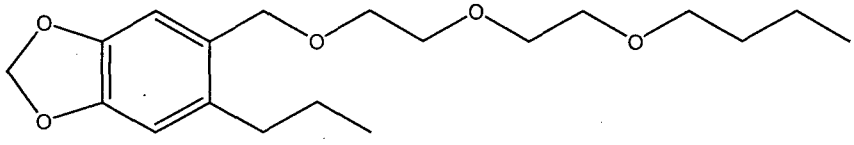
INTRODUCTION

The purpose of this method is to describe procedures to be employed for the analysis of PBO and degradates, PBO-alcohol, PBO-aldehyde and PBO-acid in soil, sediment, ground water and surface water. This method has been validated at Ricerca Biosciences under study 032384.

MATERIALS AND EQUIPMENT

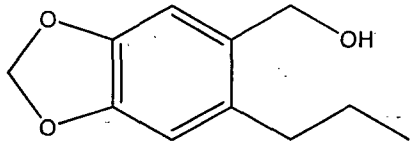
Reference Standards

- PBO



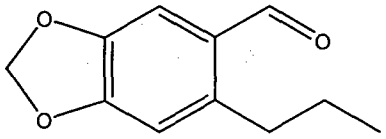
Common Name:	PBO
Chemical Name:	5-[2-(2-butoxyethoxy) ethoxymethyl]-6-propyl-1,3-benzodioxole
CAS No:	51-03-6
Molecular Formula:	$C_{19}H_{30}O_5$
Lot/Batch No:	R1309008
Purity:	95.0%
Molecular Weight:	338.4 g/mole

- **PBO-alcohol**



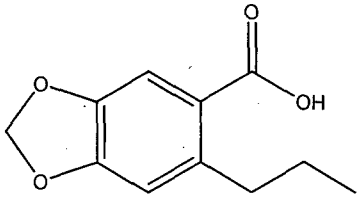
Common Name:	PBO-alcohol
Chemical Name:	(6-propylbenzo[d][1,3]dioxol-5-yl) methanol
CAS No:	21809-60-9
Molecular Formula:	$C_{11}H_{14}O_3$
Lot No:	425-13
Purity:	98.3%
Molecular Weight:	194.23 g/mole

- **PBO-aldehyde**



Common Name:	PBO-aldehyde
Chemical Name:	6-propylbenzo[d][1,3]dioxol-5-carbaldehyde
CAS No:	34827-22-0
Molecular Formula:	$C_{11}H_{12}O_3$
Lot No:	427-13
Purity:	99.2%
Molecular Weight:	192.21 g/mole

- **PBO-acid**



Common Name:	PBO-acid
Chemical Name:	6-propy-lbenzo[1,3]dioxol-5-carboxylic acid
CAS No:	23505-33-1
Molecular Formula:	$C_{11}H_{12}O_4$
Lot No:	182-13
Purity:	98.0%
Molecular Weight:	208.21 g/mole

REAGENTS

Equivalent substitutions may be made for the following reagents.

Acetonitrile (ACN)	Optima Grade, Fisher Scientific
Water	HPLC Grade, Fisher Scientific
Ammonium acetate	≥99.99, Sigma Aldrich
Formic acid	ACS Reagent ≥96%, Sigma Aldrich
Methanol	HPLC Grade, Fisher Scientific
Hydrochloric acid (HCl)	Certified ACS Plus, Fisher Scientific

EQUIPMENT

Equivalent substitutions may be made for the following equipment.

Analytical balance	Sartorius ME 215P and Mettler AE200
Mechanical shaker	Eberbach E6010 2-speed
Pipets	Rainin EDP3 100 µL to 1 mL Gilson Microman 10 µL to 100 µL
Centrifuge	Jouan C14 and Eppendorf 5417R
Filter	Whatman 25 mm, GDX, 0.25 µm, PVDF
Vials	1.5 mL amber glass for autosampler
Vial inserts	100 uL inserts for autosampler vials
Scintillation vials	20 mL, amber with caps
Vortexer	Fisher Genie II
Centrifuge tubes	50 mL Corning Falcon polypropylene with caps
SPE collection tubes	Reusable, glass, graduated 10 mL
SPE manifold	12 port Burdick and Jackson
SPE cartridges	Waters Oasis HLB 6cc 200 mg pn# WAT106202
HPLC column	Phenomenex Luna C8(2) 3u, 30 x 2 mm
Guard column cartridge	Phenomenex Security Guard C18
HPLC pumps	Shimadzu LC-10ADvp or LC-10ATvp
HPLC system controller	Shimadzu SCL-10Avp
HPLC autosampler	CTC Analytical Combi PAL with refrigerated tray
Mass spectrometer	AB Sciex API 4000

SOLUTIONS AND REAGENTS

Miscellaneous Solutions and Mobile Phase

The following solution preparation procedures may be changed for optimization or adjusted proportionally to accommodate larger or smaller quantities of reagents needed. All changes will be properly documented.

Mobile Phase A-1: Ammonium acetate (10 mM, pH 5.5)

Add 0.77 g ammonium acetate to 1 liter of water. Adjust the pH with a pH meter to 5.5 using 6 N HCl. Store ambient. Expires in two weeks.

Mobile Phase B-1: Acetonitrile

Store ambient. Expires in two years.

Mobile Phase A-2: 0.1% formic acid in water
Add 1.0 mL formic acid to 1 liter of water. Store ambient. Expires in two weeks.

Mobile Phase B-2: 0.1% formic acid in acetonitrile
Add 1.0 mL formic acid to 1 liter of acetonitrile. Store ambient. Expires in two weeks.

Needle Wash Solution 1: Acetonitrile
Store ambient. Expires in two years.

Needle Wash Solution 2: Water
Store ambient. Expires in two years.

6 N HCl
Combine 5 mL water with 5 mL HCl and mix. Store ambient. Expires in three months.

50/50 Water/acetonitrile, v/v
Combine 50 mL water with 50 mL acetonitrile and mix. Store ambient. Expires in three months.

90/10 Water/acetonitrile, v/v
Combine 90 mL water with 10 mL acetonitrile and mix. Store ambient. Expires in three months.

Extraction Solution: 10/90 Water/acetonitrile, v/v
Combine 100 mL water with 900 mL acetonitrile and mix. Store ambient. Expires in three months.

0.1% formic acid in methanol
Add 1.0 mL formic acid to 1 liter of methanol. Store ambient. Expires in three months.

Stock Solution Preparation

Stock solutions for the four analytes will be prepared in duplicate in vessels using the appropriate weight for the lot purity correction. The primary stock solutions will be stored in amber glass vials and stored in a refrigerator at approximately 4 °C. A three month expiry was assigned to all stock solutions, however no expiry time intervals were experimentally determined.

The following series of stock solutions will be prepared in volumetric flasks. Dilutions will be generally made as described below; however, the weights, volumes and stock solution concentrations may vary.

Preparation of Primary Stock Solutions

PBO Primary Stock Solution (1,000 µg/mL)

Accurately weigh an amount of PBO and transfer to a 10 mL volumetric flask using acetonitrile. Bring to volume with acetonitrile. Correct the weighing for purity. Calculate the exact concentration of the stock solution. Two separate primary stock solutions will be prepared. Once demonstrated that the two stocks are equivalent, either stock solution will be used to prepare additional stock solutions.

PBO-alcohol Primary Stock Solution (1,000 µg/mL)

Accurately weigh an amount of PBO-alcohol and transfer to a 10 mL volumetric flask using acetonitrile. Bring to volume with acetonitrile. Correct the weighing for purity. Calculate the exact concentration of the stock solution. Two separate primary stock solutions will be prepared. Once demonstrated that the two stocks are equivalent, either stock solution will be used to prepare additional stock solutions.

PBO-aldehyde Primary Stock Solution (1,000 µg/mL)

Accurately weigh an amount of PBO-aldehyde and transfer to a 10 mL volumetric flask using acetonitrile. Bring to volume with acetonitrile. Correct the weighing for purity. Calculate the exact concentration of the stock solution. Two separate primary stock solutions will be prepared. Once demonstrated that the two stocks are equivalent, either stock solution will be used to prepare additional stock solutions.

PBO-acid Primary Stock Solution (1,000 µg/mL)

Accurately weigh an amount of PBO-acid and transfer to a 10 mL volumetric flask using 50/50 water/acetonitrile. Bring to volume with 50/50 water/acetonitrile. Correct the weighing for purity. Calculate the exact concentration of the stock solution. Two separate primary stock solutions will be prepared. Once demonstrated that the two stocks are equivalent, either stock solution will be used to prepare additional stock solutions.

Preparation of Secondary Stock Solutions for Soil and Sediment Analysis

Secondary Mixed Stock Solution (5,000 ng/mL)

Transfer 0.050 mL of each of the four 1,000 µg/mL primary stock solutions to a 10 mL volumetric flask. Dilute to volume with 50/50 water/acetonitrile and mix well. (The volume of the primary stock solutions transferred should be adjusted to produce the desired concentration.)

Secondary Mixed Stock Solution (1,000 ng/mL)

Transfer 0.20 mL of the 5,000 ng/mL secondary mixed stock solution to a 1.5 mL autosampler vial. Add 0.80 mL of 50/50 water/acetonitrile to the vial and mix.

Secondary Mixed Stock Solution (100 ng/mL)

Transfer 0.020 mL of the 5,000 ng/mL secondary mixed stock solution to a 1.5 mL autosampler vial. Add 0.980 mL of 50/50 water/acetonitrile to the vial and mix.

Preparation of Secondary Stock Solutions for Ground Water and Surface Water Analysis

Secondary Stock Solution PBO-acid Stock Solution (100,000 ng/mL)

Transfer 1.0 mL of the 1,000 µg/mL PBO-acid primary stock solutions to a 10 mL volumetric flask. Dilute to volume with 50/50 water/acetonitrile and mix well. (The volume of the primary stock solutions transferred should be adjusted to produce the desired concentration.)

Secondary Stock Solution PBO, PBO-alcohol, PBO-aldehyde Stock Solution (10,000 ng/mL)

Transfer 0.1 mL of the 1,000 µg/mL PBO primary stock solution, 1,000 µg/mL PBO-alcohol primary stock solution, and 1,000 µg/mL PBO-aldehyde primary stock solution to a 10 mL volumetric flask. Dilute to volume with 50/50 water/acetonitrile and mix well. (The volume of the primary stock solutions transferred should be adjusted to produce the desired concentration.)

Secondary Stock Solution PBO, PBO-alcohol, PBO-aldehyde Stock Solution (1,000 ng/mL), and PBO-acid (10,000 ng/mL)

Transfer 1.0 mL of the 10,000 µg/mL PBO, PBO-alcohol, PBO-aldehyde secondary stock solution, and 1.0 mL of the 100,000 ng/mL PBO-acid secondary stock solution to a 10 mL volumetric flask. Dilute to volume with 50/50 water/acetonitrile and mix well.

Secondary Stock Solution PBO, PBO-alcohol, PBO-aldehyde Stock Solution (100 ng/mL), and PBO-acid (1,000 ng/mL)

Transfer 1.0 mL of the 1,000 ng/mL PBO, PBO-alcohol, PBO-aldehyde and 10,000 ng/mL Secondary Stock Solution to a 10 mL volumetric flask. Dilute to volume with 50/50 water/acetonitrile and mix well.

Preparation of Calibration Standards for Soil and Sediment Analysis

The following standards were prepared in 90/10 water/acetonitrile solution in amber glass autosampler vials.

Standard (ng/mL)	Source Solution Concentration (ng/mL)	Aliquot Volume (mL)	Solvent Volume (mL)	Final Volume (mL)
50	1,000	0.05	0.95	1.0
25	1,000	0.025	0.975	1.0
10	100	0.10	0.90	1.0
5.0	50	0.10	0.90	1.0
2.0	25	0.08	0.92	1.0
1.4	10	0.14	0.86	1.0
1.0	10	0.10	0.90	1.0
0.40	5.0	0.08	0.92	1.0
0.30	5.0	0.06	0.94	1.0
0.20	5.0	0.04	0.96	1.0

Preparation of Calibration Standards for Surface Water and Ground Water Analysis

The following standards were prepared in 90/10 water/acetonitrile solution in amber glass autosampler vials.

PBO, PBO-alcohol, PBO-aldehyde Conc. (ng/mL)	PBO-acid Conc. (ng/mL)	Source Solution Conc. PBO, PBO-alcohol, PBO-aldehyde/PBO-acid (ng/mL)	Aliquot Volume (mL)	Solvent Volume (mL)	Final Volume (mL)
10.0	100.0	100/1,000	0.10	0.90	1.0
7.5	75	100/1,000	0.075	0.925	1.0
5.0	50	100/1,000	0.050	0.95	1.0
2.0	20	100/1,000	0.020	0.98	1.0
1.0	10	10.0/100	0.10	0.90	1.0
0.50	5.0	10.0/100	0.050	0.95	1.0
0.350	3.50	10.0/100	0.035	0.965	1.0
0.250	2.50	10.0/100	0.025	0.975	1.0
0.10	1.0	1.0/10.0	0.10	0.90	1.0
0.060	0.60	1.0/10.0	0.060	0.94	1.0
0.040	0.40	1.0/10.0	0.040	0.96	1.0

Note: The preparation of the calibration standards may be scaled up or down by adjusting the constituents proportionately. Alternate source solutions may be used to generate the target concentrations.

Sample Sets
Soil samples will be analyzed in sets that can be extracted in one day. A method validation set will consist of one reagent blank, two untreated controls, five controls fortified at the LOQ and five controls fortified at ten times the LOQ (10xLOQ). For soil and sediment sets, the reagent blank consists only of extraction solvent in the extraction jar. For ground water and surface water sets, HPLC water may be processed through the method as a reagent blank.

Processing a sample set will result in one set of extracts for PBO, and another set of extracts for the three degradates. One set of extracts can be analyzed on the day of extraction, and the second set of extracts can be analyzed the day after extraction. The extracts should be stored refrigerated.

The injections of the run will be arranged to have the samples interspersed among the calibration standards, with the run beginning and ending with calibration standards. A single injection will be made of each sample and calibration standard. There will be a minimum of six calibration standards that bracket the concentration range of the QC samples. The concentration of the lowest calibration standard will correspond to 70% or less of the lowest QC fortification concentration. The concentration of the highest calibration standard will correspond to 120% or more of the highest QC fortification concentration.

The table below lists the calibration standards used for the analyses. Each calibration range consisted of 7 or 8 standards.

Matrix	Analytes	Calibration Standard (ng/mL)
Soil and sediment	PBO	0.2, 0.3, 0.4, 1.0, 2.0, 5.0, 10 ng/mL
	PBO-alcohol, PBO-aldehyde, PBO-acid	1.0, 1.4, 2.0, 5.0, 10, 25, 50 ng/mL
Ground water and surface water	PBO	0.04/0.4, 0.06/0.6, 0.1/1.0, 0.25/2.5, 0.5/5.0, 1.0/10, 2.0/20, 5.0/50 ng/mL[1]
	PBO, PBO-alcohol, PBO-aldehyde, PBO-acid	0.25/2.5, 0.35/3.5 0.5/5.0, 1.0/10, 2.0/20, 5.0/50, 7.5/75, 10/100 ng/mL[1]

[1]The lower concentration is for PBO, PBO-alcohol and PBO-aldehyde. The higher concentration is PBO-acid.

FORTIFICATION OF SAMPLES

The table below lists the fortification volumes and solutions for the LOQ and 10xLOQ levels.

Matrix	Fortification Concentration	Fortification volume and solution
Soil and sediment	10 ng/g all analytes	0.04 mL of 5,000 ng/mL PBO, PBO-alcohol, PBO-aldehyde, PBO-acid
	100 ng/g all analytes	0.4 mL of 5,000 ng/mL PBO, PBO-alcohol, PBO-aldehyde, PBO-acid

Ground water and surface water	0.1 ng/mL PBO, PBO-alcohol, PBO-aldehyde; 1.0 ng/mL PBO-acid	0.04 mL of 100 ng/mL PBO, PBO-alcohol, PBO-aldehyde and 1,000 ng/mL PBO-acid
	1.0 ng/mL PBO, PBO-alcohol, PBO-aldehyde; 10.0 ng/mL PBO-acid	0.04 mL of 1,000 ng/mL PBO, PBO-alcohol, PBO-aldehyde and 10,000 ng/mL PBO-acid

EXTRACTION PROCEDURE FOR SOIL AND SEDIMENT

1. Weigh 20 +/- 0.2 g (dry weight) soil into a glass wide mouth jar. (Note: The soil and sediment were weighed as is. They were not dried before weighing.)
2. Fortify the recovery samples with the four analytes.
3. Add 50 mL Extraction Solution to the jar. Shake by hand to distribute the solvent and soil.
4. Place the jars on a shaker for at least 4 hours.
5. Place the jars on the bench to allow the soil to settle.
6. Centrifuge an aliquot (1-2 mL) to clear the extract.
7. For PBO, dilute the extract 10-fold by combining and mixing 0.1 mL of extract with 0.9 mL water into an autosampler vial and mix.
8. For PBO-alcohol, PBO-aldehyde and PBO-acid, dilute the extract 2-fold by combining and mixing 0.5 mL of extract with 0.5 mL water in an autosampler vial.
9. Place the samples in the refrigerated autosampler tray for analysis.

EXTRACTION PROCEDURE FOR GROUND WATER AND SURFACE WATER

1. Transfer 40 mL ground water or surface water to a 50 mL centrifuge tube.
2. Fortify with the four analytes and mix well.
3. Add 4.0 mL 0.1% formic acid in methanol and mix well.
4. Place 0.10 mL of each sample in glass inserts in autosampler vials for PBO analysis
5. Setup SPE cartridges in manifold over a waste collection container.
6. Condition with 3 mL methanol, then 3 mL water.
7. Note: The vacuum should be adjusted to have the solvent elute at 1-2 drops per second.
8. Add the water sample to the SPE.
9. After elution stops, dry the cartridges under high vacuum for 2 min. to remove the water.
10. Insert 10 mL glass, graduated centrifuge tubes and elute the analytes with 4.0 mL 0.1% formic acid in methanol.
11. After elution, increase the vacuum to completely collect the eluant remaining in the SPE column packing.
12. Add methanol to the tubes to bring to the 4 mL mark, if necessary.
13. Cap and mix the centrifuge tubes.
14. For PBO-alcohol, PBO-aldehyde and PBO-acid, dilute the eluant 2-fold by combining 0.5 mL extract with 0.5 mL water in an autosampler vial and vortex.

LC-MS/MS CONDITIONS FOR PBO

The following are LC-MS/MS conditions were used for the analysis of PBO in all matrices

HPLC Conditions for PBO:

HPLC column:	Phenomenex Luna C8(2) 3µ, 30 x 2 mm
Guard column:	Phenomenex Security Guard C18
Mobile phase A-1:	Ammonium acetate (10 mM, pH 5.5)
Mobile phase B-1:	Acetonitrile
Needle wash 1:	Acetonitrile
Needle wash 2:	Water
Injection volume:	5 µL
Flow rate:	0.3 mL/min
Autosampler temperature:	6 °C
HPLC column:	ambient
Diverter valve:	Divert the column flow to waste before and after peak elution

Gradient:

Time (Minute)	0	3.0	4.0	4.1	6.0
%B	30	90	90	30	Stop

MS/MS Conditions for PBO:

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Ion Pair	*m/z* 356.2/177.1
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	40 psi
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	Medium
Ion Spray Voltage (IS):	5000 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	38 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	50 V^1 & 19 V^2
Collision Gas Exit Potential (CXP):	8V

[1]CE = 50 V was used for soil and sediment analysis.
[2]CE = 19 V was used for ground water and surface water analysis.

Mass settings for ions monitored may vary slightly from instrument to instrument as quadrupole mass spectrometers operate at unit mass resolution rendering minor differences in the tenths place of mass settings of no significance.

The voltage and gas settings may be modified to optimize sensitivity. PBO has very good sensitivity by LC-MS/MS. Poor calibration curve linearity can result if the sensitivity is too high. The PBO sensitivity is easily adjusted by modifying the collision energy parameter.

LC-MS/MS CONDITIONS FOR PBO-ALCOHOL, PBO-ALDEHYDE AND PBO-ACID

The following are LC-MS/MS conditions were used for the analysis of PBO-alcohol, PBO-aldehyde and PBO-acid in all matrices.

HPLC Conditions for PBO-alcohol, PBO-aldehyde and PBO-acid:

HPLC column:	Phenomenex Luna C8(2) 3u, 30 x 2 mm
Guard column:	Phenomenex Security Guard C18
Mobile phase A-2:	0.1% Formic acid in water
Mobile phase B-2:	0.1% Formic acid in acetonitrile
Needle wash 1:	Acetonitrile
Needle wash 2:	Water
Injection volume:	20 µL
Flow rate:	0.3 mL/min
Autosampler temperature:	6 °C
HPLC column:	ambient
Diverter valve:	Divert the column flow to waste before and after peak elution

Gradient:

Time (Minute)	0	3.0	4.0	4.1	6.0
%B	30	90	90	30	Stop

MS/MS Conditions for PBO-alcohol, PBO-aldehyde and PBO-acid:

Parameter	PBO-alcohol	PBO-aldehyde	PBO-acid
Scan Type:	MRM	MRM	MRM
Polarity:	Positive	Positive	Positive
Ion Source:	Turbo Spray	Turbo Spray	Turbo Spray
Ion Pair	*m/z* 177.2/119.2	*m/z* 193.2/107.0	*m/z* 191.0/133.0
Resolution Q1	Unit	Unit	Unit
Resolution Q3	Unit	Unit	Unit
Ion Source Gas 1 (GS1):	50 psi	50 psi	50 psi
Ion Source Gas 2 (GS2):	40 psi	40 psi	40 psi
Curtain Gas (CUR):	30 psi	30 psi	30 psi
Collision Gas (CAD):	Medium	Medium	Medium
Ion Spray Voltage (IS):	5000 V	5000 V	5000 V
Temperature (TEM):	500 °C	500 °C	500 °C
Declustering Potential (DP):	50 V	70 V	60 V
Entrance Potential (EP):	10 V	10 V	10 V
Collision Energy	23 V	26 V	26 V
Collision Gas Exit Potential (CXP):	5 V	6 V	10 V

Mass settings for ions monitored may vary slightly from instrument to instrument as quadrupole mass spectrometers operate at unit mass resolution rendering minor differences in the tenths place of mass settings of no significance. The voltage and gas settings may be modified to optimize sensitivity.

ANALYTE CONCENTRATIONS IN SAMPLE EXTRACTS

The nominal concentrations of the analytes in each of the matrix extracts were calculated. These values were compared to the measured analyte concentrations to calculate the percent recoveries.

Soil:

A 20 g wet weight soil sample was extracted in 50 mL extraction solvent, and then diluted for analysis. Since the soil contained low moisture content, the volume of water contained in the 20 g soil sample was not factored into the equation to calculate the final analyte concentration.

PBO concentration in final extract:
LOQ fortification level: 0.04 mL x 5,000 ng/mL/ 50.0 mL/ 10 mL = 0.40 ng/mL

10x LOQ fortification level: 0.4 mL x 5,000 ng/mL/ 50.0 mL/ 10 mL = 4.0 ng/mL

PBO-alcohol, PBO-aldehyde, PBO-acid concentration in final extract:
LOQ fortification level: 0.04 mL x 5,000 ng/mL/ 50.0 mL/ 2 mL = 2.0 ng/mL
10x LOQ fortification level: 0.4 mL x 5,000 ng/mL/ 50.0 mL/ 2 mL = 20.0 ng/mL

Sediment:
Sediment EFS-471 used in validation set 062014 contained 17.12% moisture. The moisture content in the sediment was factored into the equation to calculate the final analyte concentrations. A 20 g dry weight sediment sample was extracted in 50 mL extraction solvent, and then diluted for analysis.

Amount of moisture in a 20 g sediment sample:
20 g + (17.12/100 x 20 g) = 3.42 g or 3.42 mL
The 3.42 mL water contained in the sediment sample was added to the volume of extraction solvent.

Analyte concentration at LOQ fortification level:
2x Dilution (PBO-alcohol, PBO-aldehyde, PBO-acid): 0.04 mL x 5,000 ng/mL/ 53.42 mL/ 2 mL = 1.87 ng/mL
10x Dilution (PBO): 0.04 mL x 5,000 ng/mL/ 53.42 mL/ 10 mL = 0.374 ng/mL

Analyte concentration at 10xLOQ fortification level:
2x Dilution (PBO-alcohol, PBO-aldehyde, PBO-acid): 0.4 mL x 5,000 ng/mL/ 53.42 mL/ 2 mL = 18.7 ng/mL
10x Dilution (PBO): 0.4 mL x 5,000 ng/mL/ 53.42 mL/ 10 mL = 3.74 ng/mL

Ground water and surface water:
A 40 mL surface water or ground water sample was combined with 4.0 mL of 0.1% formic acid in methanol and mixed. A 0.1 mL aliquot (extract A) was removed for PBO analysis. After passing the remaining water sample through the SPE procedure, the final elution volume was 4.0 mL. The extract was diluted 2-fold (extract B).

PBO concentration in final extract A:
LOQ fortification level: 0.04 mL x 100 ng/mL/ 44.0 mL = 0.0909 ng/mL
10x LOQ fortification level: 0.04 mL x 1,000 ng/mL/ 44.0 mL = 0.909 ng/mL

PBO-alcohol, PBO-aldehyde concentration in final extract B:
LOQ fortification level: 0.04 mL x 100 ng/mL/ 4.0 mL/ 2.0 mL = 0.50 ng/mL
10x LOQ fortification level: 0.04 mL x 1,000 ng/mL/ 4.0 mL/ 2.0 mL = 5.0 ng/mL

PBO-acid concentration in final extract B:
LOQ fortification level: 0.04 mL x 1,000 ng/mL/ 4.0 mL/ 2.0 mL = 5.0 ng/mL
10x LOQ fortification level: 0.04 mL x 10,000 ng/mL/ 4.0 mL/ 2.0 mL = 50.0 ng/mL

CARRYOVER

Significant carryover was not observed for any of the analytes during analytical runs. Should carryover occur, it can be mitigated by placing solvent blanks before injections predicted to be of low concentration as needed to reduce carryover from samples predicted to be of high concentration.

CONDITIONING INJECTIONS

Before starting the analytical run, inject several injections of standards to condition the system. The conditioning injections should be clearly identified.

DATA PROCESSING

Once the run is complete, copy the Analyst data files from the LC-MS/MS data system computer to the corresponding LC/MS instrument folder on the Company network. Use the Analyst quantitative analysis tools to integrate chromatographic peaks and save the results table file.

Check the calibration standards for acceptability according to the protocol acceptance criteria. If the calibration curve is acceptable, check the QC samples for acceptability according to the protocol acceptance criteria.

Examine the control and/or reagent blank. If analyte residues are detected in the control or reagent blank at a concentration above the lowest calibration standard, determine the impact to the integrity of the run.

An external standard calibration curve will be constructed using the peak areas and concentrations of the calibration standards. A weighted linear regression (1/x) will be performed to determine slope and y-intercept values. The calibration curve will not be forced through zero.

An analytical run is considered to be acceptable if the following conditions are met:

- Standards are accepted if they do not deviate by more than ±15% (±20% for the lowest concentration) of the nominal concentration. Standards that do not meet these criteria may be rejected from the calibration curve.
- The r^2 value must be ≥ 0.98.
- At least six standards meet their respective acceptance criterion, with acceptable standards bracketing the QC samples.
- The calibration range may be reduced to improve linearity provided that acceptable standards bracket the sample extract concentrations.
- The mean percent recovery of the QC samples at both concentration levels must be in the range of 70-120%, and the relative standard deviations must be less than 20%.