INTRODUCTION

The purpose of this study was to verify the performance of methodology for the analysis of Prometon in soil to be used in support of terrestrial 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). Soil validation samples were prepared and analyzed between September 24 and 26, 2014. All raw data generated by Wildlife International and the original final report are filed under Project Number 234C-116 in archives located on the Wildlife International site.

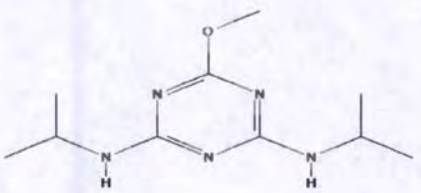
MATERIALS AND METHODS

This study was conducted according to the protocol "Validation of a Method for the Determination of Prometon in Soil for the Support of Terrestrial 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: $C_{10}H_{19}N_5O$

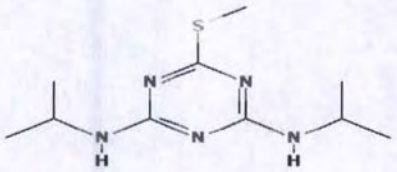
Molecular Weight: 225.29 grams/mole

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, 2013 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 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 Solvents Preparation

Methanol: 0.2% Formic Acid (v/v): measure approximately 500 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.

Extraction Solvent-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 #1: 100% HPLC Grade Water

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 soil used to prepare the method validation samples for this study was obtained from Agvise Laboratories and was characterized in compliance with GLP standards. This particular soil was chosen to best represent the soil type for the region of potential terrestrial field dissipation study. It was characterized as a sandy loam according to USDA textural class (hydrometer method). The soil characterization report is presented 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 1.00 and 0.100 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)
0.100/1.00	0.100/0.250	100	0.000100/0.00250
0.100/1.00	0.250/0.250	100	0.000250/0.00250
0.100/1.00	0.500/0.250	100	0.000500/0.00250
1.00/1.00	0.100/0.250	100	0.00100/0.00250
1.00/1.00	0.250/0.250	100	0.00250/0.00250
1.00/1.00	0.500/0.250	100	0.00500/0.00250
1.00/1.00	1.00/0.250	100	0.0100/0.00250

Analytical Method

Soil (5.00 gram aliquots) were weighed into individual 50-mL plastic graduated centrifuge tubes, fortified at two different concentrations (0.0500 and 0.500 mg/kg) and analyzed based on methodology

developed by Wildlife International. One reagent blank and two matrix blanks were prepared for analysis to evaluate potential analytical method interferences.

Twenty mL volumes of extraction solvent (methanol: 0.2% formic acid containing 0.00500 µg/mL of internal standard) were added to each soil sample and mixed well by a combination of hand shaking and vortexing. The mixture was extracted for approximately one minute using a BRANSONIC ultrasonic disruption sample processor at an amplitude setting of approximately 45%. The samples were than capped and placed securely on a gyratory shaker table at a setting of approximately 250 excursions/ minute for approximately 15 minutes. The resulting extracts were then centrifuged at approximately 4500 rpm for approximately ten minutes and the supernatant decanted into glass 100-mL graduated cylinders. Following, a second 20 mL volume of extraction solvent was added to each soil pellet. The samples were capped and placed securely on a gyratory shaker table apparatus and shaken at a setting of approximately 250 excursions/ minute for approximately 15 minutes. The extracts were centrifuged again at approximately 4500 rpm for approximately ten minutes and the supernatant combined in the same 100-mL graduated cylinders from above. The final volumes in the graduated cylinder were adjusted to 50.0 mL using extraction solvent. The final extracts were transferred to 150-mL beakers and swirled gently to ensure mixing. Each intermediate extract was diluted 1:1, v/v, by combining a 2.00 mL aliquot of each extract with a 2.00 mL volume of dilution solvent #1 (100 % HPLC Grade Bottled Water) in 20-mL scintillation vials or equivalent using a Eppendorf 2500 series reference pipettor to achieve a final extract solvent composition of methanol: water: formic acid (50:50:0.1, v/v/v) containing 0.00250 µg/mL of internal standard. The dilutions were mixed well. Each 1:1 diluted extract was filtered using an assembly of a 5-mL BD disposable plastic syringe connected to a 0.2 µm WHATMAN PURADISK 25 TF syringe filter into a 20-mL glass scintillation vial. Next, a 1.00 mL aliquot of each final filtered extract was volumetrically transferred to 15-mL plastic graduated centrifuge tube and adjusted to 5.00 mL final volume using Dilution solvent #2 (methanol: water: formic acid, 50:50:0.1, v/v/v containing 0.00250 µg/mL internal standard). The final dilutions were mixed well. A portion (~20 mL) of each original final intermediate extract from above was transferred to appropriate storage container and the remainder of each extract was discarded. Aliquots of the final diluted sample extracts were transferred to auto-sampler vials and submitted for analysis.

Concentrations of Prometon in soil sample extracts 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 soil method verification was set at 0.0500 mg/kg, 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 0.0100 mg/kg, calculated as the product of the lowest calibration standard (0.000100 µg/mL) and the dilution factor of the matrix blank samples (100). The actual LOQ was determined to be 0.00055 mg/kg, 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 (100) as shown below:

0.000100 µg/mL/ 189.05 x 10 x 100 = 0.00055 mg/kg*

Limit of Detection (LOD)

The instrumental limit of detection (LOD) was determined to be 0.0000016 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:

0.000100 µg/mL/ 189.05 x 3 x 1.00 = 0.0000016 mg/L*

*Results were generated using Excel in the full precision mode. Manual calculations may differ slightly.

Example Calculations

The Prometon analytical result and percent recovery for soil method validation sample number 234C-116-MV-S-MAS-1, nominal concentration of 0.0500 mg/kg, were calculated using the following equation:

Prometon
(mg/kg) in sample:

$$= \frac{\text{Peak Area Ratio - (Y-intercept)}}{\text{Slope}} \times \frac{\text{Final Volume}}{\text{Initial Mass}} \times \text{Secondary DF} \times \text{IS Concentration}$$

Where:

Peak Area Ratio (Analyte/IS) = 0.1219334
Y-intercept = 0.00372794
Slope = 0.625165
Initial Mass (g): 5.00
Final Volume (mL): 50.0
Secondary Dilution Factor (DF) = 10.0
Internal Standard Conc. (µg/mL) = 0.00250

$$\text{Concentration (mg/kg) in sample} = \frac{0.1219334 - 0.0037294}{0.625165} \times \frac{50.0}{10.0} \times 5.00 \times 0.00250$$

Concentration in sample (mg/kg) = 0.04727

$$\text{Percent of nominal concentration} = \frac{0.04727\ (\text{mg/kg})}{0.0500\ (\text{mg /kg})} \times 100$$

Percent of nominal concentration = 94.5%*

*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 0.0500 to 0.500 mg/kg and is suitable for the determination of residues of Prometon in soil. A Confirmatory ion transition method was also established for further identification of the Prometon test substance in soil, 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	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: 5500.00	
	GS1: 40.0	DP: 130	
	GS2 : 50.0	EP: 10.00	
	CAD: 7.00	CE: 31.00, 25.00, 31.00	
	TEM: 600.00	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 SOIL

Prepare combined calibration standards in methanol: water: formic acid (50:50:0.1, v/v/v) Containing 0.00250 µg/mL Prometryn internal standard (IS) using volumetric flasks and gas-tight syringes. **STORE REFRIGERATED**

Prometon Soil Extraction

1. Weigh 5.00 grams of soil matrix into 50-mL plastic graduated centrifuge tubes or equivalent.
2. Fortify samples as needed, using the appropriate stock solution(s).
3. Add 20 mL of **Extraction Solvent** (methanol: 0.2% formic acid extraction solvent containing 0.00500 µg/mL IS). Mix well by hand shaking/ vortexing briefly.
4. Extract samples for ~ ONE minute using a BRANSONIC ultrasonic disruption sample processor at 45% amplitude setting. Cap and place securely on a gyratory shaker table apparatus. Shake samples at a setting of ~ 250 for ~ 15 minutes.
5. Centrifuge the samples at ~ 4500 rpm for ~ 10 minutes. Decant the supernatant into a 100- mL glass graduated cylinder.
6. Add a second 20 mL volume of above extraction solvent to each soil pellet. Cap and place securely on a gyratory shaker table apparatus. Shake samples at a setting of ~ 250 for ~ 15 minutes. Centrifuge the samples at ~4500 rpm for ~ 10 minutes and combine the extract supernatant in the 100-mL graduated cylinder from above.
7. Adjust to final volume of 50.0 mL with extraction solvent. Transfer to a 100-mL beaker or equivalent and gently swirl to mix.
8. Volumetrically dilute all extracts 1:1(v/v) by combining 2.00 mL of each filtered extract above with 2.00 mL of **Dilution Solvent #1** (100% HPLC Grade Bottled Water) in 20-mL scintillation vial or equivalent using a Eppendorf 2500 series reference pipettor or equivalent to achieve an approximate final extract solvent composition of methanol: water: formic acid (50:50:0.1, v/v/v) containing 0.00250 µg/mL of IS. Mix well. Transfer a portion (~20 mL) of each remaining final intermediate extract from step 7 to appropriate storage container. Discard the remainder of each extract
9. Filter each 1:1 diluted extract from step 8 using a BD disposable 5.0-mL plastic syringe connected to a WHATMAN PURADISK 25 TF Syringe Filter into a 20-mL glass scintillation vial or equivalent.
10. Dilute sample extracts further by measuring a 1.00 mL aliquot into a 15-mL graduated centrifuge tube and adjusting to 5.00 mL final volume using **Dilution Solvent #2** (methanol: water :formic acid (50:50:0.1, v/v) containing 0.00250 µg/mL of IS). Transfer an aliquot of the final extract to an auto-sampler vial and submit for LC/MS/MS analysis.

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