INTRODUCTION

The purpose of this study was to verify the performance of methodology for the analysis of Prometon in water to be used in support of aquatic field dissipation studies. Validation samples were analyzed based upon procedures developed by Wildlife International. The study was performed based on U.S. Environmental Protection Agency Residue Chemistry Test Guideline, OPPTS 860.1340, entitled "*Residue Analytical Method* (1)", and on the procedures outlined in the European Commission Working Document SANCO/3029/99 rev.4, 11/07/00, entitled "*Residues: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414* (2)" The analysis of the samples were performed using High Performance Liquid Chromatography (HPLC) with Tandam Mass Selective Detection (MS/MS). Freshwater validation samples were prepared and analyzed between September 23 and 25, 2014. All raw data generated by Wildlife International and the original final report are filed under Project Number 234C-114 in archives located on the Wildlife International site.

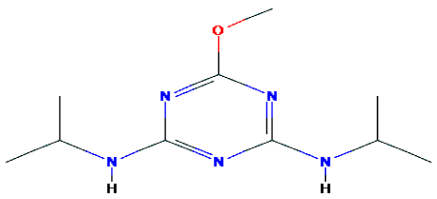
MATERIALS AND METHODS

This study was conducted according to the protocol "Validation of a Method for the Determination of Prometon in Freshwater for the Support of Aquatic Field Dissipation Studies" (Appendix 1).

Test and Reference Substances

The test substance of Prometon was received from Santa Cruz Biotechnology on June 19, 2014 and was assigned Wildlife International identification number 11776 upon receipt. This test substance was used to prepare method validation samples and calibration standards for this study. The test substance chemical structure and additional information are shown below:

Structure:



Name: Prometon

Supplier: Santa Cruz Biotechnology

Lot#: F1714

Catalog #: sc-253319

CAS Number: 1610-18-0

Molecular Formula: $C_{10}H_{19}N_5O$

Molecular Weight: 225.29 grams/mole

Purity: 99.5%

The Prometon test substance was stored under ambient conditions at the testing facility. A Certificate of Analysis for the Prometon test substance is presented in Appendix 2.

The reference substance of Prometryn was received from Sigma-Aldrich on June 12, 2014 and was assigned Wildlife International identification number 11762 upon receipt. This reference substance was used as an internal standard (IS) for aid in quantitation and to enhance the stability of the LC/MS/MS response during analyses. The reference substance chemical structure and additional information are shown below:

Structure:

Name: Prometryn

Supplier: Sigma-Aldrich

Batch#: LC07507V

Catalog #: 49087

CAS Number: 7287-19-6

Molecular Formula: $C_{10}H_{19}N_5S$

Molecular Weight: 241.36 grams/mole

Purity: 99.9%

The Prometryn internal standard reference substance was stored under ambient conditions at the testing facility. A Certificate of Analysis for the Prometryn internal standard reference substance is presented in Appendix 3.

Solvents

Methanol and HPLC Grade Water were obtained from Burdick and Jackson. Formic acid (conc.) was obtained from Sigma-Aldrich. All solvents and reagents used in this study were HPLC grade or equivalent.

Dilution Solvent Preparations

Methanol: 0.2% Formic Acid (v/v): measure approximately 998 mL of methanol into a 1000-mL graduated cylinder or equivalent. Add 2.0 mL of formic acid (conc.) to the cylinder and adjust to 1000 mL final volume using methanol. Mix well and transfer to appropriate storage container.

Methanol: HPLC Grade Water: Formic Acid (50:50:0.1, v/v/v): measure 500 mL of methanol into a 1000-mL graduated cylinder or equivalent. Add 1.0 mL of formic acid (conc.) to the cylinder and adjust to 1000 mL final volume using HPLC Grade Water. Mix well and transfer to appropriate storage container.

Dilution Solvent #1-Methanol: 0.2% formic acid (v/v) containing 0.00500 µg/mL of Prometryn (IS): Measure approximately 500 mL of Methanol: 0.2% formic acid solution into a 1000-mL class A volumetric flask. Add 0.500 mL of a 10.0 µg/mL stock of Prometryn (IS) and adjust to final volume using the methanol: 0.2% formic acid solution. Mix well. Transfer to appropriate storage container and store refrigerated for use during sample processing.

Dilution Solvent #2-Methanol: HPLC Grade Water: Formic Acid (50:50:0.1, v/v/v) containing 0.00250 µg/mL of Prometryn (IS): measure approximately 500 mL of Methanol: HPLC Grade Water: Formic Acid (50:50:0.1, v/v/v) solution into a 1000-mL class A volumetric flask. Add 0.250 mL of a 10.0 µg/mL stock of Prometryn (IS) and adjust to final volume using Methanol: HPLC Grade Water: Formic Acid (50:50:0.1, v/v/v) solution. Mix well. Transfer to appropriate storage container and store refrigerated for use during sample processing.

Equipment

Laboratory Balances

Beakers

Class A Volumetric Flasks - 10, 25, 50, 100, 1000-mL

Assorted Hamilton Gas-tight Syringes

Eppendorf 2500 Reference Pipettor and associated disposable tips

Vortex Genie Mixer

Glass Culture Tubes (15-mL)

B&D Plastic Disposable Syringes (3-mL)

WHATMAN Puradisk 25 TF Syringe Filters (0.2µm)

AB Sciex 5500 Series Triple Quad Mass Spectrometer with an Agilent Technologies 1260 Series HPLC (HPLC/MS/MS)

Alternative equipment may be substituted as long it is considered equivalent in function and generates successful method outcome.

Test System

The freshwater used to prepare the method validation samples was obtained from locally from Tuckahoe Lake, located in Ridgley, MD. The water was collected from the surface of the lake and was characterized at Wildlife International by measuring the specific conductance, hardness, alkalinity and pH. The results of this characterization are summarized in Appendix 4.

Test Substance Stock Preparation

A primary stock solution of Prometon was prepared by weighing 0.01005 grams (weight corrected for purity of 99.5%) of the test substance on an analytical balance. The test substance was transferred to a 10.0-mL class A volumetric flask, and brought to volume using methanol to achieve a 1.00 mg/mL stock solution. This primary stock solution (1000 µg/mL) was serially diluted in the same solvent solution to prepare 100, 10.0, 1.00 and 0.100 µg /mL working stock solutions. The following shows the dilution scheme for the working stocks of Prometon:

Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Stock Concentration (µg/mL)
1000	1.00	10.0	100
100	1.00	10.0	10.0
10.0	1.00	10.0	1.00
1.00	1.00	10.0	0.100

The 100 and 10.0 µg/mL stock solutions were used to prepare the method validation samples and calibration standards for this study.

Internal Standard Stock Preparation

A primary stock solution of Prometryn was prepared by weighing 0.01001 grams (weight corrected for purity of 99.9%) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 0.100 mg/mL stock solution. This primary stock solution (100 µg/mL) was serially diluted in the same solvent solution to prepare 10.0, 1.00 and 0.100 µg/mL working stock solutions. The following shows the dilution scheme for the working stocks of Prometryn reference substance:

Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Stock Concentration (µg/mL)
100	5.00	50.0	10.0
10.0	5.00	50.0	1.00
1.00	5.00	50.0	0.100

The 10.0 µg/mL stock of the internal standard was used to prepare the dilution solvents for use in sample preparation procedures and the 0.100 µg/mL stock of internal standard was used in the preparation of calibration standards.

Calibration Standards Preparation

Calibration standards of Prometon and Prometryn (IS) were prepared in methanol: water: formic acid (50:50:0.1, v/v/v) using the appropriate 1.00 and 0.100 µg/mL stock solutions. The following shows the dilution scheme for a set of calibration standards:

Prometon/IS Stock Concentrations (µg/mL)	Aliquot (mL)	Final Volume (mL)	Prometon/IS Calibration Standard Concentration (µg/mL)
0.100/0.100	0.0500/0.250	10.0	0.000500/0.00250
0.100/0.100	0.100/0.250	10.0	0.00100/0.00250
0.100/0.100	0.250/0.250	10.0	0.00250/0.00250
0.100/0.100	0.500/0.250	10.0	0.00500/0.00250
1.00/0.100	0.100/0.250	10.0	0.0100/0.00250
1.00/0.100	0.250/0.250	10.0	0.0250/0.00250
1.00/0.100	0.500/0.250	10.0	0.0500/0.00250

Analytical Method

Water (10.0 mL) was fortified at two different concentrations in quintuplet (0.100 and 1.00 mg/L) and analyzed based on methodology developed by Wildlife International. One reagent and two matrix blanks were prepared for analysis to evaluate potential analytical method interferences.

The method validation samples were processed using a direct injection approach. An aliquot (2.00 mL) of each aqueous sample was initially diluted volumetrically with an equal volume (2.00 mL) of Dilution Solvent #1 (methanol: 0.2% formic acid, v/v containing 0.00500 µg/mL of Prometryn IS) in a disposable 15-mL glass culture tube to achieve a final solvent composition of methanol: water: formic acid containing 0.00250 µg/mL IS. The solutions were vortexed briefly to mix. An aliquot of the diluted sample was then filtered through 0.2µm PTFE syringe filter assembly into a second tube. Subsequently, a 1.00 mL aliquot of the filtered dilution mixture above was volumetrically diluted further to a 25.0 mL final volume using Dilution Solvent #2 (methanol: HPLC grade water: formic acid (50:50:0.1, v/v/v) containing 0.00250 µg/mL Prometryn IS. The final solution was mixed well by inversion. An aliquot of each final dilution was transferred transferred to auto-sampler vials and submitted for analysis. Note: Alternative dilutions may be used depending on concentration levels expected in samples.

Concentrations of Prometon in water samples were determined using an Agilent Technologies 1260 Infinity Series High Performance Liquid Chromatograph (HPLC) coupled with an AB SCIEX 5500 Triple Quad Mass Spectrometer (MS/MS) using a Turbo-Ion Spray source operated in the positive ion, multiple reaction monitoring (MRM) mode. Chromatographic separations were achieved using a THERMO EC Betasil C-18 column (50 mm x 2.1 mm, 5 µm particle size), preceded by a THERMO EC Javelin Betasil

C-18 guard column (10 mm x 2.1mm) utilizing a gradient elution profile. The High Performance Liquid Chromatography/ Mass Spectrometer (HPLC/MS/MS) operating parameters are summarized in Table 1. A detailed analytical method outline is provided in Figure 1.

A calibration curve was generated from analyses of Prometon/ Prometryn (IS) standard solutions analyzed concurrently with the series of method validation samples.

Method Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) for this freshwater method verification was set at 0.100 mg/L, the lowest level fortified and analyzed during the verification set (Note: this method validation was conducted at levels well above the actual limit of quantitation for this method). Reagent blank and matrix blank samples were further screened to confirm any potential interference to be < 30% of the fortified LOQ level. The theoretical LOQ was 0.0250 mg/L, calculated as the product of the lowest calibration standard (0.000500 µg/mL) and the dilution factor of the matrix blank samples (50.0). The actual LOQ was determined to be 0.000441 mg/L, calculated as the product of the lowest calibration standard /(average signal to noise ratio) x 10 x the dilution factor of the matrix blank samples (50) as shown below:

0.000500 µg/mL/ 567.4 x 10 x 50 = 0.000441 mg/L

Limit of Detection (LOD)

The instrumental limit of detection (LOD) was determined to be 0.0000026 mg/L, calculated as the product of the lowest calibration standard /(average signal to noise ratio) x 3 x the dilution factor of the standard (1.0) as shown below:

0.000500 µg/mL/ 567.4 x 3 x 1.00 = 0.0000026 mg/L

Example Calculations

The Prometon analytical result and percent recovery for water method validation sample number 234C-114-MV-W-MAS-1, nominal concentration of 0.100 mg/L, were calculated using the following

equation:

Prometon
(mg/L) in sample:

$$= \frac{\text{Peak Area Ratio - (Y-intercept)}}{\text{Slope}} \times \frac{\text{Final Vol. (mL)}}{\text{Initial Vol. (mL)}} \times \text{Secondary Dilution Factor} \times \text{IS Concentration}$$

Where:

Peak Area Ratio (Analyte/IS) = 0.5290561
Y-intercept = 0.0350858
Slope = 0.612787
Initial Volume (mL): 2.00
Final Volume (mL): 4.00
Secondary Dilution Factor = 25.0
Internal Standard Conc. (µg/mL) = 0.00250

$$\text{Concentration (mg/L) in sample} = \frac{0.5290561 - 0.0350858}{0.612787} \times \frac{4.00}{2.00} \times 25.0 \times 0.00250$$

Concentration in sample (mg/L) = 0.101

$$\text{Percent of nominal concentration} = \frac{0.101\ (\text{mg/L})}{0.100\ (\text{mg/L})} \times 100$$

Percent of nominal concentration = 101%*

*Results were generated using Analyst Software Version 1.6.2 in the full precision mode. Manual calculations may differ slightly.

CONCLUSIONS

The method was successfully validated at concentrations of 0.100 to 1.00 mg/L and is suitable for the determination of residues of Prometon in freshwater. A Confirmatory ion transition method was also established for further identification of the Prometon test substance in freshwater, if needed.

Table 1

Typical High Performance Liquid Chromatography/ Mass Spectrometer (HPLC/MS/MS)
Operational Parameters for the Analysis of Prometon

Instrument:	Agilent Technologies 1260 Infinity Series High Performance Liquid Chromatograph (HPLC) coupled with an AB SCIEX 5500 Triple QUAD Mass Spectrometer (MS/MS) operated in the positive ion multiple reaction monitoring (MRM) mode.
Analytical Column:	THERMO EC Betasil C-18 (50 x 2.1 mm, 5 µm particle size)
Guard Column:	THERMO EC Javelin Betasil C-18 (10 x 2.1 mm)
Column Oven Temperature:	40°C
Mobile Phases:	A - 0.1% formic acid in water B – 0.1% formic acid in Acetonitrile

Gradient Elution Profile :

Time (min)	Flow Rate (µL/min.)	Percent A	Percent B
0.00	350	90.0	10.0
1.00	350	90.0	10.0
4.00	350	10.0	90.0
5.00	350	10.0	90.0
5.01	350	90.0	10.0
9.00	350	90.0	10.0

Injection Volume:	5.0 µL		
Ion Source:	Turbo-V Ion Spray, positive mode		
Parameter Table:	CUR: 30.0	IS: 5500	
	GS1: 40.0	DP: 130	
	GS2 : 50.0	EP: 10.00	
	CAD: 7.00	CE: 31.00, 25.00, 31.00	
	TEM: 600	CXP: 12.00, 14.00, 14.00	
Monitored Transition(s):	226 → 142 m/z – Quantitation (dwell time 250 msec)		
	226 → 184 m/z – Confirmation (dwell time 250 msec)		
	242 → 158 m/z – Internal Standard (dwell time 250 msec)		
Approximate Retention Time:	~4.32 minutes - Prometon		
	~5.26 minutes - Prometryn Internal Standard		

METHOD OUTLINE FOR THE ANALYSIS OF PROMETON IN FRESHWATER

Prepare Prometon calibration standards in methanol: HPLC Grade Water: formic acid (1:1:0.1, v/v) and fortify with 0.00250 µg/mL Prometryn internal standard using volumetric flasks and gas-tight syringes from methanol stock solutions, **STORE REFRIGERATED**.

↓

Prepare matrix fortification samples in freshwater matrix using Prometon stock solutions, volumetric flasks and gas-tight syringes. Using an appropriate sized reference pipettor or equivalent, combine an aliquot of each aqueous sample with an equal volume of **Dilution Solvent #1** (i.e.; 2.00 mL H20 + 2mL of dilution solvent #1 = 4.00 mL final volume = 2X dilution factor) in a disposable 15-mL glass culture tubes or equivalent container. Vortex briefly to mix.

↓

Filter an aliquot of each diluted sample through a 0.2um PTFE syringe filter assembly into a second disposable 15-mL glass culture tube or equivalent container.

↓

Volumetrically dilute above filtered samples further with **Dilution Solvent #2** (i.e. 1.00 mL to 25.0 mL final volume) using Class A volumetric flasks and appropriate size reference pipettor or equivalent. (Alternative volumes may be used depending on sample concentration levels). Mix well by inversion.

↓

Transfer aliquots of final sample dilutions and calibration standards to auto-sampler vials and submit for analysis by HPLC/MS/MS.

Figure 1. Analytical method outline for the analysis of Prometon in Freshwater.