SUMMARY

A residue method for quantitation of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone in water test system was validated.

The test substance containing a mixture of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone (1:1:1) was analyzed using external standardization by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS). Water samples were fortified with 0.1 ppb (LOQ) and 1 ppb (10X LOQ) of each compound. The limit of detection was defined as approximately 20% LOQ using the current methodology.

The experiment for water matrix was conducted with two reagent blank, two untreated controls and five control samples spiked for each fortification level: one at LOQ level and another at 10X LOQ level. Target compounds were extracted from water samples, followed by liquid partition and solvent exchange. The final concentrated extract was reconstituted with methanol and analyzed by LC-MS/MS.

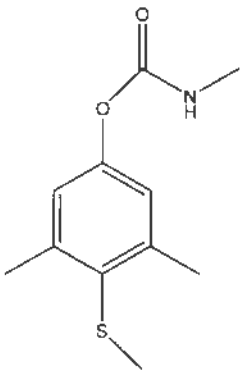
Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone contents were quantitated against separate 1/x weighted linear curves of the reference substances Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone whose concentrations ranged from 2 ng/mL to 100 ng/mL for each compound. The calibration for each compound yielded acceptable linearity (correlation coefficient $r > 0.9995$) over the range examined. The quantitation of each compound was based on the peak area response and concentration of the calibration standards. The amount of Methiocarb was determined with the quantitation MS/MS ion transition from m/z 226 to m/z 169 and the confirmation MS/MS ion transition from m/z 226 to m/z 121. The amount of Methiocarb sulfoxide was determined with the quantitation MS/MS ion transition from m/z 242 to m/z 185 and the confirmation MS/MS ion transition from m/z 242 to m/z 170. The amount of Methiocarb sulfone was determined with the quantitation MS/MS ion transition from m/z 258 to m/z 107 and the confirmation MS/MS ion transition from m/z 258 to m/z 202. Recoveries from fortified samples were determined by calculating the found concentration of individual compound and averaging by the relevant fortification level.

The LOD in water matrix is estimated to be 0.02 ppb of each compound using either MS/MS transition.

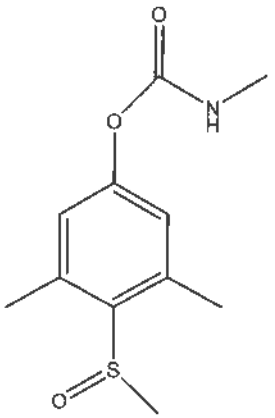
MATERIAL AND METHODS

Test and Reference Substances

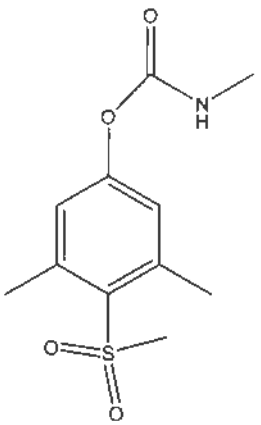
Name:	Methiocarb
Supplier:	Ricerca Biosciences, LLC
Lot No.:	436200075
IUPAC name:	3,5-Dimethyl-4-(methylthio)phenyl methylcarbamate
CAS No.:	2032-65-7
Molecular formula:	$C_{11}H_{15}NO_2S$
Molecular weight:	225.3 grams/mole
Purity:	98.97%
Expiration Date:	November 2014
Structure:	



Name:	Methiocarb sulfoxide
Supplier:	Sigma-Aldrich
Lot No.:	SZBC248XV
IUPAC name:	3,5-Dimethyl-4-(methylsulfinyl)phenyl methylcarbamate
CAS No.:	2635-10-1
Molecular formula:	$C_{11}H_{15}NO_3S$
Molecular weight:	241.3 grams/mole
Purity:	99.5%
Expiration Date:	September 4, 2017
Structure:	



Name:	Methiocarb sulfone
Supplier:	ChemService
Lot No.:	2352100
IUPAC name:	3,5-Dimethyl-4-(methylsulfonyl)phenyl methylcarbamate
CAS No.:	2179-25-1
Molecular formula:	$C_{11}H_{15}NO_4S$
Molecular weight:	257.3 grams/mole
Purity:	98.7%
Expiration Date:	June 30, 2015
Structure:	



The reference and test substances were stored in freezer when not in use for long-term. Certificates of Analysis for the reference and test substances are provided in Appendix B.

Other Chemicals

HPLC grade water, methanol, hexanes, dichloromethane and ethyl acetate were obtained from Burdick & Jackson; formic acid, sodium sulfate and sodium chloride were obtained from Fisher Scientific.

Equipment List

Laboratory Balances
Thermometers
Pasteur pipettes
Beakers
Glass funnels (6 cm diameter)
Graduated glass cylinders
Hamilton glass precision syringes
Volumetric flasks
Pipetmen with plastic disposable tips
Separatory funnels (250 mL capacity)
Glass conical tubes (15 mL capacity)
Amber bottles and vials with Teflon® lined caps
Vortex mixer
Büchi rotavapor with water bath
Turbovap® LV nitrogen evaporator

AB Sciex API 4000 Series Triple Quad Mass Spectrometer with Thermo Scientific Dionex Ultimate 3000 Liquid Chromatograph (LC-MS/MS)

Test System

Source of Test System

Water test system is a natural surface water collected in Richmond, CA. The water sample (Inventory No. 2465W-004) was stored refrigerated (typically < 4°C) in the dark when not in use.

Characterization of the Test System

The water (Inventory No. 2465W-004) used in the study was characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). The characterization reports and methods of characterization are presented in Appendix C.

Test Method

The analytical method for the analysis of three compounds validated at PTRL West by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) was based on the analytical method developed in this study.

The water samples were spiked with known concentrations of three compounds (Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone (1:1:1)). Compounds were extracted from samples, followed by liquid partition and solvent exchange. The final concentrated extract was reconstituted with methanol and analyzed by LC-MS/MS. The percent recovery was determined using external standardization where linear curves for calibration standards were analyzed along with the samples.

Preparation of Methiocarb Stock Solution

A stock solution of the Methiocarb reference substance was prepared by weighing an aliquot (26.14 mg) of the reference substance (Inventory No. 2465W-001) onto glass boat and then into a 50 mL volumetric flask. The stock solution was dissolved and diluted with 51.7 mL acetonitrile to yield a nominal concentration of 0.500 mg/mL. The concentration of stock solution was corrected for the purity of the reference substance (98.97%). The stock solution was transferred into an amber bottle and stored in the freezer (typically < -10°C) when not in use.

Preparation of Methiocarb sulfoxide Stock Solution

A stock solution of the Methiocarb sulfoxide reference substance was prepared by weighing an aliquot (25.44 mg) of the reference substance (Inventory No. 2465W-002A) onto glass boat and then into a 50 mL volumetric flask. The stock solution was dissolved and diluted with 50.6 mL acetonitrile to yield a nominal concentration of 0.500 mg/mL. The concentration of stock solution was corrected for the purity of the reference

substance (99.5%). The stock solution was transferred into an amber bottle and stored in the freezer (typically < -10°C) when not in use.

Preparation of Methiocarb sulfone Stock Solution

A stock solution of the Methiocarb sulfone reference substance was prepared by weighing an aliquot (25.62 mg) of the reference substance (Inventory No. 2465W-003A) onto glass boat and then into a 50 mL volumetric flask. The stock solution was dissolved and diluted with 50.6 mL acetonitrile to yield a nominal concentration of 0.500 mg/mL. The concentration of stock solution was corrected for the purity of the reference substance (98.7%). The stock solution was transferred into an amber bottle and stored in the freezer (typically < -10°C) when not in use.

Preparation of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone Mixed Stock and Fortification Solutions

The 100 µg/mL mixed stock solution was prepared by measuring 5 mL of each stock solution into 25 mL volumetric flask. Final solution was diluted to the mark with methanol. This mixed stock solution contains 100 µg/mL of each compound.

The 10 µg/mL mixed stock solution was prepared by measuring 1 mL of 100 µg/mL mixed stock solution into 10 mL volumetric flask. Final solution was diluted to the mark with methanol. This mixed stock solution contains 10 µg/mL of each compound.

The 1 µg/mL mixed fortification solution was prepared by measuring 1 mL of 10 µg/mL mixed stock solution into 10 mL volumetric flask. Final solution was diluted to the mark with methanol. This mixed fortification solution contains 1 µg/mL of each compound.

The 100 ng/mL mixed fortification/calibration solution was prepared by measuring 0.1 mL of 10 µg/mL mixed stock solution into 10 mL volumetric flask. Final solution was diluted to the mark with methanol. This mixed fortification/calibration solution contains 100 ng/mL of each compound.

The mixed stock and fortification solutions were vortexed to mix, transferred into amber bottle and stored in the freezer (typically < -10°C) when not in use.

Preparation of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone Mixed Standard Solutions

Due to the instability of Methiocarb sulfone, it is recommended that the calibration solutions be freshly prepared for the analysis. Six additional calibration standard solutions were prepared by mixing an appropriate volume of 100 ng/mL mixed fortification/calibration solution via pipetman with an appropriate volume of methanol. Final calibrants were transferred into autosampler vials and vortexed to mix, for LC-MS/MS analysis. The standard solutions ranged from 2 ng/mL to 75 ng/mL can be prepared as shown below:

Theoretical Conc. (ng/mL) Each compound	Volume of 100 ng/mL Solution (µL)	Volume of Methanol (µL)	Final Volume (mL)
75	750	250	1
50	500	500	1
25	250	750	1
10	100	900	1
5	50	950	1
2	20	980	1

Fortification Procedure

Fortification of untreated water samples was conducted at two fortification levels as shown below:

Fortification Level (ppb or µg/L)	Each compound Solution
0.1	0.100 mL of 100 ng/mL in 100 mL of water
1.0	0.100 mL of 1 µg/mL in 100 mL of water

Fortification was conducted to determine the percent recovery within the method validation. This procedure was performed in quintuplicate during method validation at each fortification level for each matrix.

Extraction Method for Methiocarb, Methiocarb Sulfoxide and Methiocarb Sulfone in Water

1. Using a graduated cylinder, aliquot 100 mL of water sample into 250mL separatory funnel.
2. Fortify the sample as necessary.
3. Add 50 mL of dichloromethane and 1 g of sodium chloride to the separatory funnel containing the water.
4. Shake funnel vigorously for 2 min.
5. Allow the phases to separate for about 5 min.
6. Collect the lower dichloromethane layer a into 250 mL concentration flask.
7. Add 50mL ethyl acetate to the separatory funnel containing the aqueous.
8. Shake funnel vigorously for 2 min. Allow the phases to separate for 5 min. Drain the lower aqueous phase into a suitable container.
9. Combine the upper ethyl acetate layer into the same 250 mL concentration flask.
10. Return the aqueous to the separatory funnel. Rinse the container with 50mL ethyl acetate and then add to the separatory funnel. Repeat Step 8, discarding the lower aqueous layer.
11. Rinse the separatory funnel with 5 mL of dichloromethane; drain and combine in the same 250 mL concentration flask.
12. Roto-evaporate combined extract to ~5 mL at 30°C. Transfer to a 15mL disposable glass tube; rinse the flask with 5 mL of ethyl acetate and combine into the same 15mL disposable glass tube.
13. Turbo-evaporate (N_2) to a small volume, ~0.2 mL, at 30°C. Manually evaporate to dryness with nitrogen.
14. Reconstitute with 1.0 mL of methanol. Sonicate well. Aliquot to autosampler vial for analysis by LC-MS/MS. Due to the instability of Methiocarb sulfone, it is recommended that the sample be analyzed on the same day as it is prepared.

A schematic diagram of the water extraction method is presented in Figure 1.

Liquid Chromatography with Tandem Mass Spectrometry Analytical Method (LC-MS/MS)

LC conditions

Column: Agilent Zorbax® SB-CN, 3.5µm, 4.6mm X 75mm (S/N USMC004586)
Injection volume: 10 µL
Autosampler tray temperature: 8 °C
Flow rate: 0.5 mL/min
Run time: 12.5 minutes
Mobile Phase:

- *A: 0.05% Formic acid in HPLC grade water*
- *B: 0.05% Formic acid in HPLC grade Methanol*

Gradient Program:

Time (minutes)	%A	%B	Flow rate (mL/min)
0.0	80	20	0.5
0.5	80	20	0.5
4.0	10	90	0.5
6.5	10	90	0.5
7.0	80	20	0.5
12.5	80	20	0.5

MS conditions

APCI Positive mode

APCI Parameters

Collision Gas (CAD)	9
Curtain Gas (CUR)	40
Gas 1 (GS1)	90
Gas 2 (GS2)	40
Temperature (TEM)	300
Declustering Potention (DP)	50
Exit Potential (EP)	10
MS1 resolution	unit
MS2 resolution	low

MRM Parameters

Compound name	Precursor ion	Product ion	Collision Energy (CE)	Cell Exit Potential (CXP)	Dwell (msec)
Methiocarb	226	169	14	14	100
Methiocarb	226	121	27	10	100
Methiocarb sulfoxide	242	185	21	9.8	100
Methiocarb sulfoxide	242	170	32	15	100
Methiocarb sulfone	258	107	54	9	100
Methiocarb sulfone	258	202	12.5	11	100

LC-MS/MS Analysis

Samples were analyzed interspersed between the calibrants so as to assess the response of the calibrants if they had been affected by matrix samples (signal suppression or enhancement). Since separate linear curves were prepared for each compound, samples were interspersed between each calibration standards. Calibrants and samples were analyzed in single injection. Due to the instability of Methiocarb Sulfone, the analysis should be done as soon as the samples are prepared.

Methods of Calculation

Quantitation

Separation of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone was achieved by LC-MS/MS. The compounds were identified by the coincidence of their retention times with their respective reference standards and MS characteristics. The quantitation of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone was conducted by peak area of each compound relative to the theoretical concentration of the calibrants. The content of Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone in samples was quantitated against separate 1/x weighted linear curves ($y = mx + b$) of *Methiocarb, Methiocarb sulfoxide and Methiocarb sulfone* calibrants where:

y = peak area
x = ng/mL compound injected
m = slope
b = intercept

Weighting of the calibration curve of each compound was applied so as to provide better curve fit at the lower concentration levels of each compound. The calculation of weighted curve equations (linear regression) and concentration (ng/mL) present in samples and calibrants was conducted using Analyst® software.

Recoveries from fortified samples were determined by averaging the found concentration of both compounds and dividing by the relevant fortification level.

Transcriptions (spreadsheets) of the raw data to support calculations for this study are presented in Appendix D.

Calibration Range

The calibration curve was generated by Analyst® software range from 2 ng/mL to 100 ng/mL of each compound for water validation.

Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.1 ppb for each in water which represented 10 ng/mL of each compound in calibration standard solution as validated in this study.

Limit of Detection

The limit of detection (LOD) was defined as approximately 20% of LOQ which represented 2 ng/mL of each compound in calibration standard solution. So the LOD for water is estimated to be 0.02 ppb of each compound.

Time Required for Completion of a Sample Set

A sample set can be divided into two subsample sets for efficient handling. A subsample set consisted of a reagent blank, one controls (untreated water samples) and five fortified water samples (at one level i.e. LOQ). Time required for one subsample set from initiation of extraction until the completion of instrumental analysis and data evaluation is as follows:

- Sample preparation takes approximately 6 hours

- LC-MS/MS analysis and data processing (one or two MS/MS transitions for each compound) take approximately 6 hours

TOTAL = approximately 12 hours for one analyst to complete a subsample set (approximately one a half calendar days) or 24 hours (3 calendar days) to complete two subsample sets to satisfy the validation requirements.

Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression were the only statistical methods employed in this study.

Figure 1. Schematic Diagram of the Analytical Method-Water.