INTRODUCTION

EAG Laboratories performed an independent laboratory validation (ILV) of a method for the determination of residues of Triallate and TCPSA in ground and surface water. The protocol for this study titled "Independent Laboratory Validation of a Method for the Determination of Triallate and TCPSA in Aqueous Matrices by LC/MS/MS" is presented in Appendix I. The Sponsor provided analytical method report entitled; "Validation of the Analytical Method for the Determination of Triallate and TCPSA in Aqueous Matrices by LC/MS/MS" for independent validation 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 860 - Residue Chemistry Test Guidelines, OCSPP 850.6100, *Environmental 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 EAG Laboratories analytical chemistry facility in Easton, Maryland. The experimental portion of the study was conducted between April 11 and June 14, 2017. Raw data and a copy of the final report are archived at the EAG Laboratories-Easton site under project number 334C-134.

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

Ground and surface water were fortified with Triallate and TCPSA at two concentrations and analyzed according to the method supplied by the Sponsor. The LOQ for each analyte was set at 0.100 µg/L. The higher concentration was ten-fold the LOQ, i.e., 1.00 µg/L. Reagent and matrix blanks (controls) were analyzed concurrently to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Ground and Surface Water - Origin

Ground (well) water control matrix used for this study was obtained locally from EAG Laboratories aquatic testing facility in Easton, MD. The water was stored under refrigerated conditions in the dark following collection and when not in use. The EAG Laboratories ground well water was characterized internally and the mean results for the 4 week period immediately preceding its use is summarized to Appendix IV.

Surface water control matrix was obtained from a local source, Tuckahoe Lake located in Ridgely, MD. The water was collected on March 29, 2016 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 V.

Analytical Reference Substances

A reference substance Triallate was received from Smithers on July 25, 2016 and was assigned the EAG Laboratories-Easton Identification Number 13191. The material was a liquid and was identified on the label as Tri-allate; Lot# 5125900; Purity 99.5%; Expiration Date 06/17/2018. This reference substance was stored under ambient conditions. A certificate of analysis is presented in Appendix III.

A reference substance of TCPSA was received from Gowan on March 29, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13701. The material was a solid and was identified on the label as TCPSA; Lot# SP15-106-1-1; Purity 99.8%; Expiration Date 06/16/2018. This reference substance was stored under ambient conditions. A certificate of analysis is presented in Appendix III.

The reference substances above were used to prepare separate primary analytical stocks and subsequently various combined secondary fortification/calibration stocks and standards.

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

A primary stock solution of the Triallate reference standard was prepared by weighing a 50.3 mg aliquot into a beaker. The reference material was transferred to a 50-mL volumetric flask, dissolved and adjusted to final volume using acetonitrile to yield a final nominal stock concentration of 1000 µg/mL (corrected for purity). This Triallate primary stock was diluted in acetonitrile to prepare an intermediate stock at 100 µg/mL.

A primary stock solution of TCPSA reference standard was prepared by weighing a 10.02 mg aliquot onto a piece of weigh paper. The reference material was transferred to a 100-mL volumetric flask, dissolved and adjusted to final volume using acetonitrile: HPLC grade water (1:1, v/v) to yield a final nominal stock concentration of 100 µg/mL (corrected for purity).

Combined secondary fortification stocks of Triallate and TCPSA analytes were prepared at 10.0, 0.100, 0.0100, and 0.00100 µg/mL in acetonitrile solvent from the above 100 µg/mL stocks as shown below:

Stock Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Standard Conc. (µg/mL)
100 (Triallate)	1.00	10.0	10.0
100 (TCPSA)	1.00	--	--
10.0 (combined)	0.100	10.0	0.100
0.100 (combined)	1.00	10.0	0.0100
0.0100 (combined)	1.00	10.0	0.00100

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

For the analysis of Triallate (both quantitation and confirmation analyses – Xbridge C18 Column) and TCPSA (quantitation analysis only – T3 column) combined working calibration standards ranging in concentration from 0.00500 to 0.500 µg/L were prepared in acetonitrile: HPLC grade water (1:1, v/v) from the combined secondary fortification stocks above as shown below:

Combined Secondary Fortification Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Calibration STD Conc. (µg/L)
0.0100	0.500	10.0	0.500
0.0100	0.250	10.0	0.250
0.0100	0.100	10.0	0.100
0.0100	0.0500	10.0	0.0500
0.0100	0.0250	10.0	0.0250
0.00100	0.100	10.0	0.0100
0.00100	0.0500	10.0	0.00500

For the analysis of TCPSA in ground water and the initial trial in surface water (confirmation analysis only – HILIC column) combined working calibration standards ranging in concentration from 0.00500 to 0.500 µg/L for were prepared in acetonitrile: HPLC grade water (9:1, v/v) from the combined secondary fortification stocks above as shown below:

Combined Secondary Fortification Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Calibration STD Conc. (µg/L)
0.0100	0.500	10.0	0.500
0.0100	0.250	10.0	0.250
0.0100	0.100	10.0	0.100
0.0100	0.0500	10.0	0.0500
0.0100	0.0250	10.0	0.0250
0.00100	0.100	10.0	0.0100
0.00100	0.0500	10.0	0.00500

Upon analysis of surface water samples for TCPSA (confirmation analysis – HILIC column), the above solvent based calibration standards resulted in inconsistent retention times and chromatographic peak shapes between the samples and calibration standards due to suspected matrix effects on the HILIC analytical column used for this analysis. As a consequence, the calibration standards for the surface water HILIC column confirmation analysis only were prepared using matrix-matched acetonitrile: surface water (9:1, v/v) as the dilution solvent using the same dilution scheme above. This modification alleviated the chromatographic differences mentioned above using the solvent based calibration standards. The matrix-matched dilution solvent was prepared using a scaled up version of the dilution scheme employed during sample processing. Initially, a 100 mL volume of surface water matrix was combined with a 100 mL volume of acetonitrile and mixed well. Subsequently, 50 mL of this 1:1 mixture was further diluted with acetonitrile solvent to a 250 mL final volume in a 250-mL graduated cylinder to match the matrix and solvent composition of samples.

The combined calibration standard solutions were stored under refrigerated conditions when not in use. All solutions were prepared using volumetric flasks and gas-tight syringes.

Analytical Method – Ground/Surface Water

The analytical method developed for ground/surface water matrices and provided for validation for this Independent Laboratory Validation (ILV) study utilized a dilution/direct injection technique. The method was divided into three separate analyses for Triallate and TCPSA analytes. The assignments of each analyte to its associated dilution solvent, and analytical column are summarized below:

Analyte/Analysis:	Dilution Solvent	Column
Triallate(quantitation/confirmation)	Acetonitrile: HPLC grade water (1:1, v/v)	XBridge C18
TCPSA(quantitation)	Acetonitrile: HPLC grade water (1:1, v/v)	Atlantis T3
TCPSA(confirmation)	Acetonitrile: HPLC grade water (9:1, v/v) or Acetonitrile: matrix water (9:1, v/v)	Atlantis HILIC

All aqueous samples were initially diluted with acetonitrile solvent, and secondarily diluted with either acetonitrile: HPLC grade water (1:1, v/v) solution for Triallate (primary quantitation and secondary confirmation analysis) and TCPSA (quantitation analysis) or with 100% acetonitrile to achieve a final solvent: aqueous composition of 9:1, v/v for TCPSA confirmation analysis only. Note: the provided methodology used a dilution solvent of Acetonitrile: HPLC grade water (9:1, v/v) for TCPSA confirmation analysis, however it was determined that a dilution with 100% acetonitrile was necessary for these method validations due to chromatographic difficulties encountered with the HILIC column analyses for this analyte component. Final quantitation of samples was performed utilizing High Performance Liquid Chromatography with tandem mass spectrometric detection (HPLC/MS/MS).

Fortification of Recovery Samples

For each matrix validated, one reagent blank, two unfortified matrix blanks, five fortified control matrix samples at the LOQ, and five fortified control matrix samples at 10X the LOQ were prepared in ground and surface water as shown below:

Surface/Ground Water Fortification

Analyte (s)	Nominal Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Combined Stock Conc. (µg/mL)
Triallate/TCPSA	0.100 (LOQ)	0.0500	5.00	0.0100
	1.00 (10X LOQ)	0.0500	5.00	0.100

All fortified samples were prepared with combined fortification solutions that were prepared compensating for the purity of the reference materials. Therefore, all concentration levels, expressed in µg/L, are equivalent to the expression as µg/L active ingredient (µg/L a.i.).

Processing and Analysis of Triallate and TCPSA in Ground/Surface Water

For analysis, 5.00-mL volumes of either control ground/surface water were measured into twelve individually labeled 20-mL glass scintillation vials, five of which were fortified at the LOQ (0.100 µg/L) and five at 10x the LOQ (1.00 µg/L) with combined secondary fortification stocks of the reference substances prepared as described above, adjusting for fortification volumes. A single reagent blank

consisting of all reagents except matrix, and the two matrix blanks of unfortified control matrix and carried through the methodology for each matrix validation. All samples were subsequently analyzed initially by methodology in Appendix II, with minor adjustments to dilution scheme for the TCPSA analyses only. Slight adjustments in the HPLC/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 specific details of the method are presented in Appendix II, a more general description is provided here.

Five milliliter (5.00 mL) volumes of acetonitrile solvent were added to each aqueous sample in vials above and the resulting solutions were mixed well.

For Triallate processing (both quantitation and confirmation analysis using the XBridge C18 column), the blanks and LOQ samples did not require any further dilution. Aliquots of the 10X LOQ samples were volumetrically diluted 0.500 mL to 5.00 mL final volume using a solution of acetonitrile: HPLC grade water (1:1, v/v).

For TCPSA processing (quantitation analysis only using the T3 column), the blank and LOQ samples were diluted 0.250 mL to final volume of 1.25 mL, and the 10 X LOQ samples were diluted 0.500 mL to 5.0 mL final volume also using a solution of acetonitrile: HPLC grade water (1:1, v/v). For the surface water validation only for TCPSA quantitation analysis, the blanks and LOQ samples were reprocessed and analyzed without the secondary dilution due to dilution related artifact peaks at the retention time of the TCPSA analyte observed during initial analysis. This dilution modification gave acceptable results for the blanks and LOQ samples which are reported for this particular validation.

For the TCPSA processing (confirmatory analysis only using the HILIC column) in ground water validation, the blank, LOQ, and 10 X LOQ samples were initially diluted 1.00 mL to 5.00 mL, 1.00 to 5.00 mL, and 0.500 mL to 5.00 mL volumes, respectively, using a solution of acetonitrile: HPLC grade water (9:1, v/v) as per methodology provided. The trial #1 validation failed due to inconsistent chromatography, variable instrumental responses, and associated chromatographic differences encountered between the samples and calibration standards using the HILIC column. After consulting with the Sponsor and developing laboratory, it was suggested that the secondary dilutions be performed using 100% acetonitrile, and to also increase the acetonitrile composition in the mobile phase as the HILIC columns tend to be very sensitive sample composition. As a consequence of this discussion, a second trial of the validation for TCPSA (confirmation analysis only) was performed employing a secondary dilution of scheme of 1.00 mL to 5.00 mL final volumes for all samples using 100% acetonitrile as dilution solvent to achieve a final sample dilution composition of acetonitrile: HPLC grade water (9:1, v/v). The isocratic mobile phase acetonitrile composition was also increased from 90:10% to 95:5%. Both these method adjustments resulted in acceptable results for this portion of the validation in ground water.

For the TCPSA (confirmatory analysis only using the HILIC column) in surface water validation, the blank, LOQ, and 10 X LOQ samples were initially diluted 1.00 mL to 5.00 mL final volumes using a solution of 100% acetonitrile as adjusted to during the ground water method validation. Once again, this trial #1 validation failed due to inconsistent chromatography, variable and non-linear instrumental responses and associated chromatographic differences encountered between the samples and calibration standards using the HILIC column. After consulting with the Sponsor and developing laboratory, it was suggested that issues may be matrix related as well, i.e. pH, salt and mineral content etc. As a consequence of this potential scenario, the performing laboratory matrix-matched the calibration

standards to the exact composition of the final diluted samples for this particular method validation in an attempt to eliminate any differences in final composition between matrix samples and calibration standards, and its effect on the HILIC column's sensitivity to differences in sample constituents. A second trial of the surface water validation for TCPSA (confirmation analysis only) was performed employing the same modified secondary dilution of scheme and mobile phase adjustments from trial #1, with the additional adjustment of using matrix-matched calibration standards. The matrix-matched dilution solvent preparation is discussed in the calibration standards section of this report. The addition of matrix-matched calibration standards to the methodology seemed to alleviate the chromatographic effects observed previously and resulted in acceptable results for this portion of the validation in surface water.

Aliquots of each final sample dilution were transferred to low-binding micro-centrifuge tubes and centrifuged at approximately 13,000 RPM for 5 minutes. A portion of each of the supernatants was transferred to auto-sampler vials and submitted for HPLC/MS/MS analysis. The remainders of each sample initial dilution (acetonitrile: aqueous matrix, 1:1, v/v) were transferred to 20-mL glass vials and stored under refrigerated conditions.

Quantitation of Triallate and TCPSA by HPLC/MS/MS

An Agilent Technologies Model 1200 Infinity Series High Performance Liquid Chromatograph connected to an AB /MDS Sciex API 5000 Mass Spectrometric Detector (HPLC/MS/MS) was used to analyze samples. An acidified (0.1% formic acid) acetonitrile: water gradient elution profile was used for the analysis of Triallate and an isocratic elution profile was used for the analysis of TCPSA.

Quantitation was performed using the responses of the primary quantitation ion transitions for each analyte and associated analytical column specific method. Confirmation analysis was performed using the response of a secondary confirmation ion transition for Triallate, and using the same primary quantitation ion transition with a secondary analytical column and method for TCPSA analyses. The ion transitions monitored and specific analytical columns used for each are summarized below:

Analyte	Primary(Quantitation)/ Column	Secondary(Confirmation)/ Column
Triallate	304.1→85.8 amu XBridge BEH C18	304.1→142.8 amu/ XBridge BEH C18
TCPSA	224.9→79.8 amu/ Atlantis T3	224.9→79.8 amu Atlantis HILIC Silica

Specific details of the HPLC/MS/MS instrumentation and operational parameters are presented in Tables 1-3.

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 (µg/L) 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 analyses) were determined by substituting peak area responses of the samples into the re-arranged weighted (1/x) regression equation as follows:

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

Using the data from the ground water method validation sample 334C-134-GWVMAS-1, 0.100 µg/L shown below, the Triallate (quantitation analysis) analytical result and percent recovery was calculated as follows using the software algorithms of Analyst version 1.6 of the AB /MDS Sciex API 5000 mass spectrometer system in full precision mode. Note: manual calculations shown here may differ slightly than reported.

Where:

Peak area = 17258
Y-intercept = 1335.22
Slope = 326328

The concentration of Triallate 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:

$$\text{Concentration at instrument (µg/L): } = \frac{17258 - (1335.22)}{326328}$$

Concentration at instrument (µg/L): = 0.04879

The residue concentration (µg/L) for Triallate in the fortified water recovery sample was determined as the product of the at instrument solution concentration determined above and the overall dilution factor as follows:

$$\text{Concentration in µg/L} = \text{Triallate Concentration at Instrument} \times \frac{\text{(Final Volume)}}{\text{(Initial Volume)}} \times \text{DF}$$

Where: Initial Volume= 5.00 mL
Final Volume = 10.0 mL
Secondary Dilution (DF) = 1.00

Using the nominal concentration (µg/L) from above, the concentration of Triallate in water sample was calculated as follows:

Concentration in sample (µg/L) = 0.04879 x 2.00 x 1.00

Concentration in sample (µg/L) = 0.09758

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:

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

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

$$\text{Recovery (\%)} = \frac{0.09758 \text{ µg/L Found}}{0.100 \text{ µg/L Added}} \text{ x } 100$$

Recovery (%) = 97.6%

The same calculation procedure was applied for the Triallate confirmation analyses and TCPSA quantitation and confirmation analyses for both matrices in this study.

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 for quantitation and confirmation analyses.

Table 1. Triallate HPLC/MS/MS Instrumentation and Operational Parameters (Quantitation and Confirmation Analysis)

Instrumentation	Agilent Technologies Model 1200 Series High Performance Liquid Chromatograph with a AB /MDS Sciex API 5000 Mass Spectrometer (HPLC/MS/MS) and Turbo-V Ion Spray Source
Analytical Column	Waters XBridge BEH C18 2.5 µm (2.1 mm x 50 mm)
Guard Column	None
Injector/Needle Wash	Flush Port – Acetonitrile: HPLC Grade Water (1:9, v/v) -10 seconds
Mobile Phases	A1: 0.1% Formic Acid in HPLC-grade water B1: 0.1% Formic Acid in Acetonitrile Gradient Elution Program: Time (min) / %A1 / %B1 / Flow Rate (µL/min) / Temp (°C) 0.00 / 75.0 / 25.0 / 350 / 40.0 0.500 / 75.0 / 25.0 / 350 / 40.0 4.00 / 0.00 / 100 / 350 / 40.0 6.00 / 0.00 / 100 / 350 / 40.0 6.10 / 75.0 / 25.0 / 350 / 40.0 7.50 / 75.0 / 25.0 / 350 / 40.0
Diverter Valve (Valco)	Time (min) / Position 0.00 / A 1.00 (2.5 surface water) / B
Injection Volume	100 µL
Period 1-Experiment 1	Scan Type/Polarity: MRM/Positive: GS1 = 30, GS2 = 30, CUR = 30.0, CAD = 4, IS = 5000, TEM = 500, DP = 50, EP = 10 Primary Quantitation: (304.1/85.8 amu), CE = 25, CXP = 36 Secondary Confirmation: (304.1/142.8 amu), CE = 39, CXP = 16
Triallate Retention Time	Approximately 4.9 minutes (ground water) Approximately 5.8 minutes (surface water)

Table 2. TCPSA HPLC/MS/MS Instrumentation and Operational Parameters (Quantitation Analysis)

Instrumentation	Agilent Technologies Model 1200 Series High Performance Liquid Chromatograph with a AB /MDS Sciex API 5000 Mass Spectrometer (HPLC/MS/MS) and Turbo-V Ion Spray Source
Analytical Column	Waters Atlantis T3 3.0 µm (4.6 mm x 100 mm)
Guard Column	None
Injector/Needle Wash	Not used - Ground Water Trial #1 Flush Port – Acetonitrile: HPLC Grade Water (1:9, v/v) -10 seconds (Surface Water Trial #1)
Mobile Phases	A1: 0.1% Formic Acid in HPLC-grade water B1: 0.1% Formic Acid in Acetonitrile Isocratic Elution Program: Time (min) %A1 %B1 Flow Rate (µL/min) Temp (°C) 0.00 50.0 50.0 1200 40.0 3.00 50.0 50.0 1200 40.0
Injection Volume	100 µL
Period 1-Experiment 1	Scan Type/Polarity: MRM/Negative: GS1 = 30, GS2 = 30, CUR = 30.0, CAD = 4, IS = -4500, TEM = 500, DP = -50, EP = -10 Quantitation: (224.9/79.8 amu), CE = -50, CXP = -15
TCPSA Retention Time	Approximately 1.9 minutes (ground water) Approximately 2.0 minutes (surface water)

Table 3. TCPSA HPLC/MS/MS Instrumentation and Operational Parameters (Confirmation Analysis)

Instrumentation	Agilent Technologies Model 1200 Series High Performance Liquid Chromatograph with a AB/MDS Sciex API 5000 Mass Spectrometer (HPLC/MS/MS) and Turbo-V Ion Spray Source
Analytical Column	Waters Atlantis HILIC Silica 3.0 µm (3.0 mm x 100 mm)
Guard Column	None
Injector/Needle Wash	Wash/Rinse Vial (Ground Water only) – acetonitrile : HPLC grade water (9 :1, v/v) dilution solvent as carry-over evaluation only Flush Port - Acetonitrile: HPLC Grade Water (1:9, v/v) -10 seconds (Surface Water only)
Mobile Phases	A1: 0.1% Formic Acid in HPLC-grade water B1: 0.1% Formic Acid in Acetonitrile Isocratic Elution Program: Time (min) / %A1 / %B1 / Flow Rate (µL/min) / Temp (°C) 0.00 / 5.00 / 95.0 / 300 / 40.0 5.00 / 5.00 / 95.0 / 300 / 40.0
Injection Volume	50.0 µL
Period 1-Experiment 1	Scan Type/Polarity: MRM/Negative: GS1 = 30, GS2 = 30, CUR = 30.0, CAD = 4, IS = -4500, TEM = 500, DP = -50, EP = -10 Confirmation: (224.9/79.8 amu), CE = -50, CXP = -15
TCPSA Retention Time	Approximately 2.0 minutes (ground water) Approximately 3.1 minutes (surface water)