Raw data and a copy of the final report are archived at the Wildlife International site under project number 334C-121.

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

Surface water 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 0.100 µg/L, the method LOQ. The higher concentration was ten-fold the LOQ, i.e., 1.00 µg/L. Matrix blanks (controls) were analyzed concurrently to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Surface Water - Origin

Control matrix was obtained from a local source, Tuckahoe Lake located in Tuckahoe State Park, Ridgely, MD. The water was collected on September 19, 2014 and was logged in and stored under refrigerated conditions at the testing facility upon receipt. A summary of surface water characterization results 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 (non-matrix matched for quantitation purposes during this study).

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, 1.00 and 0.100 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
10.0 (Combined)	0.100	10.0	0.100*

*Note: the 0.100 µg/mL combined standard was used as the high-level calibration standard in the scheme below.

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 0.100 µg/mL combined secondary fortification stock as shown below:

Combined Secondary Fortification Stock Concentration (µg/mL)	Aliquot (mL)	Dilution Solvent Volume (mL)	Combined Calibration (ng/mL)
0.100	0.0200	0.980	2.00
0.100	0.0500	0.950	5.00
0.100	0.100	0.900	10.0
0.100	0.250	0.750	25.0
0.100	0.500	0.500	50.0
0.100	0.750	0.250	75.0

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 suspected stability of analytes in solution. Upon the initial analysis of the validation set, the 100 ng/mL calibration

standard was inadvertently not included in the analysis scheme due to oversight by the analyst in interpretation of the provided method. The following day this oversight was discovered and the analytical set was re-analyzed including the 100 ng/mL calibration standard to bracket high-level validation sample concentrations.

Fortification of Recovery Samples

Fortified surface water samples were prepared by fortification with the 0.100 and 1.00 µg/mL combined secondary fortification stocks of the analytes. Subsamples were fortified at the LOQ (0.100 µg/L) and 10x the LOQ (1.00 µg/L). 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/L, are equivalent to the expression as µg active ingredient/L (µg a.i./L).

Extraction and Analysis of Methiocarb, Methiocarb Sulfoxide, and Methiocarb Sulfone from Surface Water

For analysis, subsamples of control surface water were measured into twelve individually labeled 250-mL separatory funnels, five of which were fortified at an LOQ of 0.100 µg/L and five at 1.00 µg/L (10x the LOQ) with combined secondary fortification stocks of the reference substances prepared above. 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.

One hundred (100) mL volumes of control surface water were measured into 250-mL separatory funnels and fortified as shown below:

Nominal Conc. (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Combined Stock Conc. (µg/mL)
0.100	0.100	100	0.100
1.00	0.100	100	1.00

Fifty (50 mL) of dichloromethane (DCM) extraction solvent and 1 gram of sodium chloride was added to each separatory funnel. Each funnel was shaken vigorously for 2 minutes and the phases allowed to separate. The lower organic layer (DCM) was collect into 250-mL concentration flask. A second 50 mL volume of ethyl acetate (EtoAc) extraction solvent was added to the aqueous layer in the funnel and the mixture was shaken as above. After phases separation the lower aqueous layer was drained into a suitable container, and the upper organic layer (EtoAc) was combined into the same 250-mL concentration flask. The extraction was repeated a third time with an additional 50 mL of EtoAc, discarding the lower aqueous layer and combining the upper organic layer into the same 250-mL concentration flask. The combined extracts were evaporated by rotary-evaporation at ~30°C to a volume of approximately 5 mL, and following transfer to 15-mL tubes, were reduced to near dryness using nitrogen evaporator and then to dryness manually using nitrogen. The final residue was

reconstituted in 1.00 mL volumes of methanol solvent. Each final extract was 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 Technolgies 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 analysis was performed using the responses of the secondary confirmation ion transitions for each analyte. The ion transitions monitored are summarized below:

Transition	Methiocarb	Sulfoxide	Sulfone
Primary(Quantitation)	226→169 amu	242→185 amu	258→107 amu
Secondary (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 water method validation sample 334C-121-WVMAS-1, 0.100 µg/L 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 = 426070
Y-intercept = 3627.37
Slope = 46865.9

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:

$$\text{Concentration at instrument (ng/mL): } = \frac{426070 - (3627.37)}{46865.9}$$

Concentration at instrument (ng/mL): = 9.0139

The residue concentration (µg/L) for Methiocarb in the fortified water 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:

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

Where: Initial Volume= 100 mL
Final Volume = 1.00 mL
CF (ng/mL to µg/L) = 1µg/1000ng x 1000mL /1 L = µg/L

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

$$\text{Concentration in sample (µg /L)} = 9.0139 \times 0.0100 \times \frac{1\ \mu g}{1000\ ng} \times \frac{1000\ mL}{1\ L}$$

Concentration in sample (µg /L) = 0.090139

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:

$$\text{Recovery (\%)} = \frac{\text{µg/L Found}}{\text{µg/L Added}} \times 100$$

For the above 0.100- µg /L fortified sample, the percent recovery of Methiocarb was calculated as:

$$\text{Recovery (\%)} = \frac{0.090139\ \mu g\ /L\ \text{Found}}{0.100\ \mu g\ /L\ \text{Added}} \times 100$$

$$\text{Recovery (\%)} = 90.1\%$$

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 Technologies Model 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