1.0 INTRODUCTION

Methodology provided by Smithers Viscient (DeVellis, 2015) was validated 29 June to 8 July 2015 to quantify the concentration of aldicarb, aldicarb-sulfone and aldicarb-sulfoxide present in soil. This method was utilized to perform an independent laboratory validation (ILV) for aldicarb and its metabolites in soil conducted on 8 September 2015. This independent laboratory validation (ILV) study is required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one group, can be independently validated by a second group with no major interaction between the two groups. This method was validated by fortification of soil with aldicarb and its metabolites (aldicarb-sulfone and aldicarb-sulfoxide) at concentrations of 10.0 (limit of quantification, LOQ) and 100 µg/kg (10X LOQ). Recovery samples were extracted twice with 0.1% formic acid in acetonitrile and were diluted into the calibration standard curve with 20:80 acetonitrile:purified reagent water (v:v) prior to analysis. Samples were then analyzed using liquid chromatography with mass spectrometry (LC/MS/MS).

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled "Independent Laboratory Validation (ILV) of the Analytical Method: Aldicarb and metabolites Aldicarb Sulfone and Aldicarb Sulfoxide - Validation of the Analytical Method for the Determination of Test Substances in Soil" (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100: Environmental Chemistry Methods

and Associated Independent Laboratory Validation (U.S. EPA, 2012) and OSCPP Guideline 850.7100: Data Reporting for Environmental Chemistry Methods (U.S. EPA, 1996).

2.2 Test Substances

The test substance, aldicarb PESTANAL®, was received on 1 September 2015 from Sigma Aldrich Inc., Allentown, Pennsylvania. The following information was provided:

Name:	aldicarb PESTANAL®
Batch No.:	SZBE307XV
CAS No.:	116-06-3
Purity:	99.9%
Expiration Date:	3 November 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 7864) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, aldicarb-sulfoxide PESTANAL®, was received on 11 September 2014 from Sigma Aldrich Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	aldicarb-sulfoxide PESTANAL®
Synonym:	aldicarb-sulfoxide
Batch No.:	SZBD049XV
CAS No.:	1646-87-3
Purity:	99.2%
Expiration Date:	18 February 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 7285) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, aldicarb-sulfone PESTANAL®, was received on 11 September 2014 from Sigma Aldrich Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	aldicarb-sulfone PESTANAL®
Synonym:	aldicarb-sulfone
Batch No.:	SZBB343XV
CAS No.:	1646-88-4
Purity:	99.5%
Expiration Date:	9 December 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7284) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
2.	Formic acid:	EMD, reagent grade
3.	Purified reagent water:	prepared from a Millipore Milli-Q® Direct 8 system (meeting ASTM Type II requirements)
4.	Dimethylsulfoxide:	BDH, reagent grade
5.	Methanol	EMD, reagent grade

2.4 Equipment

1.	Instrument:	AB Sciex API 5000 mass spectrometer equipped with an AB Sciex Turbo V ESI Ion Spray source Acquity Sample Manager autosampler Acquity Binary Solvent Manager binary pump Acquity Column Compartment column oven, and Analyst 1.6 software for data acquisition
2.	Balance:	Mettler PG-2002-S, Mettler Toledo XSE205DU, Mettler Toledo Moisture Balance HB43-S
3.	Centrifuge:	Beckman Allegra X-12
4.	Shaker table:	VWR Analog 3500 STD
5.	Laboratory equipment:	volumetric flasks, disposable glass pipets, disposable glass vials, positive displacement pipets, Nalgene centrifuge tubes, autosampler vials, and amber glass bottles with Teflon®-lined caps

2.5 Test Soil

The soil used for this ILV analysis was Rochester Sandy Loam soil (SMV Lot No. 021814, Sample ID 2014 100 ROCH LOAM) from Rochester, Massachusetts. The soil was stored refrigerated in the dark until needed for analysis. Prior to testing, soil moisture content of the soil was determined to be 13.61% using a Mettler Toledo HB43-S moisture analyzer.

2.6 Preparation of Stock Solutions

Primary stock solutions were typically prepared as per the table below. All volumes and masses can be scaled up or down as necessary.

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as Active Ingredient	Final Volume (mL)	Solvent Stock	Primary Stock Concentration (mg/L)	Primary Stock Uses
7864A	0.0251	0.0251	25.0	Acetonitrile	1000	Secondary stock solutions
7284D	0.0251	0.0250	25.0		999	
7285-2B	0.0253	0.0251	25.0		1000	

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)	Solvent Stock	Final Stock Concentration (mg/L)	Stock ID	Stock Uses
7684A	1000	5.00	50.0	Acetonitrile	100	7684A-2	Sub-stock solutions
7284D	999	5.00	50.0		99.9	7284D-2	
7285-2B	1000	5.00	50.0		100	7285-2B-2	

Mixed sub-stock solutions were typically prepared as summarized in the table below.

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Solvent Stock	Final Stock Concentration (mg/L)	Stock ID	Stock Uses
7684A-2	100	0.100	10.0	Acetonitrile	1.00	Mix-STK 1	Sub-stock, LOQ- and high-level recovery samples
7284D-2	99.9	0.100					
7285-2B-2	100	0.100					
Mix-STK 1	1.00	1.00	10.0		0.100	Mix-STK 2	Calibration standards
Mix-STK 2	0.100	1.00	10.0		0.0100	Mix-STK 3	Calibration standards

All primary and secondary stock solutions were stored refrigerated in amber glass bottles fitted with Teflon®-lined caps until use. The mixed sub-stock solutions were prepared on the day of use and discarded after use.

2.7 Reagent Solution and Mobile Phase Preparation

A 0.1% formic acid in purified reagent water mobile phase solution was typically prepared by adding 2.00 mL of concentrated formic acid to 2000 mL of purified reagent water. The mobile phase was mixed using a stir bar and stir plate for five minutes and degassed under vacuum with sonication for 10 minutes.

A 0.1% formic acid in acetonitrile mobile phase solution and was typically prepared by adding 2.00 mL of concentrated formic acid to 2000 mL of acetonitrile and mixed well. The mobile phase was mixed using a stir bar and stir plate for five minutes and degassed under vacuum with sonication for 10 minutes.

A 20:80 acetonitrile:purified reagent water (v:v) liquid reagent solution was typically prepared by combining 400 mL of purified reagent water with 100 mL of acetonitrile. The solution was mixed using a stir bar and stir plate for five minutes.

A 0.1% formic acid in acetonitrile liquid reagent solution was typically prepared by adding 0.900 mL of concentrated formic acid to 900 mL of acetonitrile. The solution was mixed well using a stir bar and stir plate for five minutes.

A 30:30:40 acetonitrile:methanol:dimethyl sulfoxide (v:v:v) autosampler wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol and 2000 mL of dimethyl sulfoxide.

A 90:10 purified reagent water:acetonitrile (v:v) autosampler purge solution was typically prepared by combining 400 mL of acetonitrile and 3600 mL of purified reagent water.

2.8 Preparation of Calibration Standards

Calibration standards were prepared in 20:80 acetonitrile:purified reagent water (v:v) by fortifying with the 0.0100 and 0.100 mg/L mixed sub-stock solutions as described in the following table.

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Calibration Standard Concentration (µg/L)
Mix –STK 3	0.0100	0.0500	10.0	0.0500
		0.100	10.0	0.100
Mix-STK 2	0.100	0.0500	10.0	0.500
		0.0750	10.0	0.750
		0.125	10.0	1.25
		0.175	10.0	1.75

2.9 Sample Fortification and Preparation

All soil recovery samples (5.00 g dry weight) were weighed into individual 50.0-mL Nalgene® centrifuge tubes. Five replicates of each concentration were dosed with the 1.00 mg/L mixed sub-stock solution at 10.0 and 100 µg/kg (dry weight). The dosing procedure is detailed in the following table:

Sample ID	Stock ID	Fortifying Stock Concentration (mg/L)	Fortification Volume (mL)	Dry weight (g)	Nominal Concentration (µg/kg)
Reagent Blk	NA[a]	NA[a]	NA[a]	NA[a]	0.00
CTL A, B, C, D & E	NA[a]	NA	NA	5.00	0.00
LOQ A, B, C, D & E	Mix-STK1	1.00	0.0500	5.00	10.0
High A, B, C, D & E			0.500	5.00	100

[a] NA = Not Applicable

Five additional 5.00 g samples were prepared and left unfortified to serve as controls. One additional sample was extracted using only extraction solvents to serve as the reagent blank.

2.10 Soil Extraction and Dilution

A 20.0-mL aliquot of 0.1% formic acid in acetonitrile was added to the soil recovery samples (5.00 g dry weight). The samples were placed on an orbital shaker table for 30 minutes at 150 rpm. The samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to labeled 50.0-mL volumetric flasks. The extraction and centrifugation procedure was repeated with an additional 20.0-mL aliquot of 0.1% formic acid in acetonitrile. The second extract was combined with the first in the appropriate volumetric flasks and taken to a final volume of 50.0 mL with 0.1% formic acid in acetonitrile and mixed well. Samples were further diluted into the calibration standard range with 20:80 acetonitrile:purified reagent water (v:v). The extraction and dilution procedures are detailed below.

Sample ID	Fortified Concentration (µg/kg)	Dry weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Secondary Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blk	0.00	NA[c]	20.0	50.0	1.00	10.0	100
CTL A, B, C, D & E	0.00	5.00	20.0	50.0	1.00	10.0	100
LOQ A, B, C, D & E	10.0	5.00	20.0	50.0	1.00	10.0	100
High A, B, C, D & E	100	5.00	20.0	50.0	0.500	10.0	200

[a] Extracted and diluted with 0.1% formic acid in acetonitrile.
[b] Dilution solvent: 20:80 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: X-Bridge™ C18, 2.5 µm, 2.1 × 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 (%)
0.00	0.450	98.0	2.0
1.20	0.450	98.0	2.0
2.00	0.450	0.0	100.0
3.00	0.450	0.0	100.0
3.20	0.450	98.0	2.0
4.50	0.450	98.0	2.0

Injection volume: 50 µL
Column oven: 30 °C
Sample temperature: 5 °C
Retention Time: Approximately 2.02 minutes (for aldicarb)
Approximately 1.83 minutes (for aldicarb sulfone)
Approximately 1.77 minutes (for aldicarb sulfoxide)

MS parameters:

Instrument: Instrument:MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan type: MRM
Q1/Q3 Resolution: Unit/Unit
Dwell Time: 200 milliseconds
Source Temperature: 500 °C
Curtain Gas: 10.00
Ion Source – Gas 1/Gas 2: 70.00/70.00
Collision Gas: 4.00

Parameter	Aldicarb	Aldicarb sulfone	Aldicarb sulfoxide
Q1/Q3 Masses	213.10/116.10	223.10/148.00	207.10/132.10
Collision Energy	17.00	14.00	18.20
Collision Cell Entrance Potential	6.00	10.00	8.00
Collision Cell Exit Potential	25.00	7.00	24.00
Declustering Potential	110.00	110.00	15.00

Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each 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.

2.11.2 Method Differences

The method used for this independent laboratory validation was modified slightly from the method validation (DeVellis, 2015) as follows: the injection volume was decreased from 100 µL to 50 µL and the HPLC conditions on the instrumentation were optimized.

2.12 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the low- and high-level recovery samples. Recoveries of 70 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the low- and high-level recovery samples for each analyte. The retention time should have an RSD of less than or equal to 2%. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. Specificity of the method was determined by examination of the control samples for peaks at the same retention time as aldicarb, aldicarb-sulfone and aldicarb-sulfoxide which might interfere with the quantitation of the analytes. Interferences with peak areas that are less than 50% at the limit of detection (LOD) are not considered significant. Linearity of the method was determined

by the correlation coefficient (r^2), y-intercept and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient not less than 0.990. The precision of the method at the LOQ was reported in terms of the relative standard deviation or coefficient of variation of the observed recovery values. A polynomial calibration curve was used for this testing due to the nature of the LC/MS/MS detection. This calibration curve was evaluated based on the correlation coefficient (r^2) and the recoveries of the calibration standards.

2.13 Communications

Communications occurred with the Sponsor Monitor to discuss items such as:
1) clarification/approval of the protocol and method, 2) acquisition of analytical standard and control matrix and 3) pre-validation evaluation and method establishment including calibration curve linearity. A complete list of communications is maintained in the study raw data.

No communications occurred between the groups performing the independent laboratory validation and the method developers.

2.14 Time Required for Analysis

A normal batch of samples consists of ten fortified and five unfortified samples, 1 reagent blank and 6 solvent standards (22 samples total). A single analyst completed a set of 22 samples in one working day (8 hours) with LC/MS/MS analysis performed overnight.

3.0 Calculations

A calibration curve was constructed by plotting the analyte concentration (µg/L) in the calibration standards against the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the

quadratic equation, the peak area of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = ax^2 + bx + c$$

$$(2) \qquad DC(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

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

where:

y	=	detector response (peak area) for analyte
a, b and c	=	regression constants
DC (x)	=	detected concentration (µg/L) in the sample
C	=	constant c minus the peak area; C = (c - y)
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of detection (LOD) was set at the lowest concentration in soil samples which can possibly be detected based on the lowest concentration in the calibration standards and was calculated using the following equation.

$$4) \quad LOD = LOD_{LCAL} \times DF_{CNTL}$$

Where (for example):

LOD_{LCAL}	=	the lowest concentration calibration standard (0.0500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 100)
LOD	=	limit of detection for the analysis of soil (0.0500 µg/L × 100 = 5.00 µg/L)

PROTOCOL DEVIATIONS

No deviations from the protocol occurred during this study.

REFERENCES

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