INTRODUCTION

Wildlife International performed an independent laboratory validation (ILV) of an analytical method for the determination of Methiocarb, and its metabolites Methiocarb sulfoxide and Methiocarb sulfone in soil. The protocol for this study titled "Independent Laboratory Validation of Methods for the Determination of Methiocarb and its metabolites Methiocarb Sulfoxide and Methiocarb Sulfone in Soil by LC/MS/MS" is presented in Appendix I. The analytical method, "Development and Validation of a Method for the Determination of Methiocarb, Methiocarb Sulfoxide, and Methiocarb Sulfone" is presented in Appendix II.

This study was performed to satisfy regulatory requirements for independent laboratory validation of methods as set forth by the U.S. Environmental Protection Agency Series 850 - Residue Chemistry Test Guidelines, OCSPP 850.6100, *Enironmental Chemistry Methods and Associated Independent Laboratory Validation* (1) and U.S. Environmental Protection Agency, 1996. Pesticide Regulation (PR) Notice 96-1: Notice to Manufacturers, Formulators, Producers and Registrants of Pesticides Products, *Tolerance Enforcement Methods - Independent Laboratory Validation By Petitioner* (2). The study was performed at the Wildlife International analytical chemistry facility in Easton, Maryland. The experimental portion of the study was conducted between October 13 and 22, 2014. Raw data and a copy of the final report are archived at the Wildlife International site under project number 334C-126.

PURPOSE

This study was conducted to fulfill EPA requirements set forth in guideline OCSPP 850.6100 and PR Notice 96-1. This study provides validation data demonstrating that an independent researcher could reproduce the results of the analytical method with minimal contact with the method developers.

EXPERIMENTAL DESIGN

Soil was fortified with Methiocarb, and its metabolites Methiocarb sulfoxide and Methiocarb sulfone, at two concentrations and analyzed according to the methods supplied by the Sponsor. The lower concentration was 1.00 µg/kg, the method LOQ. The higher concentration was ten-fold the LOQ, i.e., 10.0 µg/kg. Matrix blanks (controls) were analyzed concurrently to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Soil - Origin

Control soil matrix was obtained and characterized from Agvise laboratories, in Northwood, ND. The soil was identified as RMN-PF and was collected from the 0-6" horizon from a site in Grand Forks, ND. The soil was received at Wildlife International on January 13, 2014, logged in and stored

under refrigerated conditions at the testing facility upon receipt. A copy of the soil characterization report is presented in Appendix III.

Analytical Reference Substances

A reference substance of Methiocarb was received from Chem Service, Inc. on October 02, 2014 and was assigned the Wildlife International Identification number 11917. The material was a solid and was identified on the label as Methiocarb; Lot# 2848000; Purity 99.5%; CAS Number 2032-65-7; Expiration Date 04/30/2020. This reference substance was stored under ambient conditions. A certificate of analysis is presented in Appendix IV.

A reference substance of Methiocarb sulfoxide was received from Sigma-Aldrich on October 02, 2014 and was assigned the Wildlife International Identification number 11916. The material was a solid and was identified on the label as Methiocarb sulfoxide; Lot# SZBD225XV; Purity 99.8%; CAS Number 2635-10-1; Expiration Date 08/13/2018. This reference substance was stored under refrigerated conditions. A certificate of analysis is presented in Appendix IV.

A reference substance of Methiocarb sulfone was received from Chem. Service on October 02, 2014 and was assigned the Wildlife International Identification number 11918. The material was a solid and was identified on the label as Methiocarb sulfone; Lot# 2978900; Purity 99.5%; CAS Number 2179-25-1; Expiration Date 04/30/2019. This reference substance was stored under ambient conditions. A certificate of analysis is presented in Appendix IV.

All three reference substances above were used to prepare separate primary analytical stocks and subsequent combined (1:1:1) fortification standards and calibration standards (both non-matrix matched for trial #1 and matrix-matched for trial #2).

Preparation of Primary Analytical Stocks and Secondary Combined Fortification Stocks and Calibration Standards

Separate primary stock solutions of each reference standard of Methiocarb, Methiocarb sulfoxide, and methiocarb sulfone were prepared in acetonitrile at a concentration of 0.500 mg/mL (active ingredient/mL) by compensating for the purity of each analyte. Combined secondary fortification stocks were then prepared at 100, 10.0, and 1.00 µg/mL in methanol dilution solvent as shown below:

Stock Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Standard Conc. (µg/mL)
500 (Methiocarb)	5.00	25.0	100
500 (Sulfoxide)	5.00		
500 (Sulfone)	5.00	--	--
100 (Combined)	1.00	10.0	10.0
10.0 (Combined)	1.00	10.0	1.00

All solutions were prepared using volumetric flasks, pipettes, and gas-tight syringes and were stored under frozen conditions when not in use.

Combined working calibration standards (Methiocarb, Methiocarb sulfoxide, Methiocarb sulfone) ranging in concentration from 2.00 to 100 ng/mL were prepared in methanol dilution solvent from the 1.00 µg/mL combined secondary fortification stock as shown below:

Combined Secondary Fortification Stock Concentration (µg/mL)	Aliquot (µL)	Dilution Solvent Volume (µL)	Combined Calibration Standard (ng/mL)
1.00	2.00	998	2.00
1.00	5.00	995	5.00
1.00	10.0	990	10.0
1.00	25.0	975	25.0
1.00	50.0	950	50.0
1.00	75.0	925	75.0
1.00	100	900	100

Calibration standard solutions were prepared freshly upon analysis directly in auto-sampler vials and were not stored other than on LC/MS/MS system while being analyzed due to suspected stability of analytes in solution. The first trial was performed using solvent (methanol) standards and was unsuccessful due to suspected matrix suppression effects. The second trial was performed using matrix-matched calibration standards in methanol to eliminate matrix suppression effects observed during the first trial and was successful.

Fortification of Recovery Samples

Fortified soil samples were prepared by fortification with the 1.00 and 10.0 µg/mL combined secondary fortification stocks of the analytes. Subsamples were fortified at the LOQ (1.00 µg/kg) and 10x the LOQ (10.0 µg/kg). All fortified samples were prepared with fortification solutions that were prepared compensating for the purity of the reference/test materials. Therefore, residue fortification and recovery levels, expressed in µg/kg, are equivalent to the expression as µg active ingredient/L (µg a.i./kg).

Extraction and Analysis of Methiocarb, Methiocarb Sulfoxide, and Methiocarb Sulfone from Soil

For analysis, subsamples of control soil were weighed into twelve individually 125-mL plastic bottles, five of which were fortified at a LOQ of 1.00 µg/kg and five at 10.0 µg/kg (10x the LOQ) with combined secondary fortification stocks of the reference substances prepared above and shown

below. The remaining two samples were not fortified and served as blanks. All samples were subsequently analyzed by methodology presented in the provided method presented in Appendix II. Slight deviations in the LC/MS/MS source optimization parameters were utilized and were considered to be equivalent values related to inherent differences in instrumental performance and not a limitation of the methodology. Since details of the method are presented in the Appendix, only a general description is provided here.

Fortification/Processing Scheme:

Nominal Conc. (µg/kg)	Fortification Volume (mL)	Sample Mass (g)	Combined Stock Conc. (µg/mL)
1.00	0.0250	25.0	1.00
10.0	0.0250	25.0	10.0

Forty (40 mL) of acetone extraction solvent and 10 mL of water was added to each 125-mL bottle. The bottles were placed on a gyratory shaker table at a setting of ~ 300 rpm for ~ 30 minutes. Following shaking, the extracts were filtered through a Whatman #4 filter contained in a fritted funnel connected to a 125-mL round-bottomed flask. The filter cakes and bottles were rinsed with 10 mL of acetone and collected in same 125-mL flasks. The filtrates were transferred to a 250-mL separatory funnel. The 125-mL flasks were rinsed with 50 mL of dichloromethane: hexane solvent (1:1, v/v) with sonication and transferred to the separatory funnels containing the corresponding filtrate. The funnels were shaken vigorously by hand for approximately two minutes and the layers allowed to separate. The lower aqueous layer was drained back into the 125-mL collection flask and the upper organic solvent layer was collected into a 250-mL concentration flask. The aqueous layer was returned to the separatory funnel and the previous extraction was repeated, combining the final organic layer in the same 250-mL concentration flask. The combined extracts were rotary-evaporated to dryness at ~ 30°C and the residues reconstituted with 3 mL of ethyl acetate: hexane (1:4, v/v) solution with the aid of sonication. An appropriate number of SPE cartridges (20-mL/5g FL, Agilent Mega-Bond Elut) were then conditioned with ~ 10 mL of 1:4 solution from above. Each reconstituted sample was loaded onto the prepared SPE cartridges, and the eluate collected into a clean 100-mL concentration flask. The 250-mL flasks and subsequently the SPE cartridges were rinsed with 30 mL of 1:4 solution and a final flask and SPE cartridge rinse was performed using 10 mL of acetone, all collected into the same 100-mL flasks. The final extracts were rotary-evaporated to dryness at ~ 30°C and reconstituted in 2.50 mL of methanol. Each final extract was also sonicated well to ensure adequate dissolving of residues, followed by transfer to auto-sampler vials and submission for LC/MS/MS analysis.

Quantitation of Methiocarb, Methiocarb sulfoxide, and Methiocarb sulfone by LC/MS/MS

An Agilent Technologies Model 1260 High Performance Liquid Chromatograph connected to an AB Sciex Triple Quad 5500 Mass Spectrometric Detector (LC/MS/MS) was used to analyze samples. An acidified (0.05M formic acid) methanol: water gradient was used.

Quantitation was performed using the responses of the primary ion transitions for each analyte. Confirmation ion transitions were also monitored for each analyte. The ion transitions monitored are summarized below:

Transition	Methiocarb	Sulfoxide	Sulfone
Quantitation	226→169 amu	242→185 amu	258→107 amu
Confirmation	226→121 amu	242→170 amu	258→201 amu

Specific details of the LC/MS/MS instrumentation and operational parameters are presented in Table 1.

Example Calculations

For each analyte, a regression equation was derived from the chromatographic peak area responses of the analytes determined in calibration standard solutions versus the respective nominal concentrations of the standards. Standard curves were generated by plotting this function with analyte concentration (ng/mL) ratio on the abscissa and the respective analyte peak area response on the ordinate. The applied regression was weighted 1/x with respect to concentration and expressed as a linear regression as follows:

$$y = mx + b$$

Where:
Y = peak area
m = slope
b = Y-intercept
x = analyte concentration

Concentrations of analytes in the samples (quantitation and confirmation methods) were determined by substituting peak area responses of the samples into the re-arranged regression equation as follows:

$$\text{Analyte Concentration} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}}$$

Using the data from the soil method validation sample 334C-126-SVMAS-11, 1.00 µg/kg shown below, the analytical result and percent recovery was calculated as follows using the software algorithms of Analyst version 1.6.2 of the AB Sciex Triple Quad 5500 mass spectrometer system in full precision mode. Note: manual calculations shown here may differ slightly than reported.

Where: Peak area = 174820
Y-intercept = -4744.98
Slope = 20359.1

The concentration of Methiocarb at instrument was determined by substituting the resulting analyte peak area response into the above equation. Using the values above, the concentration in the final sample solution was calculated as:

$$\text{Concentration at instrument (ng/mL): } = \frac{174820 - (-4744.98)}{20359.1}$$

Concentration at instrument (ng/mL): = 8.820

The residue concentration (µg/kg) for Methiocarb in the fortified soil recovery sample was determined as the product of the at instrument solution concentration determined above and the dilution factor and units of conversion factor (CF) for the sample as follows:

$$\text{Concentration in µg/kg} = \text{Methiocarb Concentration} \times \frac{\text{(Final Volume)}}{\text{(Initial mass)}} \times \text{CF}$$

Where: Initial Mass= 25.0 g
Final Volume = 2.50 mL
CF (ng/g to µg/kg) = 1µg/1000ng x 1000g /1 kg = µg/kg

Using the nominal concentration (ng/mL) from above, the concentration of methiocarb in soil sample was calculated as follows:

$$\text{Concentration in sample (µg /kg)} = 8.820 \times \frac{(2.50)}{(25.0)} \times \frac{1\ \mu g}{1000\ ng} \times \frac{1000\ g}{1\ kg}$$

Concentration in sample (µg /kg) = 0.8820

The percent recovery was determined by dividing the concentration of the analyte recovered in the fortified sample by the nominal concentration added as shown below:

$$\text{Recovery (\%)} = \frac{\mu g/kg \text{ Found}}{\mu g/kg \text{ Added}} \times 100$$

For the above 1.00 µg /kg fortified sample, the percent recovery of Methiocarb was calculated as:

$$\text{Recovery (\%)} = \frac{0.8820 \ \mu g \text{ /kg Found}}{1.00 \ \mu g \text{ /kg Added}} \times 100$$

$$\text{Recovery (\%)} = 88.2\%$$

The same calculation procedure was applied for the quantitation and confirmation of Methiocarb sulfoxide, and Methiocarb sulfone metabolites for this study as well.

Statistical Treatment of Data

Mean recoveries for each analyte for each fortification level were calculated by dividing the sum of the percent recoveries by the total number of fortified samples. The standard deviation and relative standard deviation (coefficient of variation) for the recoveries for each analyte were also determined and reported.

Table 1. LC/MS/MS Instrumentation and Operational Parameters.

Instrumentation	Agilent Technolgies 1260 High Performance Liquid Chromatograph with a AB Sciex Triple QUAD 5500 Mass Spectrometric Detector (LC/MS/MS) and Turbo-V Ion Spray Source
Analytical Column	Agilent ZORBAX SB-CN (75mm x 4.6 mm, 3.5-µm particle size)
Guard Column	NONE
Mobile Phases	A2: 0.05% Formic Acid in HPLC-grade water B2: 0.05% Formic Acid in Methanol Gradient Elution Program: Time (min) / %A2 / %B2 / Flow Rate (µL/min) / Temp (°C) 0.00 / 80.0 / 20.0 / 500 / 40.0 0.50 / 80.0 / 20.0 / 500 / 40.0 4.00 / 10.0 / 90.0 / 500 / 40.0 6.50 / 10.0 / 90.0 / 500 / 40.0 7.00 / 80.0 / 20.0 / 500 / 40.0 12.5 / 80.0 / 20.0 / 500 / 40.0
Diverter Valve (Valco)	Time (min) Not Used.
Injection Volume	10 µL
Total Run Time	12.5 minutes
Period 1	Scan Type/Polarity: MRM/Positive GS1 = 90, GS2 = 40.0, CUR = 30.0, CAD = 9, IS = 5500, TEM = 300, DP = 50, EP = 10,
Methiocarb	Quantitation: (226/169 amu), CE = 14, CXP = 14 Confirmation: (226/121 amu), CE = 27, CXP = 10 Retention Time: Approximately 6.9 minutes
Methiocarb Sulfoxide	Quantitation: (242/185 amu), CE = 21, CXP = 9.8 Confirmation: (242/170 amu), CE = 32, CXP = 15 Retention Time: Approximately 5.8 minutes
Methiocarb Sulfone	Quantitation: (258/107 amu), CE = 54, CXP = 9.0 Confirmation: (258/201 amu), CE = 12.5, CXP = 11 Retention Time: Approximately 6.1 minutes