2.0 INTRODUCTION

The purpose of this study was to develop and validate an extraction method for the analysis of propargite in drinking and surface water at the Limit of Quantitation (LOQ) 0.01 µg/L and (10X LOQ) 0.1 µg/L. Samples are partitioned by hexane, concentrated to dryness, reconstituted in acetonitrile and analyzed by LC-MS/MS in the positive ionization mode monitoring the parent-daughter ion transitions (MRMs) 368.1 m/z → 231.6 m/z (quantitation) and 368.1 m/z → 81.4 m/z (confirmation).

This study was performed in compliance with EPA Good Laboratory Practice Standards (40 CFR Part 160). The study initiation date was February 25, 2016. The experimental start date was April 4, 2016 (standard preparation) and experiments were terminated on May 17, 2016 (end of analytical work). The protocol used for this study is provided in Appendix A.

3.0 MATERIALS AND METHODS

3.1 Reference Substance and Reference Substance Stability

The propargite reference substances was provided by Arysta LifeScience Canada Inc. Stock solutions in acetonitrile were prepared at concentrations of 1 mg/mL, and were found to be stable over the period of the study. The stability of calibration solutions were established by comparing calibration solutions made fresh from a separately prepared freezer stored stock solution with calibration solutions prepared at the beginning of the experimental phase. The calibration solutions had been stored under refrigeration conditions for at least 21 days. The percent difference of the calculated concentrations for each individual calibrant was < 20% with an average percent difference of 11% across the entire calibration curve, indicating stability. The certificate of analysis is provided in Appendix B.

Compound: **Propargite**
IUPAC Name: 2-(4-tert-butylphenoxy)cyclohexyl prop-2-ynyl sulfite
CAS No.: 2312-35-8
Purity: 93.5%
Molecular Weight: 350.47 g/mole
Lot Number: 2757-25-RRG
Date Received: March 28, 2016
Expiration Date: October 31, 2017
Storage Conditions: Refrigerate

3.2 Test Systems

The test systems were obtained by PTRL West and characterized by Agvise Laboratories. The bulk water samples were stored under refrigeration conditions when not in use. (Inventory Nos. 2706W-055 Drinking water, and 2706W-049 Surface water). The water characterization reports are presented in Appendix C.

3.3 Method

The method for the analysis of propargite in drinking and surface water was developed by PTRL West, and derived from "Development and Validation of an Analytical Method for the Determination of Propargite in Reagent Water, Raw Surface Water and Finished Drinking Water", Wood, Brenda J., November 13, 2003. Reference 1.

The determination of propargite in the above matrices was validated by spiking known concentrations into control matrix samples. A complete description of the analytical method is provided in Appendix D.

3.4 Preparation of Stock Standard and Fortification Solutions

Duplicate stock standard solutions were prepared in acetonitrile on April 4, 2016 at a concentration of 1 mg/mL. Approximately 10 mg of standard (adjusted for purity) was transferred to a 10 mL volumetric flask and diluted to volume with acetonitrile. From the stock standard solutions, serial dilutions were made in volumetric flasks to generate fortification solutions on April 4, 2016. The 1 µg/mL intermediate fortification solutions were prepared by removing a 0.1 mL aliquot of the 1 mg/mL stock standard solutions and

diluting to 100 mL with acetonitrile. The 100 ng/mL fortification solutions were prepared by removing a 1 mL aliquot of the 1 µg/mL fortification solution and diluting to 10 mL with acetonitrile. The 10 ng/mL fortification solutions were prepared by removing a 1 mL aliquot from the 100 ng/mL fortification solutions and diluting to 10 mL with acetonitrile. Stock standard solutions were transferred to glass amber vials, labeled with concentration and designated as "Stock A" or "Stock B", and placed in the freezer for storage. The fortification solutions were transferred to glass amber vials, labeled with concentration and designated as "A" or "B", and placed in the freezer for storage. Details of recommended methods of preparation are provided in Appendix D.

3.5 Preparation of Calibration Solutions

The 1 mg/mL stock "A" solution was used to prepare seven solvent-based calibrants ranging from 0.04 ng/mL to 5 ng/mL, these calibrants were used to confirm linearity and determine sample concentrations over the course of the study. The calibration solutions were prepared on April 13, 2016 by diluting the 10 and 100 ng/mL "A" fortification solutions into a 10 mL volumetric flask. The calibration solutions were diluted to volume with a acetonitrile, transferred to 12 mL amber glass containers and stored in the freezer when not in use. Additionally, in order to indicate stability of calibrants over the course of the study, the 1 mg/mL "B" stock solution was removed from the freezer and diluted to generate a stock "B" calibration curve on May 2, 2016. Details of recommended methods of preparation are provided in Appendix D.

3.6 Fortification Procedure

100 mL aliquots of control water (drinking or surface) were placed in 250 mL separatory funnels and fortified with the appropriate fortification solution to achieve fortification levels of 0.01 µg/L, and 0.1 µg/L. For details on the fortification procedure see Appendix D.

3.7 Extraction Procedure

Aliquots of drinking and surface water (100 mL) were partitioned with 25 mL of hexane (3x). The samples were shaken by hand and allowed to settle for approximately 10 minutes between each partition. The partition of the organic solvent and the aqueous

phase was induced by sodium chloride. After settling the aqueous layer was drained into a beaker and the hexane layer was transferred to a 125 mL concentration flask by being passed through a filter funnel containing glass wool and 5 g of sodium sulfate. The aqueous layer was then transferred back into the separatory funnel and the partition was repeated two additional times, combining each partition in the concentration flask. The combined partitions were then evaporated to near dryness under increased pressure (230 mbar) in a water bath set at 40°C. The remaining partition was then placed under a stream of nitrogen and concentrated to dryness. The samples were then reconstituted to 10 mL with acetonitrile, using sonication and mixing. Approximately 500 µL of the final sample was transferred to a 0.45 µm microfilterfuge tube and centrifuged, the supernatant was then transferred to an autosampler vial for LC/MS-MS analysis. The remaining sample extact was transferred to a glass storage vial and placed under freezer conditions for storage. A schematic of the extraction procedure is presented in Figure 1 with details provided in Appendix D.

3.8 Calibration/Sample Analysis

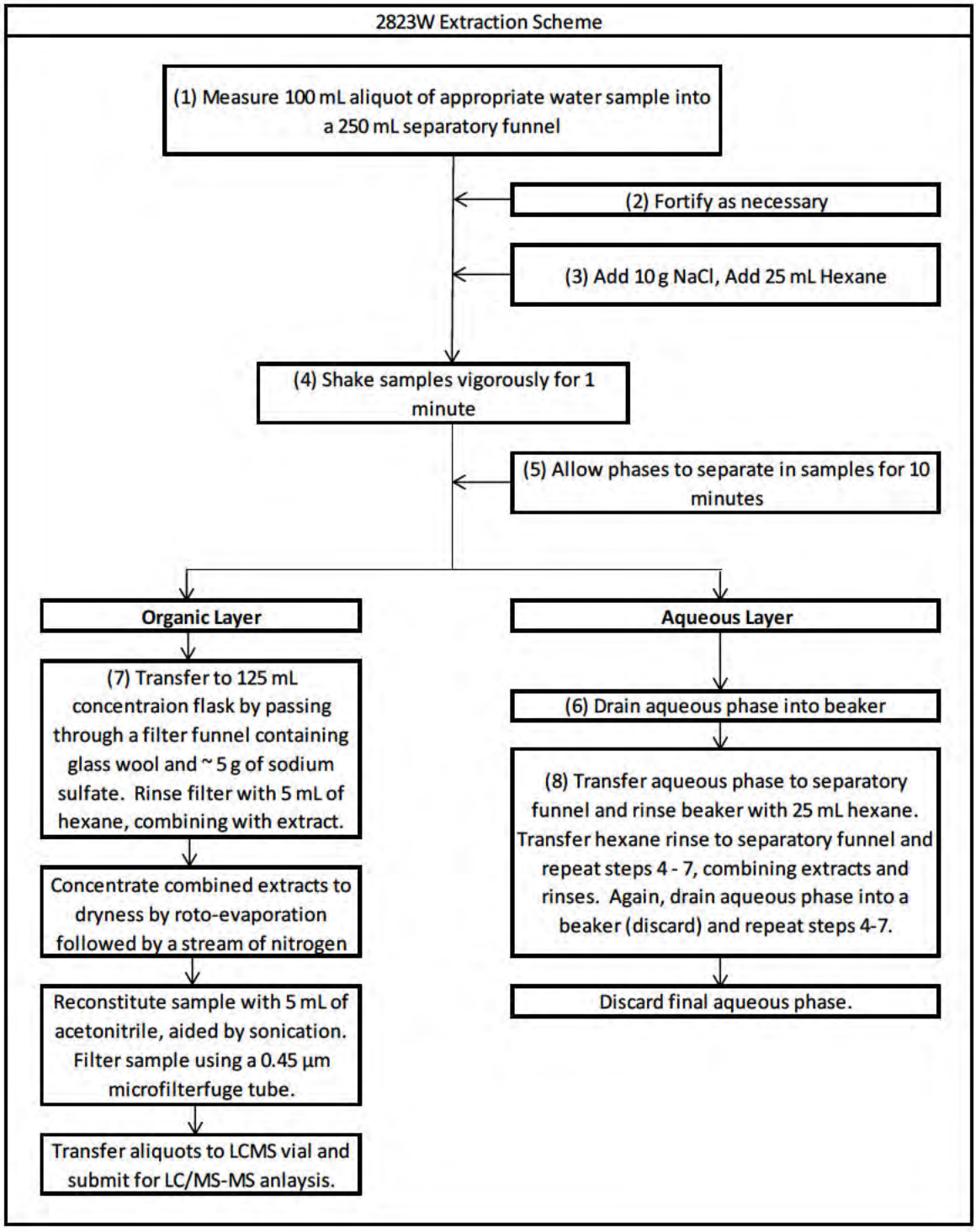
Separation of propargite was achieved by HPLC using a Phenomenex Gemini, 3 µ C18 110Å (50 x 2.0 mm) column and a gradient mobile phase system using water and methanol each with 10 mm ammonium acetate. A tandem mass spectrometer was used with electrospray ionization (ESI) in positive polarity mode and Multiple Reaction Monitoring. Two ion transitions were monitored for propargite. The analyte was identified by the coincidence of its retention time with the reference standard and MS characteristics, and each ion transition was quantitated separately by integration of peak areas. Calibrants were interspersed with samples and ranged in nominal concentrations from 0.04 to 5 ng /mL. Linearity curves calculated from least squares regression with 1/x weighting were used to quantify each ion transition. Details of the analysis method is provided in Appendix D. Representative calibration curves and LC-MS/MS chromatograms for propargite calibrants and samples are presented in Figure 2 through Figure 18.

3.9 Statistical Analysis

The method validation data included the following statistical calculations: averages, standard deviations, relative standard deviations, percent variance and linear least squares

regression analyses with 1/x weighting factor (linearity curves for this study were generated using Applied Biosystems/MDS SCIEX Instruments with Analyst Software).

Figure 1. Schematic of Extraction Procedure



Propargite Analytical Method as Described in
"Development and Validation of a Method for the Determination of Propargite in Surface and Drinking Water"
Dan Keenan, PTRL Study No. 2823W

The method described herein is used for the determination of propargite in surface and drinking water matrices. The method for these matrices was validated under this study.

LIMITS OF DETECTION AND QUANTITATION

The limit of quantitation (LOQ) is defined as the lowest concentration validated for each matrix. The LOQ for drinking and surface water is 0.01 µg/L. Using the current methodology this is equivalent to an LOQ of 0.4 ng/mL for both drinking and surface water. The limit of detection (LOD) was determined to be 0.002 µg/L for drinking water and 0.003 µg/L for surface water. Data for the LOD determination is presented in Table VIII.

MATERIALS AND METHODS

Equipment

Balances, capable of measuring grams to two and at least four decimal places.

250 mL separatory funnels

125 mL concentration flasks

Graduated cylinder, various sizes

Glass Beakers, various sizes

Pipetman, various sizes

Microfilterfuge tubes, 0.45µm Nylon

Pasteur pipettes, various sizes

Vials, amber (various sizes) with Teflon®-lined cap

Volumetric flask, various sizes

Reagents and Standards

HPLC Grade from Fisher Scientific or VWR (unless otherwise specified)

Acetonitrile

Hexane

Sodium Chloride

Sodium Sulfate

CO_2 (dry ice)

Water

Standard Reference Substances

The propargite reference substance was provided by Arysta LifeScience Canada Inc. Stock solutions of the reference substance was prepared at 1 mg/mL in acetonitrile, and was determined to be stable over the duration of this study. The certificate of analysis is provided in Appendix B of this report.

Compound: **Propargite**

IUPAC name: 2-(4-tert-butylphenoxy)cyclohexyl prop-2-ynyl sulfite

Molecular Formula: $C_{19}H_{26}O_4S$

Molecular Weight: 350.47 grams/mole

PTRL West Number: 2823W-001

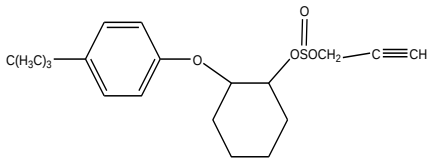
Lot Number: 2757-25-RRG

Purity: 93.5 (±0.67)

Expiration: 10-31-17

Storage: Refrigerate

Structure:



Sample Preparation

The test systems were obtained by PTRL West and characterized by Agvise Laboratories. The bulk water samples were stored under refrigeration conditions when not in use. (Inventory Nos. 2706W-055 Drinking water and 2706W-049 Surface water).

Preparation of Stock Standards

Duplicate stock standard solutions were prepared in acetonitrile at a concentration of 1 mg/mL. Approximately 10 mg of standard (adjusted for purity) was transferred to a 10 mL volumetric flask and diluted to volume with acetonitrile. From the stock standard solutions, serial dilutions were made in volumetric flasks to generate fortification solutions. The 100 µg/mL intermediate fortification solutions were prepared by removing a 1 mL aliquot of the 1 mg/mL stock standard solutions and diluting to 100 mL with acetonitrile. The 10 µg/mL fortification solutions were prepared by removing a 1 mL aliquot of the 100 µg/mL fortification solution and diluting to 10 mL with acetonitrile. The 1 µg/mL fortification solutions were prepared by removing a 100 µL aliquot from the 100 µg/mL fortification solutions and diluting to 10 mL with acetonitrile. Stock standard solutions were transferred to glass amber vials, labeled with concentration and designated as "Stock A" or "Stock B", and placed in the freezer for storage. The fortification solutions were transferred to glass amber vials, labeled with concentration and designated as "A" or "B", and placed in the freezer for storage. Details of recommended methods of preparation are provided below.

Stock Solutions:

PTRL West No.	Stock ID	Standard Name	Weight (mg)	Final Volume (mL)	Purity (%)	Concentration (mg/mL)
2823W-001	Stock A	Propargite	10.86	10.154	93.5	1.0
2823W-001	Stock B	Propargite	11.23	10.5	93.5	1.0

Intermediate Fortification Solutions:

Concentration of Standard Solution Used	Volume Taken	Final Volume	Final Concentration
1.0 mg/mL Stock A	0.1 mL	100 mL	1.0 µg/mL
1.0 mg/mL Stock B	0.1 mL	100 mL	1.0 µg/mL

Fortification Solutions:

Concentration of Standard Solution Used	Volume Taken	Final Volume	Final Concentration
1.0 µg/mL A	1.0 mL	10 mL	100 ng/mL
100 ng/mL A	1.0 mL	10 mL	10 ng/mL

Fortification Check Solutions:

Concentration of Standard Solution Used	Volume Taken	Final Volume	Final Concentration
1.0 µg/mL B	1.0 mL	10 mL	100 ng/mL
100 ng/mL B	1.0 mL	10 mL	10 ng/mL
10 ng/mL B	1.0 mL	10 mL	1.0 ng/mL

Preparation of Calibration Solutions

The 100 ng/mL and 10 ng/mL fortification solutions were used to prepare six solvent-based calibrations standards ranging from 0.1 ng/mL to 5 ng/mL, the 0.1 ng/mL calibration standard was used to prepared a 0.04 ng/mL calibration solution. These calibrants, ranging from 0.04 ng/mL to 5 ng/mL were used to confirm linearity and determine sample concentrations over the course of the study. The calibration solutions were prepared from the stock "A" fortification solutions and diluted into separate 10 mL volumetric flask. The calibration solutions were diluted to volume with acetonitrile, transferred to 12 mL amber glass containers and stored in the freezer when not in use. Details of recommended methods of preparation are provided below.

Calibration Standards:

Concentration of Standard Solution Used	Volume Taken	Final Volume	Final Concentration
100 ng/mL A	0.5 mL	10 mL	5 ng/mL (Cal 7)
100 ng/mL A	0.2 mL	10 mL	2 ng/mL (Cal 6)
100 ng/mL A	0.1 mL	10 mL	1 ng/mL (Cal 5)
10 ng/mL A	0.5 mL	10 mL	0.5 ng/mL (Cal 4)
10 ng/mL A	0.2 mL	10 mL	0.2 ng/mL (Cal 3)
10 ng/mL A	0.1 mL	10 mL	0.1 ng/mL (Cal 2)
2 ng/mL Calibrant	0.2 mL	10 mL	0.04 ng/mL (Cal 1)

Fortification Procedure

Measure a 100 mL aliquot of the water specimen into a 250 mL separatory funnel and fortify with the appropriate fortification solutions just prior to extraction as follows:

Matrix	Aliquot (mL)	Fortification Level	Fortification Solution	Volume of Fort. Solution added
Drinking/Suface	10.0 g	0.01 mg/kg	1.0 µg/mL	100 µL

Water		0.1 mg/kg	10.0 µg/mL	100 µL

Extraction Method(s)

A schematic of the extraction procedure presented at the end of this appendix.

1. Measure 100 mL aliquots of the water specimen into 250-mL separatory funnel.
2. Fortify as needed (LOQ is 0.01 µg/L).
3. Add 10g of NaCl then 25mL of hexane to each separatory funnel. Shake as vigorously as possible for 1 minute. Allow separatory funnels to sit for 10 minutes.
4. Drain the aqueous layer into a beaker; do not discard.
5. Transfer the hexane extract to a 125-mL concentration flask, passing solvent through a filter funnel containing glass wool and approximately 5g of sodium sulfate. Rinse filter with 5 mL of hexane.

   Note: Do not allow any water to transfer to the concentration flask.
6. Pour aqueous layer back into the separatory funnel. Rinse the beaker with 25 mL of hexane, and add the rinse to the separatory funnel.
7. Shake as vigorously as possible for 1 minute. Allow separatory funnels to sit for 10 minutes.
8. Repeat Steps 6-7 once more, passing solvent through the same filter funnel each time. Discard the aqueous layer.

Concentration:

9. Roto-vap extracts to near dryness, setting the water bath to 40°C, and pressure to 230 mbar.
10. Use a gentle nitrogen stream to bring extract to dryness.

Reconstitution:

11. Reconstitute the extract with 10 mL of ACN, thoroughly sonicating the concentration flask for 1 minute.

12. Transfer extract to a glass vial for storage as final extract. Aliquot approximately 500 µL of extract to a 0.45 µm microfilterfuge, and centrifuge. Aliquot filtered final extract to an autosampler vial for analysis.

LC-MS/MS CHROMATOGRAPHY

The following LC-MS/MS method is used to determine propargite residues in samples of drinking and surface water.

LC Components and Parameters:

Pump: Agilent 1260 Series Binary Pump or equivalent

Autosampler: Agilent 1260 series or equivalent

Micro-Degasser: Agilent 1100 series, model G1379A or equivalent

Column Compartment: Agilent 1100 series, model G1316A or equivalent

Column: Phenomenex Gemini 3µ C18 110Å, (50 mm x 2.0 mm)

Column Temperature: 55°C

Injection Volume: 12 µL

Mobile Phase System: A) 10 mM Ammonium Acetate in HPLC Grade water
B) 10 mM Ammonium Acetate in HPLC Grade methanol

Time (min.)	Flow Rate (µL/min.)	% A	% B
0.00	200	70	30
1.0	200	5	95
5.0	200	5	95
5.1	200	70	30
8.0	200	70	30

Retention time: propargite: ~ 5.0 minutes

Mass Spectrometry Components and Parameters:

Electrospray Ionization Mass Spectrometry

An Applied Biosystems MDS/SCIEX API 4000 tandem mass spectrometer is used with electrospray ionization (ESI) in negative positive mode to acquire data by Multiple

Reaction Monitoring (MRM). The instrument management and data collection software for the LC-MS/MS systems is Analyst® version 1.4.2 (Applied Biosystems/MDS SCIEX Instruments) or the equivalent.

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential	Collision Energy	Collision Cell Exit Potential
Propargite	368.1	231.6	250	45	15	13
	368.1	81.4	250	45	47	6

The optimized settings are as follows:

Nebulizer Temperature (TEM):	300°C
Nebulizer Gas (GS1):	40
Ion spray (GS2):	40
Curtain Gas (CUR):	30
Negative Ion Spray Voltage (IS):	1250
Entrance Potential	8
Collision Activated Dissociation Gas (CAD):	12

A diverter valve is set to waste from time zero to 4.5 minutes, to mass spec. from 4.5 minutes to 5.9 minutes then to waste for the remainder of the run.

Separation of the analyte is achieved by HPLC. The analyte is identified by the coincidence of its retention time with the reference standard and MS characteristics, and each ion transition is quantitated separately by integration of peak areas and comparison to a linear least squares regression curve with 1/x weighting. Calibrants are interspersed with samples. A repeat injection of a calibrant solution is made at the end of a sample set to establish peak area response stability.

Limit of Detection and Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration validated for each matrix. The LOQ for drinking and surface water is 0.01 µg/L. Using the current methodology this is equivalent to an LOQ of 0.4 ng/mL for both drinking and surface water. The limit of detection (LOD) was determined to be 0.002 µg/L for drinking water and 0.003 µg/L for surface water. Data for the LOD determination is presented in Table VIII.

Time Required For Analysis

A sample set consisting of 12 samples can be extracted and readied for analysis by one chemist in 6 hours. Analysis by LC-MS/MS requires approximately 10 hours of continuous operation (unattended). Data processing requires one chemist 2 hours.

Statistical Methods

The method validation data included the following statistical calculations: averages, standard deviations, relative standard deviations, percent variance and linear least squares regression analyses with 1/x weighting factor (linearity curves for this study were generated using Applied Biosystems/MDS SCIEX Instruments with Analyst Software).

METHODS OF CALCULATION

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
P = Chemical purity of neat standard
FC = Final Concentration (mg/mL)

Recoveries

Two ion transitions were monitored for propargite. Each ion transition is used to determine individual ion recovery data. Recovery data from fortified samples is calculated as follows:

A linear regression from solvent based calibrant solution responses is determined:

Linear regression formula from calibration curve $y = mx + b$

Where the x values are represented by the concentrations (ng/mL) of the calibrants, and the y values are represented by the corresponding peak area counts.

Note: the calibration curve includes a 1/x weighting factor, curves are determined by Applied Biosystems/MDS SCIEX Instruments Analyst Software version 1.4.2 or the equivalent.

The calculated concentration in the final extract is determined as follows:

$$\text{ng/mL analyte} = \frac{y - b}{m}$$

where y = Sample peak area
b = Calibration intercept
m = Slope

The residue of the analyte in the sample is determined as follows:

$$\text{Residue (µg/L)} = \frac{\text{ng/mL analyte x Final Extract Volume (mL) x Dil. Factor}}{\text{1,000 ng/µg x Initial Volume (L)}}$$

where µg/L = or ppb and

Initial Volume = 0.1 L (volume of water sample)

The Percent Recovery of a fortified sample is determined as follows:

$$\frac{\text{Residue (}ug\text{/L) - Average Residue of Controls (}ug\text{/L)}}{\text{Fortification Level (}ug\text{/L)}} \text{ x 100}$$

An example calculation for the recovery of propargite (m/z 368.1/231.6 ion transition) in drinking water fortified at 0.01 µg/L (sample designated F1A) follows:

Linear regression equation: y = 2.87E +4 x + -3.06E +2 (r = 0.9999)

The calculated concentration in F1A final extract:

$$\text{ng propargite/mL} = \frac{5.79E+3+3.06E+2}{2.87E+4} = 0.212 \text{ ng/mL}$$

where 5.79E +3 is the peak area for F1A propargite (m/z 368.1/231.6)

The propargite residue (µg/L) for F1A =

$$\frac{0.212\ \text{ng/mL} \times 5\ \text{mL (Final Volume)} \times 1\ \text{(Dil. Factor)}}{1{,}000\ \text{ng/µg} \times 0.1\ \text{L (Initial Volume)}}$$

= 0.0106 µg/L

The percent recovery of fortified sample FIA (propargite):

{[0.0106 µg/L – 0.000 µg/L (avg. control residue)] ÷ 0.01 µg/L (fort. level)}x 100%

= 106%

Note: Due to rounding, hand calculated values may differ from reported results.