptable recovery range of 70-120% with RSD of <20%.

5.0 PROCEDURE FOR THE INDENTIFICATION OF THE TEST SYSTEM

The test system will be defined as the fortified recovery samples. The fortified recovery samples will be labeled as defined in section 4.0 and each sample replicate will be assigned a unique identifier. Processing of fortified recovery samples will be performed at a lab station labeled with the study number.

6.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples and replicate analysis.

7.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

8.0 REPORTING

The raw data generated at Smithers Viscient will be peer-reviewed and the final report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The Quality Assurance Unit will inspect the final report to confirm that the methods, procedures, and observations are accurately and completely described, that the reported results accurately and completely reflect the raw data generated at Smithers Viscient and to confirm adherence with the study protocol. A single copy of the draft report will be submitted to the Sponsor for review. The report will be finalized according to standard operating procedures. The final report will meet the formatting requirements of EPA's PR Notice 2011-3. All reports will include, but will not be limited to reporting requirements presented in Ecological Effects Test Guidelines OCSPP 850.6100 (U.S. EPA, 2012) and California DPR Guide for Analytical Method Development (CA DPR, 2017) and the following information:

- The report and project numbers from Smithers Viscient and Sponsor Study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program Coordinator (if applicable), Study Director and Principal Investigator.
- Identification of the test substance including chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).
- A full description of the experimental design and procedures followed and a description of the test equipment used.
- The determined specificity, linearity, accuracy, precision, LOQ, LOD and MDL, and confirmation of identification.
- The mathematical equations and statistical methods used in generating and analyzing the data as well as calculations using these equations. Tabular and graphical representations (if appropriate) of the data.
- Description of any problems experienced and how they were resolved.

- Good Laboratory Practice (GLP) Compliance Statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of raw data and report.
- A copy of the study protocol and study amendments, if any.

9.0 PROTOCOL AMENDMENTS

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any.

10.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide, Fungicide and Rodenticide Act (40 CFR, Part 160) and as compatible with OECD Principles of Good Laboratory Practice (OECD, 1998)

11.0 REFERENCES

OECD, 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997). Environment Directorate Chemicals Group and Management Committee. ENV/MC/CHEM(98)17. OECD Paris. France. 41 pp.

U.S. EPA. 2011. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160); FR: 7/1/2011; pp. 34052. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA, 2011. Pesticide Registration (PR) Notice 2011-3 Standard Format for Data Submitted Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Certain Provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA). US Environmental Protection Agency Office of Pesticide Programs. November 30, 2011.

U.S. EPA, 2012. Office of Chemical Safety and Pollution Prevention. Ecological Effects Guideline, OCSPP 850.6100. Environmental Chemistry Methods and Associated Independent Laboratory Validation. EPA 712-C-001. January 2012. U.S. Environmental Protection Agency, Washington, D.C.

CA DPR, 2017. California Department of Pesticide Regulation. 2017. Guide for Analytical Method Development, SOP#QAQC0.12.0. January 2017. California Department of Pesticide Regulation, Sacramento, CA.

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 1

Protocol Title: Validation of the Analytical Method for the Determination of Veratridine and its Major Metabolite in Soil and Sediment by LC-MS/MS

Study Sponsor: McLaughlin Gormley & King, MGK®

Test Substance(s): Veratridine & 3,4-Dimethoxybenzoic acid (Veratric Acid)

Smithers Viscient Study No.: 14012.6139

Amendment:

Protocol Currently States:

Test Substances: Name: Veratridine
Purity: 90.6%

Address: McLaughlin Gromley & King, MGK®
8810 Tenth Avenue North
Minneapolis, MN 55427

Update Protocol to State:

Test Substances: Name: Veratridine
Purity: **94.1%**

Address: McLaughlin **Gormley** & King, MGK®
8810 Tenth Avenue North
Minneapolis, MN 55427

Reason for Change: The purpose of this amendment is to correct typographical error in the sponsor's name and to update the purity based on the updated purity.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Johnson I. Jutson
Smithers Viscient Study Director

18 May 2018
Effective Date

Study Sponsor Representative

22-May-2018
Date

Page 1 of 1

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.:	2
Protocol Title:	Validation of the Analytical Method for the Determination of Veratridine and its Major Metabolite in Soil and Sediment by LC-MS/MS
Study Sponsor:	McLaughlin Gormley & King, MGK®
Test Substance(s):	Veratridine & 3,4-Dimethoxybenzoic acid (Veratric Acid)
Study No.:	14012.6139
Amendment:	

Section 2.0:
Justification of Test System
This study is conducted to support the registration of the test substance.

The method validations described in this protocol are designed to conform to EPA guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as **compatible** with OECD principles on GLP.

Revise protocol to state:

The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as **accepted by** OECD principles of Good Laboratory Practice (OECD, 1998).

Reason for Change: To clarify language of OECD principles according to GLPs.

Smithers Viscient LLC
www.smithersviscient.com
790 Main Street | Wareham, MA 02571 | p 508.295.2550
2900 Quakenbush Road | Snow Camp, NC 27349 | p 336.376.0141

Section 3.2:Reference Standards

Name:	Veratridine
Synonym:	3-veratroylveracevine
Batch/Lot No.:	SLBF5557V
CAS No.:	71-62-5
Purity:	To be added by amendment
Recertification Date:	To be added by amendment

Update protocol to State:

Purity:	**94.1%**
Recertification Date:	**12 April 2020**

Reason for Change: To update purity and recertification date.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Johnson I. Jutson
Smithers Viscient Study Director

20 NOV 2018
Effective Date

Sponsor Approval

20-Nov-2018
Date