INTRODUCTION

EAG Laboratories-Easton performed an independent laboratory validation (ILV) of a method for the determination of Propargite and its metabolite *tert*-butylphenoxycyclohexanol (TBPC). The protocol for this study titled "Independent Laboratory Validation of Methods for the Determination of Propargite and its Metabolite TBPC in Soil by GC/MS" is presented in Appendix I.

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 – Ecological Effects Test Guidelines, OCSPP 850.6100, *Environmental Chemistry Methods and Associated Independent Laboratory Validation* (1) and 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 EAG Laboratories analytical chemistry facility in Easton, Maryland. The experimental portion of the study was conducted between March 23 and April 1, 2017. Raw data and a copy of the final report are archived at the EAG Laboratories-Easton site under project number 443C-127.

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 both Propargite and TBPC at two different concentrations and analyzed according to a method supplied by the Sponsor (3). The limit of quantitation (LOQ) for Propargite and TBPC was set at 0.0100 mg a.i./kg. The higher concentration was ten-fold the LOQ, i.e., 0.100 mg a.i./kg, respectively. The limit of detection (LOD) for Propargite and TBPC was set at 0.00500 mg a.i./kg. Reagent and matrix blanks (controls) were prepared and analyzed concurrently with the fortified samples to evaluate potential analytical interferences.

MATERIALS AND METHODS

Test Substance (Analytical Standard)

Propargite, CAS # 2312-35-8, was received from the Sponsor on February 14, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13555. The material was a liquid and was identified as Propargite Standard on the label and Propargite on the certificate of analysis; Lot# 2757-25-RRG; Purity 93.5%; Expiration Date 31 October, 2017. This substance was stored under refrigerated conditions. The certificate of analysis is presented in Appendix III.

Metabolite Test Substance (Analytical Standard)

TBPC (chemical name: cyclohexanol, 2-[4-(1,1-dimethylethyl)phenoxy]-), a metabolite of Propargite, was received from the Sponsor on February 14, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13554. The material was a solid and was identified on the label and certificate of analysis as 2-(4-tert-butylphenoxy)cyclohexanol; Lot# 2761-87-RRG; Purity 99%; Expiration Date 24

February, 2021. This reference substance was stored frozen. The certificate of analysis is presented in Appendix III.

Reference Substance for Propargite (Internal Standard)

An internal standard for Propargite, O-63, was received from the Sponsor on February 28, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13597. The material was a liquid and was identified on the label and certificate of analysis as O-63; Lot# 3336-127-3; Purity 99.6%; Expiration Date January 31, 2020. This substance was stored frozen. The Certificate of Analysis is presented in Appendix III.

Reference Substance for TBPC (Internal Standard)

An internal standard for TBPC, O-66, was received from the Sponsor on February 28, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13596. The material was a liquid and was identified on the label and certificate of analysis as O-66; Lot# 3336-125-5; Purity 99.0%; Expiration Date January 31, 2020. This substance was stored frozen. The certificate of Analysis is presented in Appendix III.

Test System

The test system, loamy sand soil, ID: PD-SOIL-PF-0-6" collected in North Dakota, was received from Agvise Laboratories, Northwood, North Dakota on February 24, 2017 and was stored refrigerated upon receipt. The soil was characterized by AGVISE (Appendix II).

Preparation of Stock Solutions and Calibration Standards

A primary stock solution of Propargite standard was prepared by weighing 0.05350 g of the test substance on an analytical balance. The material was transferred to a 50-mL volumetric flask and brought to volume using acetone to yield a final nominal stock concentration of 1.00 mg a.i./mL, when adjusted for purity. The primary stock solution (1.00 mg a.i./mL) was diluted in acetone to prepare 0.0100 and 0.00100 mg a.i./mL stock solutions as shown below:

Primary Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (mL)	Secondary Stock Concentration (mg a.i./mL)
1.00	0.500	50.0	0.0100
0.0100	5.00	50.0	0.00100

A primary stock solution of TBPC standard was prepared by weighing 0.0505 g of the test substance on an analytical balance. The material was transferred to a 50-mL volumetric flask and brought to volume using acetone to yield a final nominal stock concentration of 1.00 mg a.i./mL, when adjusted for purity. The primary stock solution (1.00 mg a.i./mL) was diluted in acetone to prepare 0.0100 and 0.00100 mg a.i./mL stock solutions as shown below:

Primary Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (mL)	Secondary Stock Concentration (mg a.i./mL)
1.00	0.500	50.0	0.0100
0.0100	5.00	50.0	0.00100

A primary stock solution of Propargite internal standard (O-63) was prepared by weighing 0.04998 g of the test substance on an analytical balance. The material was transferred to a 50-mL volumetric flask and brought to volume using hexane to yield a final nominal stock concentration of 1.00 mg/mL. The primary stock solution (1.00 mg/mL) was diluted in hexane to prepare 0.0100 and 0.00100 mg/mL stock solutions as shown below:

Stock Conc. (mg/mL)	Aliquot (mL)	Final Volume (mL)	Fortification Stock Concentration (mg/mL)
1.00	0.500	50.0	0.0100
0.0100	5.00	50.0	0.00100

A primary stock solution of TBPC internal standard (O-66) was prepared by weighing 0.05000 g of the test substance on an analytical balance. The material was transferred to a 50-mL volumetric flask and brought to volume using hexane to yield a final nominal stock concentration of 1.00 mg/mL. The primary stock solution (1.00 mg/mL) was diluted in hexane to prepare 0.0100 and 0.00100 mg/mL stock solutions as shown below:

Stock Conc. (mg/mL)	Aliquot (mL)	Final Volume (mL)	Fortification Stock Concentration (mg/mL)
1.00	0.500	50.0	0.0100
0.0100	5.00	50.0	0.00100

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

Calibration standards ranging in concentration from 0.0500 to 0.200 mg a.i./L for Propargite and TBPC were prepared in hexane from the secondary stocks above. To each calibration standard, 200 µL of the 0.0100 mg/mL Propargite internal standard stock and 100 µL of the 0.0100 mg/mL TBPC internal standard stock were added prior to dilution to volume. The calibration standards preparation is as shown below:

Propargite Stock		TBPC Stock			
Concentration (mg a.i./mL)	Aliquot (µL)	Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.0100	50.0	0.0100	50.0	10.0	0.0500
0.0100	70.0	0.0100	70.0	10.0	0.0700
0.0100	100	0.0100	100	10.0	0.100
0.0100	150	0.0100	150	10.0	0.150
0.0100	200	0.0100	200	10.0	0.200

Limit of Quantitation (LOQ)

The limit of quantification (LOQ) for both Propargite and TBPC was set at 0.0100 mg a.i./kg, defined as the lowest fortification concentration at which acceptable recovery data were obtained.

Limit of Detection (LOD)

The limit of detection (LOD) for both Propargite and TBPC was set at 0.00500 mg a.i./kg, defined as the product of the lowest calibration standard concentration (0.0500 mg a.i./L) and the dilution factor of the matrix blank sample (0.100).

Fortification of Samples

For each of the matrix validations, two unfortified matrix blanks, five samples at the LOQ, and five samples at 10X the LOQ were prepared in soil as shown below:

Propargite Fortification Table

Nominal Concentration (mg a.i./kg)	Propargite Fortification Volume (µL)	Propargite Fortification Stock Concentration (mg a.i./mL)	TBPC Fortification Volume (µL)	TBPC Fortification Stock Concentration (mg a.i./mL)	Sample Quantity (g)
0.0100 (LOQ)	100	0.00100	100	0.00100	10.0
0.100 (10XLOQ)	100	0.0100	100	0.0100	10.0

Analytical Method

For analysis, 10.0 g of blank soil were weighed into 50 mL centrifuge tubes and fortified using gas-tight syringes or equivalent. Within a time period of 5 minutes, 25 mL of acetonitrile was added to each sample. The tubes were then capped and agitated on a vortex mixer for about 30 seconds, followed by 10 minutes in a sonicator bath. The tubes were centrifuged at 4415 RCF (Relative Centrifugal Force) for 10 minutes. The supernatants were transferred to 50-mL centrifuge tubes. This process was repeated and the supernatant from the second extraction was combined with the first.

The tubes containing acetonitrile extracts were placed into the N-EVAP evaporator with the water bath set at about 50°C. The supernatant was reduced to about 5 mL volume, transferred to 15-mL conical glass centrifuge tubes and then reduced again to about 1 mL. Then, 5 mL of 10% NaCl solution was added to the extract. To this, 5.00 mL of hexane was added to each tube. The tubes were mixed with vortex action for about 30 seconds and centrifuged at 218 RCF for 2 minutes. Using a glass pipette, 4 mL of the upper (hexane) phase was transferred into clean centrifuge tubes, and then another 5.00 mL of hexane was added to tubes containing the original extracts. The tubes were vortexed for 30 seconds and centrifuged at 218 RCF for about 2 minutes. Using a glass pipette, 5 mL of the upper (hexane) phase was transferred into the centrifuge tubes containing the first extract.

The requisite volume of internal standard stocks was added to each low level fortification sample, which were capped and mixed well. The matrix blank and low level fortification samples were placed into the N-EVAP evaporator with the water bath set at 50°C. The combined hexane extract volume was reduced to 1 mL.

The requisite volume of internal standard stocks was added to each high level fortification sample and brought to volume using hexane. These were capped and mixed well.

An aliquot of each standard and sample was transferred to autosampler vials and analyzed by GC/MS. The analytical method outline is presented in Table 1.

Quantitation of Propargite by GC/MS

An Agilent Model 7890 Gas Chromatograph connected to an Agilent Model 5975 Mass Selective Detector was used to analyze samples.

Quantitation was performed using the response of the primary ion for propargite and TBPC. The ions monitored are summarized below:

Analyte	Primary
Propargite	350.00 amu
TBPC	248.00 amu

Specific details of the GC/MS instrumentation and operational parameters are presented in Table 2.

Example Calculations

For each analyte, a regression equation was derived from the chromatographic peak area ratios of the analyte over internal standard determined in calibration standard solutions versus the respective nominal concentrations of the analyte in standards. Standard curves were generated by plotting this function with analyte concentration (mg a.i./L) on the abscissa and the respective analyte peak area ratios on the ordinate.

The applied regression was 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 analyses) 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 Propargite soil method validation sample 443C-127-MAS-1, 0.0100 mg a.i./kg shown below, the analytical result and percent recovery were calculated as follows using the software algorithms of Excel 2010 in full precision mode. Manual calculations using values shown here may differ slightly.

Where: Peak area ratio = 0.386
Y-intercept = -0.0098
Slope = 3.9193

The concentration of Propargite 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:

$$\text{Concentration at instrument (mg a.i./L): } = \frac{0.386 - (-0.0098)}{3.9193}$$

Concentration at instrument (mg a.i./L): = 0.101

The concentration (mg a.i./kg) for Propargite in the soil sample was determined as the product of the instrument solution concentration determined above and the overall dilution factor as follows:

$$\text{Concentration in mg a.i./kg} = \text{Propargite Concentration at Instrument} \times \frac{\text{(Final Volume)}}{\text{(Initial Mass)}}$$

$$= 0.101 \text{ mg a.i./L} \times \frac{(0.00100 \text{ L})}{(0.0100 \text{ kg})}$$

$$= 0.0101$$

Where: Initial Mass = 10.0 g = 0.01 kg
Final Volume = 1.00 mL = 0.001 L

The same calculation procedure was applied for the analysis of TBPC for this study as well.

Statistical Treatment of Data

Mean recoveries for Propargite and TBPC 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 were also determined and reported.

Table 1. Analytical Method Outline for the Analysis of Propargite and its Metabolite TBPC in Soil

OUTLINE FOR THE ANALYSIS OF PROPARGITE AND ITS METABOLITE TBPC IN SOIL

1. Prepare calibration standards in hexane using appropriate stock solutions of the test substance using gas-tight syringes and volumetric flasks or equivalent.
2. Weigh 10.0 g of blank soil sample into 50-mL centrifuge tubes and fortify samples using gas-tight syringes or equivalent as appropriate. Set aside the fortified samples for no more than five minutes before processing with the extraction.
3. Add 25 mL of acetonitrile to each sample using a graduated cylinder.
4. Cap the tubes and vigorously agitate the samples on a vortex mixer for about 30 seconds and place into a sonicator bath for about 10 minutes.
5. Place the tubes into a centrifuge and spin at 4415 RCF for about 10 minutes.
6. Decant the supernatant into clean 50-mL centrifuge tubes.
7. Repeat steps 3-5.
8. Combine the supernatant from the second extraction with the first.
9. Place the tubes into the "N-EVAP" evaporator with the water bath set at about 50 °C. Reduce the supernatant to about 5 mL volume.
10. Transfer the ACN extract to 15-mL conical glass centrifuge tubes. Rinse the 50-mL tubes with about 0.5 mL of ACN. Place the 15-mL conical glass centrifuge tubes to the "N-EVAP" with the water bath set at about 50 °C. Under a stream of nitrogen, reduce the supernatant volume to about 1 mL.
11. Add about 5 mL of 10% NaCl solution to the extract using a pipettor.
12. Add 5.00 mL of hexane using a volumetric pipette to each tube and vortex the tubes for about 30 seconds. Centrifuge tubes at 218 RCF for about 2 minutes.
13. Using a disposable glass pipette, transfer **4** mL of the upper (hexane) phase into clean 15-mL conical glass centrifuge tubes.
14. Repeat step 12.
15. Using a disposable glass pipette, transfer **5** mL of the upper (hexane) phase into the15-mL conical glass centrifuge tubes containing the first extract.

Table 1. Analytical Method Outline for the Analysis of Propargite and its Metabolite TBPC in Soil (continued)

OUTLINE FOR THE ANALYSIS OF PROPARGITE AND ITS METABOLITE TBPC IN SOIL

16. For the low level fortification samples, add the requisite volume of internal standard stocks to each sample using a gas-tight syringe or equivalent. Cap and mix well. For matrix blank and low level fortification samples, uncap and place tubes into the "N-EVAP" evaporator with the water bath set at about 50 °C. Reduce the combined hexane extract volume to 1 mL.

17. For the high level fortification samples, add the requisite volume of internal standard stocks to each sample using a gas-tight syringe or equivalent. Add hexane to bring the volume to the 10-mL mark. Cap and mix well.

18. Transfer an aliquot of each standard and sample to autosampler vials and submit for analysis by GC/MS.