SUMMARY

An analytical method for the quantitation of prometon in a water, sediment, and soil test system were independently validated. The methods are described in "Validation of a Method for the Determination of Prometon in Freshwater for Support of Aquatic Field Dissipation Studies", Wildlife International, Study Number 234C-114, "Validation of a Method for the Determination of Prometon in Sediment for Support of Aquatic Field Dissipation Studies", Wildlife International, Study Number 234C-115, and "Validation of a Method for the determination of Prometon in Soil for Support of Terrestrial Field Dissipation Studies", Wildlife International, Study Number 234C-116.

The test substance, prometon was analyzed by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS). Water was fortified at 0.1 µg/mL (LOQ) and 1.0 µg/mL, and both sediment and soil were fortified at 0.05 µg/g (LOQ) and 0.5 µg/g.

The experiments for each matrix were conducted with one reagent blank, two untreated controls, and five control samples spiked for each fortification level: one at the LOQ level and another at 10X LOQ level.

The LC gradient as described and explained in the original method validation report was used for analyte separation. Prometon content was determined from quantitation against 1/x weighted linear curves of the reference substance ratios of prometon and the internal standard prometryn for both the quantitation and confirmation ions, with concentrations ranging from 0.1 ng/mL to 50 ng/mL. The calibration for prometon for all matrix analysis methods yielded acceptable linearity (correlation coefficients $r \geq 0.995$) over the range examined. The quantitation of prometon was based on the ratio of the prometon and prometryn peak area responses and peak area ratios of prometon and prometryn calibration standards. The amount of prometon was determined with the quantitation MS/MS ion transition from m/z 226 to m/z 142 and the confirmation MS/MS ion transition from m/z 226 to m/z 184 compared against the prometryn MS/MS ion transition from m/z 242 to m/z 158. Method recovery from fortified samples was determined by calculating the found concentration of prometon and dividing the concentration by the relevant fortification level.

No interferences or residues were detected in control samples with the MS/MS transitions selected for quantitation and confirmation.

INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of prometon in water, sediment, and soil. The analysis of the test substance was performed by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) based on the methods described in "Validation of a Method for the Determination of Prometon in Freshwater for Support of Aquatic Field Dissipation Studies", Wildlife International, Study Number 234C-114, "Validation of a Method for the Determination of Prometon in Sediment for Support of Aquatic Field Dissipation Studies", Wildlife International, Study Number 234C-115, and "Validation of a Method for the determination of Prometon in Soil for Support of Terrestrial Field Dissipation Studies", Wildlife International, Study Number 234C-116.

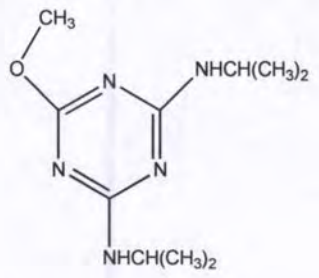
This study was designed to satisfy US EPA Guideline requirements described in OCSPP 850.6100. The study was initiated on May 29, 2015. The experimental work was conducted from June 3, 2015 through June 25, 2015 at PTRL West, 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol (Appendix A) according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160.

MATERIAL AND METHODS

Test and Reference Substance

Common Name:	**Prometon**
Chemical Name: (IUPAC):	N^2,N^4-di-isopropyl-6-methoxy-1,3,5-triazine-2,4-diamine
CAS Registry No.:	1610-18-0

Chemical Structure:



Molecular Formula:	$C_{10}H_{19}N_5O$
Molecular Mass:	225.3 g/mole
Supplier:	Wildlife International via Santa Cruz Biotechnology
Lot No.:	F1714

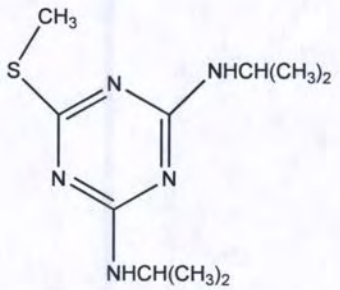
Purity:	99.5%
Storage Conditions:	Ambient Temperature

Certificate of Analysis for the test/reference substance is provided in Appendix B.

Internal Standard

Common Name:	**Prometryn**
Chemical Name: (IUPAC):	N^2,N^4-di-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine
CAS Registry No.:	7287-19-6

Chemical Structure:



Molecular Formula:	$C_{10}H_{19}N_5S$
Molecular Mass:	241.36 g/mole
Supplier:	Wildlife International via Sigma Aldrich
Lot No.:	LC07507V
Purity:	99.9%
Storage Conditions:	Ambient Temperature

Certificate of Analysis for the test/reference substance is provided in Appendix B.

Other Chemicals

HPLC grade water and acetonitrile were obtained from Burdick & Jackson; acetic acid was obtained from Fisher Scientific.

Equipment List

Laboratory Balances
Beakers
Pipetmen with plastic disposable tips
Vortex mixer

Centrifuge
Ultrasonic bath
Plastic Bottles
Wrist Action Shaker
AB Sciex API 5500 Series Tandem Mass Spectrometer with Agilent 1200 HPLC system (LC-MS/MS)

Test Systems

Source of Test Systems

The same test systems that were used in the validation studies were provided by the Wildlife International. The bulk water, soil and sediment were shipped with icepacks and stored under refrigerated conditions after receipt (Inventory Nos. 2744W-001, 002, and 003 for water, sediment, and soil, respectively).

Test Method

The analytical method for the analysis of prometon validated at PTRL West by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) was described in the method validation reports [1], [2], [3]

The test samples were spiked with known concentrations of prometon, followed by extraction and dilution with solutions containing internal standard. An aliquot of the final sample solution was injected onto the high performance liquid chromatography and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electro spray ionization. The percent method recovery was determined using external standardization where linear curve for peak area rations between prometon and prometryn for calibration standards was generated along with the samples.

Preparation of Stock Solution

A primary stock solution of prometon was prepared by weighing 0.01076 of the reference substance into a 10 mL volumetric flask. The stock solution was dissolved and diluted with methanol to yield a nominal concentration of 1.0706 mg/mL. The concentration of stock solution was corrected for the purity of the reference substance as shown in the following table. The stock solution was transferred into an amber bottle and stored in the freezer (typically < -10°C) when not in use.

Ptrl West No.	Stock ID	Standard Name	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical Conc. (µg/mL)
2744W-005	Stock C	Prometon	10.76	10	99.5	1070.62

Preparation of Working Stock Solutions

The primary stock solution (1000 µg/mL) was diluted using methanol and calibrated automatic pipets to prepare 100, 10.0, 1.00, and 0.100 µg/mL working stock solutions. The dilutions are provided below.

Theoretical Conc. (µg/mL)	Solution Used (ng/mL)	Volume of Solution (µL)	Final Volume (mL)
100	1000	934	10
10.0	1000	93.4	10
1.00	100	100	10
0.100	10	100	10

The 1.00 and 0.100 µg/mL working stock solutions were used to prepare the method validation samples and calibration standards for this study.

Preparation of Internal Standard Stock Solutions

A primary internal standard stock solution was prepared by weighing 0.01068 g of prometryn into a glass weight boat using an analytical balance. The prometryn was transferred to a 100 mL class A volumetric flask, and brought to volume using methanol to achieve a 106.69 µg/mL stock solution. Serial dilutions of the internal standard stock solution were performed in methanol using calibrated automatic pipets and appropriately sized volumetric flasks. Final preparations were stored in 12 or 60 mL glass amber bottles and stored in the freezer (typically < -10°C) when not in use. Details are provided in the table below:

Theoretical Conc. (µg/mL)	Solution Used (µg/mL)	Volume of Solution (mL) (mL)	Final Volume (mL)
10	106.69	0.9373	10
1.0	10	1.0	10
0.1	10	0.5	50

Preparation of Internal Standard Dilution Solutions

Three internal standard dilution solutions were prepared using appropriately sized volumetric flask and pipets.

Internal Standard Dilution Solution One (for water samples):

In a 1000 mL volumetric flask a 0.2 % methanol formic acid solution was prepared by combining 500 mL of methanol, 2.0 mL of formic acid, and then diluting to 1000 mL with methanol. The solution was mixed well, and approximately 200 mL was transferred to a 250 mL beaker. To the 800 mL solution 0.5 mL of the 10 µg/mL internal standard solution was added. The solution was diluted to 1000 mL with previously removed 200 mL. The final preparation was mixed well and then transferred to a 1000 mL amber glass bottle and stored under refrigerated temperatures when not in use.

Internal Standard Dilution Solution One (for sediment/soil samples):

In a 500 mL volumetric flask; combine 250 mL of methanol, 1.0 mL of formic acid, and 0.250 mL of the 10 µg/mL internal standard solution, mix will and dilute to volume with methanol. The final preparation was transferred to a 500 mL amber glass bottle and stored under refrigerated temperatures when not in use.

Internal Standard Dilution Solution Two (for water/sediment/soil samples):

In a 1000 mL volumetric flask a methanol: water: formic acid solution (50:50:0.1, v:v:v) was prepared by combining 500 mL of methanol, 1.0 mL of formic acid, and 499 mL of water. The solution was mixed well, and approximately 500 mL was transferred to a separate 1000 mL volumetric flask, in addition 0.250 mL of the 10 µg/mL internal standard solution was added and then the solution was diluted to 1000 mL with the solution prepared above. The final preparation was transferred to a 1000 mL amber glass bottle and stored under refrigerated temperatures when not in use.

Preparation of Calibration Standard Solutions

Nine calibration standard solutions were prepared by mixing appropriate volumes of working stock solutions, and the 1.0 µg/mL intermediate internal standard solution via calibrated automatic pipets with appropriate volumes of methanol: water: formic acid solution (500:500:1, v:v:v) into 100 mL volumetric flasks as described below. Final calibrants were transferred into 12 mL glass amber bottles and stored in the refrigerator when not in use. The standard solutions ranged from 0.1 ng/mL to 50 ng/mL and were prepared as shown below:

Theoretical Conc. (ng/mL)	Solution Used (µg/mL)	Volume of Solution (mL) (mL)	Final Volume (mL)
50	10	0.5	100
25	10	0.25	100
10	1.0	1.0	100
5.0	1.0	0.5	100
2.5	1.0	0.25	100
1.0	1.0	0.1	100
0.5	0.1	0.5	100
0.25	0.1	0.25	100
0.10	0.1	0.1	100

Fortification Procedure

Fortification of untreated water, sediment and soil was conducted at the following fortification levels as shown below using a Hamilton Syringe:

Fortification Level (ppm or µg/g)	Matrix	Fortification Solution
0.1	Water	0.1 mL of 10 µg /mL in 10 mL of water
1.0	Water	0.1 mL of 100 µg /mL in 10 mL of water
0.05	Sediment	0.025 mL of 10 µg /mL in 5 g of sediment
0.5	Sediment	0.025 mL of 100 µg /mL in 5 g of sediment
0.05	Soil	0.025 mL of 10 µg /mL in 5 g of soil
0.5	Soil	0.025 mL of 100 µg /mL in 5 g of soil

Fortification was conducted to determine the percent recovery, and accuracy within the method validation. This procedure was performed in quintuplicate during method validation at each fortification level.

Extraction Method for Prometon in Water

1. Transfer 10 mL of water into a 50 mL centrifuge tube.
2. Fortify the samples as necessary.
3. Transfer 2 mL to a 15 mL disposable glass centrifuge tube.
4. Add 2 mL of dilution solution one, cap tube and vortex mix.
5. Filter through a 0.2 µm nylon syringe filter.
6. Remove 1 mL and dilute to 25 mL using dilution solution two.
7. Mix well and transfer to autosampler vial for analysis by LC-MS/MS.

A schematic diagram of the water extraction method is presented in Figure 1.

Extraction Method for Prometon in Sediment/Soil

1. Weigh 5 g of sample into a 50 mL plastic centrifuge tube.
2. Fortify the samples as necessary.
3. Add 20 mL of dilution solution one and capped each tube.
4. Samples were shaken by hand and then place on a wrist action shaker for 5 minutes.
5. Samples were then centrifuged at 3500 rpm for 10 minutes to separate phases.
6. Supernatants were decanted into 50 mL graduated cylinders.
7. Repeat steps 3 through 6 combining supernatants.
8. Dilute samples to 50 mL with dilution solution one
9. Transfer to 250 mL beakers and mix well.
10. Transfer 2 mL to a small glass vial and add 2 mL of water, vortex mix.
11. Filter through a 0.2 µm nylon syringe filter.
12. Combine 1 mL of the filtered extract with 4 mL of dilution solution two.
13. Mix well and transfer ~ 1mL to an autosampler vial for analysis by LC-MS/MS.

A schematic diagram of the sediment/soil extraction method is presented in Figure 2.

Liquid Chromatography with Tandem Mass Spectrometry Analytical Method (LC-MS/MS)

LC conditions

Agilent 1200 HPLC system (LC-MS/MS)
Column: Thermo-Scientific Hypersil Gold 1.9 µ 50 x 2.1 mm,
Guard Column: Thermo-Scientific Hypersil Gold 3 µ 10 x 2.1 mm
Injection volume: 1 µL
Flow rate: 0.35 mL/min
Run time: 9 minutes

Mobile Phase:

- A: 0.1% Formic acid in HPLC H_2O
- B: 0.1% Formic acid in HPLC grade ACN

Gradient Program:

Time (minutes)	%A	%B	Flow rate (mL/min)
0	90	10	0.35
1.0	90	10	0.35
4.0	10	90	0.35
5.0	10	90	0.35
5.1	90	10	0.35
9.0	90	10	0.35

MS conditions

An Applied Biosystems API 5500 tandem mass spectrometer was used with electrospray ionization (ESI) in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM).

API 5500:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP
Prometon				
226.0	142.0	250.0	31.0	12.0
226.0	184.0	250.0	25.0	14.0
Prometryn Internal Standard				
242.0	158.0	250.0	31.0	14.0

CE = Collision Energy
CXP = Collision Cell Exit Potential

Source Dependent Settings:

Temperature (TEM):	600°C
Nebulizer Gas (GS1):	40.0
IonSpray Gas (GS2):	50.0
Curtain Gas (CUR):	30.0
Collision Activated Dissociation Gas (CAD):	7.0

Ionization Spray (IS)	5500
Entrance Potential (EP)	10.0
Declustering Potential (DP)	130.0

Post-column effluent was diverted into the mass spectrometer between 3.5 and 5.0 minutes.

LC-MS/MS Analysis

Samples were analyzed in a set consisting of a solvent blank, reagent blank, two control extracts, five low-level fortified controls, and five high-level fortified controls interspersed between the calibrants. In addition, to ensure accuracy was maintained over the course of the analytical set, quality control (QC) calibrants were added to the sequence. The final QC calibrant's response was within 10% when compared to its initial response. Calibrants and samples were analyzed in a single sequence of injections.

Methods of Calculation

Quantitation

Separation of prometon and prometryn was achieved by LC-MS/MS. The compounds were identified by the coincidence of the retention time with the respective reference standards and MS characteristics. The quantitation of prometon was conducted using the peak area ratio between prometon and prometryn relative to the theoretical concentrations of the calibrants. The content of prometon in samples was quantitated against 1/x weighted linear curve of prometon calibrants containing prometryn internal standards where:

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

y = peak area ratio
x = ng/mL ÷ concentration internal standard
m = slope
b = intercept

Weighting of the calibration curve was applied to provide better curve fit at the lower concentration levels of prometon.

The calculations of peak area ratios, weighted curve equations (linear regression) and concentrations (ng/mL) present in samples and calibrants was conducted using Analyst® software.

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

$$\text{Residue (ppm)} = \frac{\text{ng/mL x Initial Extract Volume (mL) x Dil. Factor x internal standard conc. 2.5 ng/mL}}{\text{Final Extract Volume (mL)}}$$

Where µg/mL =mg/L or ppm

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

$$\frac{\text{Residue (ppm)- Average Residue of Controls (ppm)}}{\text{Fortification Level (ppm)}} \times 100$$

An example calculation from the water analysis method for the recovery of prometon (m/z 226.0/142.0 ion transition) in water fortified at 0.1 ppm (sample designated F1-A) is given in following:

Linear regression equation: $y = 0.68145223117\,x + 0.021329731387$ ($r = 0.999795315925$)

The calculated concentration in F1-A final extract:

$$\text{ng prometon/mL} = \frac{0.582 - 0.021329731387}{0.68145223117} = 0.8228 \text{ ng/mL}$$

where 0.582 is the peak area ratio of prometon/prometryn (m/z 226.0/142.0) for F1-A

The prometon residue (ppm) for F1-A =

$$\frac{0.8228 \text{ ng/mL} \times 4 \text{ mL (Final Extract Volume)} \times 25 \text{ (Dil. Factor)} \; 2.5 \text{ ng/mL (Internal Std. Conc.)}}{2 \text{ mL (Initial Extract Volume)} \times 1000 \text{ ng/}\mu g}$$

$= 0.10285$ mg/L

The percent recovery of fortified sample F1-A (Prometon):

{[0.10285 mg/L – 0.000 mg/L (avg. control residue)] ÷ 0.1 mg/L (fort. level)} x 100%

= 103%

Calibration Range

The calibration curve, ranging from 0.1 ng/mL to 50 ng/mL, was generated by Analyst® software for the method validations.

Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.1 ppm for water and 0.05 ppm for sediment and soil, refer to references [1], [2], and [3] for determinations.

Limit of Detection

The limit of detection (LOD) was defined as approximately 20% of LOQ, refer to references [1], [2], and [3] for determinations.

Time Required for Completion of a Sample Set

A sample set consisted of a reagent blank (extraction solvent), two controls (untreated samples), and five fortified samples (at each level i.e. LOQ and 10X LOQ). Time required for one set from preparation of standard solutions, initiation of extraction, until the completion of instrumental analysis and data evaluation is as follows:

- Preparation of standard solutions takes approximately 6 hours
- Sample preparation takes approximately 4 hours
- LC-MS/MS analysis and data processing (two MS/MS transitions) take approximately 5 hours

TOTAL = approximately 15 hours for one analyst to complete a set to satisfy the validation requirements.

Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression fit were the only statistical methods employed in this study.

Method Modification

Two method modifications occurred:

The original method indicated the use of a THERMO EC Betasil C-18 (50x 2.1 mm, 5 µm particle size analytical column fitted with a THERMO EC Javelin Betasil C-18 (10x 2.1 mm) guard column. However this ILV was conducted using a Thermo-Scientific Hypersil Gold, 1.9 µ, 50 x 2.1 mm analytical column fitted with a Thermo-Scientific Hypersil Gold 3 µ, 10 x 2.1 mm guard column. This modification was made due to the availability of the THERMO EC Betasil analytical column.

The original method indicated the use of a 5 µL injection volume. However due to increased sensitivity the injection volume was decreased to 1 µL for this ILV.

Correspondence

There was no contact with the laboratory that developed the original method during the conduct of this study.

CONCLUSIONS

An independent laboratory validation for the analysis of prometon in water, sediment, and soil was successfully validated at both the LOQ and 10X LOQ levels by LC-MS/MS using the analytical method.

The recovery data for the independent laboratory validation in water, sediment, and soil at the LOQ and 10X LOQ levels demonstrated acceptable precision and accuracy of the analytical method. Therefore, the analytical method validated in this study was demonstrated to be suitable for the determination of prometon in water, sediment, and soil, as the method was designed for.

No interference residues were detected in control matrices at the two transition ions monitored for prometon. No significant matrix suppression or enhancement was observed in controls (untreated samples).

This study has met the requirements and acceptance criteria outlined in EPA guideline OCSPP 850.6100 and European Commission Directive SANCO/825/00 rev. 8.1 16/11/2010. The study was also in compliance with Good Laboratory Practices (GLP) as stated in 40 CFR Part 160 and in consideration of the OECD ENV/JM/MONO (2007) 17.

REFERENCES

1. MacGregor, Jon, 2014. Validation of a Method for the Determination of Prometon in Freshwater for Support of Aquatic Field Dissipation Studies. MANA Study No. 90017908

2. MacGregor, Jon, 2015. Validation of a Method for the Determination of Prometon in Sediment for Support of Aquatic Field Dissipation Studies. MANA Study No. 90017909

3. MacGregor, Jon, 2015. Validation of a Method for the determination of Prometon in Soil for Support of Terrestrial Field Dissipation Studies. MANA Study No. 90017910

Figure 1. Extraction Schematic Diagram: Prometon from Water.

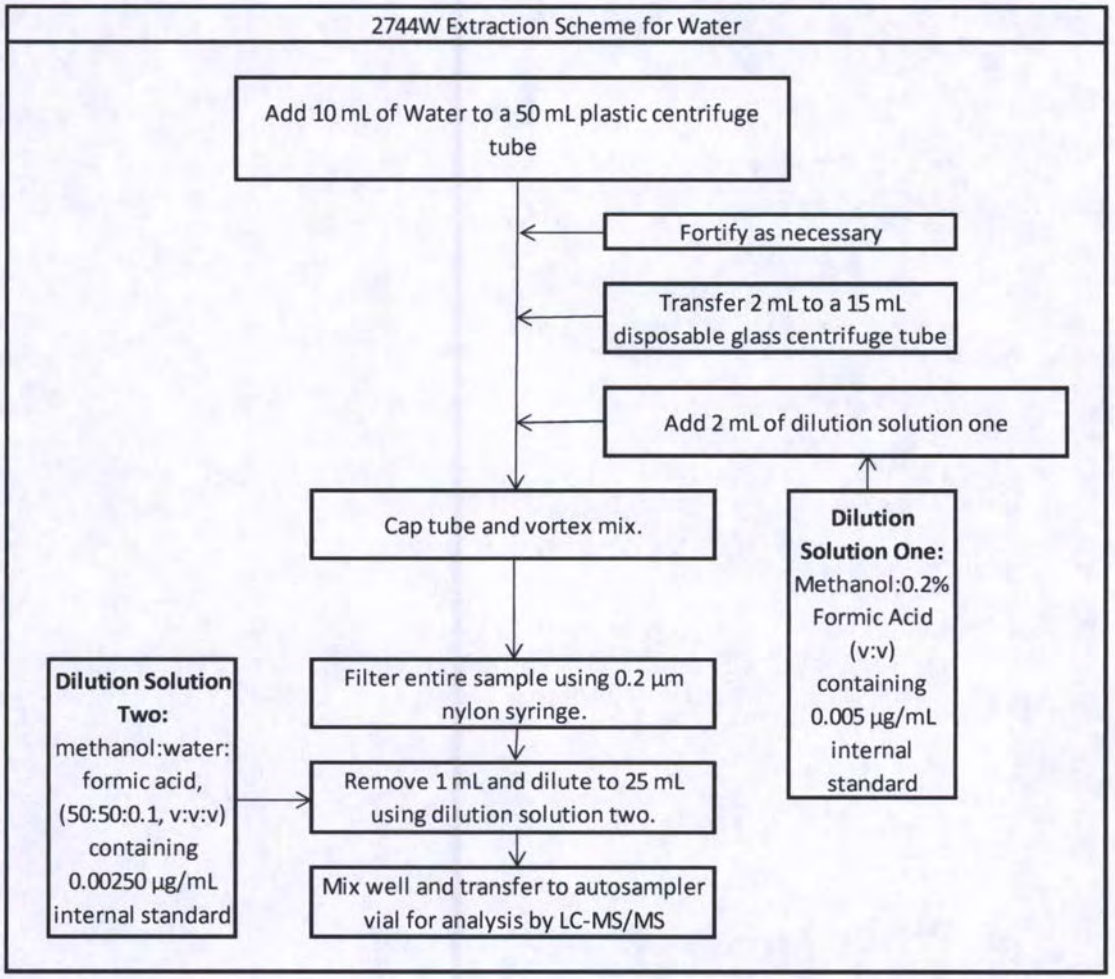


Figure 2. Extraction Schematic Diagram: Prometon from Sediment/Soil.