RETENTION OF DATA

Upon completion of the study, the complete study file including all original raw data was submitted to the Ricerca Biosciences, LLC Corporate Archives.

CONDUCT OF THE STUDY

The study was conducted at the Ricerca Biosciences, LLC AgChem Product Development Department Laboratories according to the Ricerca Biosciences, LLC protocol, Document Number 032385-0, "Independent Laboratory Validation (ILV) Study of PBO and Degradates PBO-alcohol, PBO-aldehyde and PBO-acid in Soil, Sediment, Ground Water and Surface Water" (APPENDIX A). The study director and any support staff who worked on the ILV had no prior knowledge of the method and the analysts, equipment, instruments, and supplies (e.g., glassware, solvents, reagents, standard reference materials) were distinct and operate separately and without collusion for the validation. Furthermore, the personnel conducting the independent validation do not report to the same management who was involved in developing the original method.

Personnel involved with the study were:

M. Fleshman	Associate Scientist III
K. Oden	Associate Scientist III

INTRODUCTION

This report describes the independent laboratory validation (ILV) of the analytical method for the analysis of PBO, PBO-alcohol, PBO-aldehyde and PBO-acid, in soil, sediment, ground water and surface water which had previously been validated.

OBJECTIVE/PURPOSE

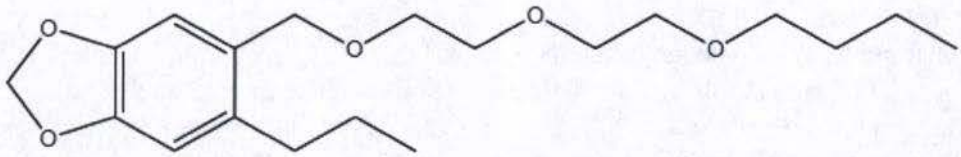
An analytical method using LC/MS for the analysis of PBO, PBO-alcohol, PBO-aldehyde and PBO-acid has been developed and validated previously (Ricerca Study Number 032384, Analytical Methods For the Determination of PBO and Degradates PBO-alcohol, PBO-aldehyde and PBO-acid in Soil, Sediment, Ground Water and Surface Water [1]). The purpose of this study was to perform an independent laboratory validation of the analytical method for analysis of PBO, PBO-alcohol, PBO-aldehyde and PBO-acid in soil, sediment, ground water and surface water.

The independent laboratory validation was conducted to satisfy the guideline requirements described in the harmonized guidelines from the OCSPP, Field Test Data Reporting Guidelines for Environmental Chemistry Methods and Associated Independent Laboratory Validation, OCSPP 850.6100 [2].

TEST SUBSTANCE AND REFERENCE STANDARD

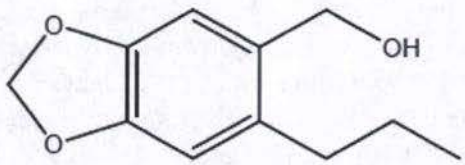
The Sponsor supplied the test substances: PBO, PBO-alcohol, PBO-aldehyde and PBO-acid. The test substances were stored refrigerated (~5 °C). The certificate of analysis for the test substances are given in APPENDIX B. Information concerning the test substance including purity is provided as follows:

- **PBO**



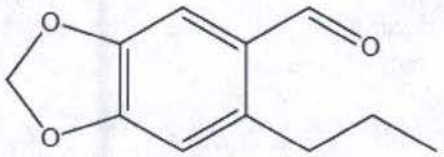
Common Name:	PBO
Chemical Name:	5-[2-(2-butoxyethoxy) ethoxymethyl]-6-propyl-1,3-benzodioxole
CAS No:	51-03-6
Molecular Formula	$C_{19}H_{30}O_5$
Batch No:	R1309008
Purity (%):	95.0%
Molecular Weight:	338.4 g/mole

- **PBO-alcohol**



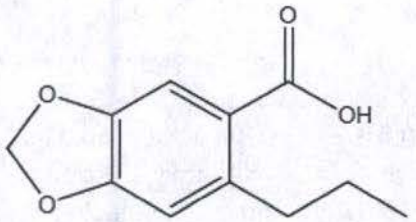
Common Name:	PBO-alcohol
Chemical Name:	(6-propylbenzo[d][1,3]dioxol-5-yl) methanol
CAS No:	21809-60-9
Molecular Formula	$C_{11}H_{14}O_3$
Batch No:	E56/13
Purity:	98.3%
Molecular Weight:	194.23 g/mol

- **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:	E58/13
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:	E57/13 (EN 01-93/6/A)
Purity:	98.0%
Molecular Weight:	208.21 g/mole

TEST SYSTEM

The test systems, untreated soil, sediment, ground water and surface water were supplied by Ricerca Biosciences, Concord, OH. The soil was collected by Research for Hire, California on 4/10/2014, and was given the Ricerca ID EFS-473. The sediment was collected from Moniteau

Creek, Missouri on 4/9/2014, and was given the Ricerca ID EFS-471. The ground water was collected from Madison, Ohio on 6/12/2014, and was given the Ricerca ID EFS-495. The surface water was collected from Moniteau Creek, Missouri on 4/9/2014, and was given the Ricerca ID EFS-471. All samples were stored in a refrigerator until use. The characterization reports for the soil, sediment, ground water and surface water are included in APPENDIX C.

SOLVENTS AND REAGENTS

Acetonitrile (ACN), Fisher Optima Grade
Formic Acid, Sigma Aldrich, ACS Grade
Water, Fisher HPLC Grade
Ammonium Acetate, Fisher HPLC Grade

STOCK, FORTIFICATION AND CALIBRATION SOLUTIONS

PREPARATION OF PRIMARY STOCK SOLUTION OF PBO, PBO-ALCOHOL, PBO-ALDEHYDE AND PBO-ACID

- PBO, R1: 0.0179g of the PBO (purity 95%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.7005 mg/ml stock solution.
- PBO, R2: 0.0113g of the PBO (purity 95%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.0735 mg/ml stock solution.
- PBO-alcohol, R1: 0.0117g of the PBO-alcohol (purity 98.3%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.15011 mg/ml stock solution.
- PBO-alcohol, R2: 0.0102g of the PBO-alcohol (purity 98.3%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.00266 mg/ml stock solution.
- PBO-aldehyde, R1: 0.0109g of the PBO-aldehyde (purity 99.2%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.08128 mg/ml stock solution.
- PBO-aldehyde, R2: 0.0123g of the PBO-aldehyde (purity 99.2%) was dissolved in a 10 mL Class A volumetric flask in ACN to give 1.22016 mg/ml stock solution.
- PBO-acid, R1: 0.0105g of the PBO-acid (purity 98.0%) was dissolved in a 10 mL Class A volumetric flask in 50/50 ACN/Water to give 1.029 mg/ml stock solution.
- PBO-acid, R2: 0.0102g of the PBO-acid (purity 98.0%) was dissolved in a 10 mL Class A volumetric flask in 50/50 ACN/Water to give 0.9996 mg/ml stock solution.

The samples were mixed to ensure the solid went into solution. These solutions were labeled with the following information: Study number, Test substance name, CACCI identification, concentration, preparation date, storage conditions, and initials and date and stored at < 5° C when not in use. The stock solutions were diluted to 1 ng/mL in 90:10 (Water:ACN) to demonstrate that the stock solutions are equivalent.

PREPARATION OF SECONDARY STOCK SOLUTION OF PBO, PBO-ALCOHOL, PBO-ALDEHYDE AND PBO-ACID

The stock solutions above were further diluted in 50/50 ACN/Water and mixed as shown below for the soil and sediment analysis.

Name	Stock Solution	Concentration of Stock (µg/mL)	Volume Taken (mL)	Final Volume (mL)	Nominal Concentration (ng/mL)
Secondary Mixed Stock Solution-1	PBO	1073.5	0.0466	10	5000
	PBO-alcohol	1002.66	0.0498		
	PBO-aldehyde	1081.28	0.0462		
	PBO-acid	1029	0.0486		
Secondary Mixed Stock Solution-2	Secondary Mixed Stock Solution-1	5000	2	10	1000
Secondary Mixed Stock Solution-3	Secondary Mixed Stock Solution-1	5000	0.2	10	100

For the ground water and surface water the secondary stock solutions were prepared as shown below.

Name	Stock Solution	Concentration of Stock (µg/mL)	Volume taken (mL)	Final volume (mL)	Nominal Concentration (ng/mL)
PBO-acid (100µg/mL)	PBO-acid	1029	0.972	10	100,000
Secondary Mixed Stock Solution-4	PBO	1073.5	0.0932	10	10,000
	PBO-alcohol	1002.66	0.0998		
	PBO-aldehyde	1081.28	0.0924		
Secondary Mixed Stock Solution-5	Secondary Mixed Stock Solution-4	10	1	10	1,000 (10,000 for Acid)
	PBO-acid	100	1		
Secondary Mixed Stock Solution-6	Secondary Mixed Stock Solution-5	1(10)	1	10	100(1,000 for Acid)

CALIBRATION SOLUTIONS

The secondary stock solutions were further diluted in 90/10 Water/ACN in amber glass vials to prepare the calibration standards for soil and sediment analysis as shown below (note: the calibration standards were prepared in 50/50 Water/ACN in Trial 2 to better represent the sample conditions).

Standard (ng/mL)	Standard solution used (ng/mL)	Volume taken (mL)	Solvent volume (mL)	Final volume (mL)
50	1000	0.5	9.5	10
25	1000	0.25	9.75	10
10	100	1	9.0	10
5.0	50	1	9.0	10
2.0	25	0.8	9.2	10
1.4	10	1.4	8.6	10
1.0	10	1	9.0	10
0.4	5	0.8	9.2	10
0.3	5	0.6	9.4	10
0.2	5	0.4	9.6	10

Ground and Surface Water calibration standards were prepared in 90/10 Water/ACN as shown below.

Standard (ng/mL)	Standard solution used (ng/mL)	Volume taken (mL)	Solvent volume (mL)	Final volume (mL)
10/100	100/1000	1	9	10
7.5/75	100/1000	0.75	9.25	10
5/50	100/1000	0.5	9.5	10
2/20	100/1000	0.2	9.8	10
1/10	10/100	1	9	10
0.5/5	10/100	0.5	9.5	10
0.35/3.5	10/100	0.35	9.65	10
0.25/2.5	10/100	0.25	9.75	10
0.1/1	1/10	1	9	10
0.06/0.6	1/10	0.6	9.4	10
0.04/0.4	1/10	0.4	9.6	10

ANALYTICAL METHODOLOGY

The validation sets were analyzed by following the instructions as detailed in the method and no changes or modifications were made to the method with the exception of changing the dilution

solvent for the sediment/soil metabolite calibration to 50:50 ACN:Water from 10:90 ACN:Water in order to improve chromatography. The first trial failed due to instrument failure and sensitivity issues. No modifications were made to the method after the first validation trial.

LC-MS/MS CONDITIONS FOR PBO

Applied Biosystems API 4000 LC/MS/MS

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:	ambient
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-1	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):	6 psi
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

Applied Biosystems API 4000 LC/MS/MS

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:	ambient
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-2	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):	6 psi	6 psi	6 psi
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.

EXAMPLE CALCULATIONS

The analyte concentrations in samples were calculated relative to the linearity curve generated with each set. Analyst version 1.4.2 software was used to generate the linear regression to calculate the analyte concentrations (ng/mL) in the matrix extracts. Below are example % recovery calculations for PBO in a soil sample, and PBO-acid in a ground water sample.

Measured PBO Concentration (ng/mL) in Soil LOQ 2 Extract:

Linear regression formula from calibration curve $y = mx + b$,

Where y = Sample peak area
b = Calibration intercept
m = Calibration slope
y = Concentration (ng/mL)

From Analyst software, the measured concentration of PBO in soil LOQ 2 extract was 0.387 ng/mL.

Nominal PBO Concentration (ng/mL) in Soil LOQ 2 Extract:

A 20 g dry weight (~21.72 g wet weight) soil sample was fortified and extracted in 50 mL extraction solvent, and then diluted 10-fold for analysis. This recovery sample was fortified at the LOQ level (10 ng/g) by fortifying with 0.04 mL of the 5,000 ng/mL standard.

PBO nominal conc. = 0.04 mL x 5,000 ng/mL/ 50 mL/ 10 mL

= 0.4 ng/mL

% Recovery = measured conc./nominal conc. x 100
= 0.387 ng/mL / 0.4 ng/mL x 100 = 96.75%

Measured PBO-alcohol Concentration (ng/mL) in Surface Water LOQ 1 Extract:

From the linear regression formula calculated by Analyst software, the measured concentration of PBO-alcohol in surface water LOQ 1extract was 0.484 ng/mL.

Nominal PBO- alcohol Concentration (ng/mL) in Surface Water LOQ 1 Extract:

The 40 mL ground water sample was fortified at the LOQ level (0.1 ng/mL) by fortifying with 0.04 mL of the 100 ng/mL standard. The sample was combined with 4.0 mL of 0.1% formic acid in methanol and mixed. After the water sample was extracted by the SPE procedure, the final elution volume was 4.0 mL. The extract was diluted 2-fold for analysis.

PBO-alcohol nominal conc. = 0.04 mL x 100 ng/mL/ 4.0 mL/ 2 = 0.5 ng/mL