1.0 INTRODUCTION

A method was validated from 29 June to 8 July 2015 to quantify the concentrations of aldicarb, aldicarb-sulfone and aldicarb-sulfoxide present in recovery samples prepared in soil. The analytical method was validated with regards to accuracy and precision, linearity, specificity and limit of quantitation (LOQ). The method was validated by fortification of soil with aldicarb and its metabolites (aldicarb-sulfone and aldicarb-sulfoxide) at concentrations of 10.0 and 100 µg/kg. Recovery samples extracted with 0.1% formic acid in acetonitrile followed by dilution into the calibration standard range with 20:80 acetonitrile:purified reagent water (v:v). All samples were analyzed by liquid chromatography with mass spectrometry detection (LC/MS/MS).

The study was initiated on 24 October 2014, 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 29 June to 8 July 2015 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 toxicity test followed those described in the Smithers Viscient protocol entitled "Aldicarb Sulfone and Aldicarb Sulfoxide – Validation of the Analytical Method for the Determination of Test Substances in Soil" (Appendix 1).

2.2 Test Substances

The test substance, aldicarb, was received on 21 April 2014 from Sigma Aldrich, Allentown, Pennsylvania. The following information was provided:

Name:	aldicarb
Batch No.:	SZBC166XV
CAS No.:	116-06-3

Purity:	99.9%
Expiry Date:	14 June 2017

Upon receipt at Smithers Viscient, the test substance (SMV No. 6962) was stored at room temperature in the original container in a dark, ventilated cabinet. 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.

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.

2.3 Reagents

1. Acetonitrile:	EMD, reagent grade
2. Formic acid:	EMD, reagent grade
3. Methanol:	EMD, reagent grade
4. Dimethyl sulfoxide:	BDH, reagent grade
5. 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 Equipment

1. Instrument:	MDS Sciex API 5000 (Serial Number: L20354655487) mass spectrometer equipped with an ESI Turbo V ion source Shimadzu 20AD/SIL-20ACHT autoinjector Shimadzu 20AD/DGU-20A3 vacuum degasser Shimadzu 20AD/DGU-20A5R vacuum degasser CBM-20A/228-40512-32 communications bus Shimadzu 20AD/LC-20AD solvent delivery pumps Shimadzu 20AD/CTO-20A column compartment Analyst 1.4.2 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 pipets, positive displacement pipets, graduated cylinders, 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 Soil

The soil used for the method validation was Rochester Sandy Loam soil (SMV Lot No. 060315, Sample ID 2014 100 ROCH LOAM) from Rochester, Massachusetts. Prior to testing, soil moisture content was determined to be 10.79% using a Sartorius MA-45 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	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
6962-2A	0.0501	0.0500	Acetonitrile	50.0	1000	Secondary stock solutions
7284C	0.0252	0.0251		25.0	1000	Secondary stock solutions
7285-1C	0.0253	0.0251		25.0	1000	Secondary stock solutions
7285-2A	0.0252	0.0250		25.0	1000	Sub-stock solution

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
6962-2A	1000	0.500	50.0	Acetonitrile	6962-2A-2	10.0	Sub-stock solution
		5.00	50.0		6969-2A-3	100	Sub-stock solution
7284C	1000	0.500	50.0		7284C-1	10.0	Sub-stock solution
		5.00	50.0		7284C-2	100	Sub-stock solution
7285-1C	1000	0.500	50.0		7285-1C-1	10.0	Sub-stock solution
		5.00	50.0		7285-1C-2	100	Sub-stock solution
7285-2A	1000	0.500	50.0		7285-2A-1	10.0	Sub-stock solution

Sub-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 (µg/L)	Stock Use
6962-2A-2	10.0	0.100	10.0	Acetonitrile	Mix Stk 1	100	Calibration Standards
7284C-1	10.0	0.100					
7285-1C-1	10.0	0.100					
6969-2A-3	100	0.100	10.0		Mix Stk 2	1000	Calibration Standards and low- and high-level recovery samples
7284C-2	100	0.100					
7285-1C-2	100	0.100					
72852A-1	10.0	0.100	10.0		Ana Stk 1	100	Calibration Standards

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

2.7 Preparation of Liquid Reagent and Mobile Phase Solutions

All volumes can be scaled up or down as necessary.

A 20:80 acetonitrile:purified reagent water (v:v) liquid reagent solution was typically prepared by combining 100 mL of acetonitrile with 400 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 acetonitrile liquid reagent solution was typically prepared by adding 0.600 mL of concentrated formic acid to 600 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 needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol and 2000 mL of dimethyl sulfoxide.

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 degassed under vacuum with sonication.

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 degassed under vacuum with sonication.

2.8 Preparation of Calibration Standards

Calibration standards for aldicarb and aldicarb-sulfone analysis were prepared in 20:80 acetonitrile:purified reagent water (v:v) by fortifying with the 100 µg/L mixed sub-stock solution to yield concentrations of 0.0500, 0.100, 0.500, 0.750, 1.25 and 1.75 µg/L.

Calibration standards for aldicarb-sulfoxide analysis were prepared in 20:80 acetonitrile:purified reagent water (v:v) by fortifying with the 100 µg/L sub-stock solution to yield concentrations of 0.0500, 0.100, 0.500, 0.750, 1.25 and 1.75 µg/L.

The methodology was intended to have all three analytes in a mixed sub-stock. However, the aldicarb-sulfoxide constituent for preparation of calibration standards was divergent due to poor chromatography.

2.9 Sample Fortification and Preparation

A total of 9 soil recovery samples (5.00 g dry weight) were weighed into individual 50.0-mL Nalgene® centrifuge tubes. Samples were dosed with the appropriate test substance stock solution at concentrations of 10 and 100 µg/kg (dry weight). Three replicates were prepared for each concentration level. In addition, three samples were left unfortified to serve as controls and were extracted in the same fashion as the low- and high-level recovery samples. The dosing procedure is detailed in the following table:

Sample ID	Mixed Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Sample Concentration (µg/kg)
Control A, B & C	NA[a]	NA	5.00	0.00
Low A, B & C	1.00	0.0500	5.00	10.0
High A, B & C	1.00	0.500	5.00	100

[a] NA = Not Applicable.

2.10 Sediment Extraction

A 20.0-mL aliquot of 0.1% formic acid in acetonitrile was added to each soil recovery sample (5.00 g dry weight) and they were placed on a 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 50.0-mL volumetric flasks. The extraction and centrifugation procedures were repeated with a 20.0-mL aliquot of 0.1% formic acid in acetonitrile. The extracts were combined, taken to volume (50.0 mL) with 0.1% formic acid in acetonitrile and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 20:80 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)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Secondary Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Control A, B & C	0.00	5.00	20.0	50.0	1.00	10.0	100
Low A, B & C	10.0	5.00	20.0	50.0	1.00	10.0	100
High A, B & C	100	5.00	20.0	50.0	0.500	10.0	200

[a] Extraction and Dilution Solvent: 0.1% formic acid in acetonitrile.
[b] Dilution solvent: 20:80 acetonitrile:purified reagent water (v:v).

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC Parameters:

Column: XBridge 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 (%)
0.50	0.250	95	5
1.50	0.250	0	100
2.50	0.250	0	100
2.60	0.250	95	5
4.10	0.250	95	5

Run time: 5.00 minutes
Injector Rinse solvent: 30:30:40 acetonitrile:methanol:dimethyl sulfoxide (v:v:v)
Column temperature: 30 °C
Sample temperature: 5 °C
Injection volume: 100 µL
Retention Time: approximately 3.2 minutes (for aldicarb)
approximately 3.1 minutes (for aldicarb-sulfone)
approximately 3.1 minutes (for aldicarb-sulfoxide)

MS Parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan type: MRM
Q1/Q3 Masses: 213.10/116.10 amu (for aldicarb)
223.10/148.00 amu (for aldicarb-sulfone)
207.10/132.10 amu (for aldicarb-sulfoxide)
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
Collision Energy: 17.00 (for aldicarb)
13.40 (for aldicarb-sulfone)
9.50 (for aldicarb-sulfoxide)
Collision Cell Entrance Potential: 6.00 (for aldicarb)
8.00 (for aldicarb-sulfone)
8.00 (for aldicarb-sulfoxide)
Collision Cell Exit Potential: 25.00 (for aldicarb)
16.00 (for aldicarb-sulfone)
24.00 (for aldicarb-sulfoxide)
Declustering Potential: 110.00 (for aldicarb)
43.90 (for aldicarb-sulfone)
15.00 (for aldicarb-sulfoxide)

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 terms of percent recovery of the low- and high-level recovery samples. The precision was reported in terms of the standard deviation and relative standard deviation for the retention time and the percent recovery values of the low- and high-level recovery samples. 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. 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 Limit of Detection

The limit of detection (LOD) was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

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 mass)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

$$(4)\ LOQ_{INST} = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

$$(5)\ LOQ = LOQ_{INST} \times DF_{CTRL}$$

where:

$Area_{LS}$	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
a, b, c	=	regression constants
C	=	regression constant ; C = (c - $Area_{LS}$)
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

The LOD was calculated using the following equation:

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

where:

SN_{ctl}	=	Mean signal to noise in height of the control samples (or Blanks)
$Resp_{LS}$	=	Mean Response in height of the two low calibration standards
$Conc_{LS}$	=	Concentration of the low calibration standard
LOD	=	limit of detection for the analysis

PROTOCOL DEVIATIONS

No deviations from the protocol occurred during this study.

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. 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160); FR: 8/17/89; pp. 34052. U.S. Environmental Protection Agency, Washington, D.C.

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 2

Protocol Title: Aldicarb Sulfone and Aldicarb Sulfoxide - Validation of the Analytical Method for the Determination of Test Substances in Soil

Study Sponsor: AgLogic LLC
121 S. Estes Dr., Suite 101, Chapel Hill, NC 27514

Test Substance:

Name:	Aldicarb Sulfone
Purity:	99.5% (A.I.)
Batch or Lot #:	SZBB343XV

Name	Aldicarb Sulfoxide
Purity	99.2% (A.I.)
Batch or Lot #:	SZBD049XV

Study No.: 14070.6103

Amendment 1

This protocol amendment expands the study to include aldicarb, aldicarb sulfone and aldicarb sulfoxide together in the same sample at 0.0100 and 0.100 mg/kg.

Test Substance Section on Cover page.

Add aldicarb to the list of test substances

Name:	Aldicarb
Purity:	99.9% a.i.
Batch or Lot #:	SZBC166XV

Reason for change:

Sponsor has requested the addition of this material in addition to the two metabolites previously included in the protocol.

Section 4.0 – VALIDATION DESIGN

The test design will consist of soils fortified with the test substances at three concentrations. The three concentrations will span the range of test concentrations to be used in studies involving the test substance. The control matrix for the validation will be selected from the matrices that will be used in the studies and will provide the worst case scenario from the point of view of interferences. The validation study levels (approximate concentrations) for test substance can be found below:

High Concentration	0.1 mg (a.i.)/kg
Low Concentration	0.01 mg (a.i.)/kg
Matrix Blank – Control Matrix	0.0 mg (a.i.)/kg

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