

## SUMMARY

A method for quantitation of Etofenprox and its metabolites  $\alpha$ -CO and 4'-OH residues in water and soil entitled "Analytical Method Validation for the Determination of Etofenprox,  $\alpha$ -CO and 4'-OH in Soil, Water and Sediment" (Reference 1) successfully underwent independent validation in surface water and soil. The matrices were fortified in replicates of five (5) at 0.01 mg/kg (the LOQ) and 0.10 mg/kg (10x LOQ) in soil and 0.05  $\mu$ g/L and 0.50  $\mu$ g/L in water. Water was repeatedly extracted from fortified matrix with dichloromethane, followed with concentration of the combined organic phase under vacuum. Soil was extracted with acetone and the organic extract was separated from matrix by Si SPE cartridge. The final residues were reconstituted in acetonitrile:water:formic acid (50:50:0.1, v:v:v) and the amount of Etofenprox,  $\alpha$ -CO and 4'-OH was determined by LC-MS/MS relative to external standard linearities.

Based on the sample size and current methodology, the limit of quantitation (LOQ) in the water and soil was equal to the lowest fortification level or 0.01 mg/kg for soil and 0.05  $\mu$ g/L in water. The limit of detection (LOD) was estimated as 1/3 LOQ or 0.0033 mg/kg for soil and 0.017  $\mu$ g/L in water, as per Reference 1.

## INTRODUCTION

Mitsui Chemical Inc. contracted with PTRL West (625-B Alfred Nobel Dr., Hercules, CA 94547) to conduct an independent laboratory validation of an analytical method for determination of residues of etofenprox and its metabolites  $\alpha$ -CO and 4'-OH in water and soil according to the method "Analytical Method Validation For The Determination of Etofenprox,  $\alpha$ -CO and 4'-OH in Soil, Water and Sediment" (Reference 1).

The study is designed to comply with the European Union guidelines for an independent laboratory validation of an analytical method. The study initiation date was November 30, 2005. The experimental termination date was March 31, 2006. See Appendix A for the study protocol.

## MATERIALS AND METHODS

### Reference Substances

Etofenprox,  $\alpha$ -CO and 4'-OH were supplied by Mitsui Chemicals, Inc. with the following lot numbers (see Appendix B) and PTRL designations:

	<u>Lot No.</u>	<u>PTRL No.</u>	<u>Purity (%)</u>	<u>Expiration Date</u>
Etofenprox	90S-01	1450W-001	99.8	Dec. 2009
$\alpha$ -CO	LS9911	1450W-003	98.9	Dec. 2007
4'-OH	043-011222-1	1450W-002	96.0	Dec. 2008

All stock and working solutions were stored at  $< 0^{\circ}\text{C}$  in amber bottles with Teflon<sup>®</sup>-lined screw-top caps. All solutions were prepared using class A volumetric flasks, syringes and pipettes. All reference substances were concluded to be stable in methanol throughout the study period based on comparison of chromatograms generated over the study period.

### Solvents and Reagents

(HPLC Grade or better)

Acetonitrile

Acetone

Dichloromethane

Formic Acid

Hydrochloric Acid

Methanol

Water, deionized

Water, HPLC

Potassium Hydroxide

### Glassware and Miscellaneous Equipment

Balances

Bottle, amber with screw cap lid

Centrifuge bottles, glass

Centrifuge, Mistral 3000E

Glass test tube, 15 mL disposable

Hamilton microliter syringes, various volumes

Nitrogen evaporator

Pipette, disposable

Rotavapor Model RE 111 with Büchi Water Bath Model 461 and Lauda RM 20  
Circulation Bath

125 mL Round Bottom Flasks

125 mL Separatory Funnels

Sonicator (Branson 2510)

SPE Cartridge Vacuum Manifold with stopcocks

Varian Bond Elut ®-Si SPE Cartridges, 500 mg, 3 mL

Vials, Amber glass (2 mL capacity) with Teflon®-lined crimp caps

Volumetric flask, class-A, various volumes

## ANALYTICAL PROCEDURES

### Preparation of Samples

Untreated control water (Lot # LA-05-428) and untreated control soil (Lot# LA-05-33) were received from Wildlife International, Ltd. on December 13, 2005. Samples were stored frozen ( $< 0^{\circ}\text{C}$ ) until sub-aliquoted for fortification and analysis.

### Preparation of Standards

Individual stock standard solutions of Etofenprox, 4'-OH and  $\alpha$ -CO in methanol at 1 mg/mL were prepared. (~100 mg in 100 mL volumetric flask). A 100  $\mu\text{g/mL}$  intermediate mixed standard solution of Etofenprox, 4'-OH and  $\alpha$ -CO was prepared by adding 10.0 mL of each stock solution to a 100 mL volumetric flask and diluting with methanol. The individual stock standards and intermediate mixed standard were stored frozen ( $< 0^{\circ}\text{C}$ ) when not in use.

### Preparation of Fortification Standards

Mixed fortification standards were prepared by dilution of the 100  $\mu\text{g/mL}$  intermediate standard with methanol (10.0 mL of 100.0  $\mu\text{g/mL}$  diluted to 100 mL) to yield a 10  $\mu\text{g/mL}$  fortification standard. The 10  $\mu\text{g/mL}$  mixed standard was then serially diluted with methanol to prepare solutions containing 1.0  $\mu\text{g/mL}$  and 0.10  $\mu\text{g/mL}$  of each compound. Additional solutions were prepared at 0.02  $\mu\text{g/mL}$  and 0.002  $\mu\text{g/mL}$  by dilution of the 1.0  $\mu\text{g/mL}$  and 0.01  $\mu\text{g/mL}$  mixed standards. Dilutions were prepared using volumetric flasks, volumetric pipettes, and Hamilton syringes. The fortification solution was prepared once during the course of the study. The fortification standards were stored at  $< 0^{\circ}\text{C}$  when not in use.

### Sample Fortification

Control matrices of water were fortified with Etofenprox, 4'-OH and  $\alpha$ -CO, in replicates of 5, at 0.05  $\mu\text{g/L}$  and 0.50  $\mu\text{g/L}$ . Control matrices of soil were fortified with Etofenprox, 4'-OH and  $\alpha$ -CO, in replicates of 5, at 0.01 mg/kg and 0.10 mg/kg as follows:

Fortification level (µg/L)	Volume of Water (mL)	Volume of Fort Std. Added
0.05	20.0	500 µL of 0.002 µg/mL
0.50	20.0	500 µL of 0.02 µg/mL
Fortification level (mg/kg)	Weight of Wet Soil (grams)	Volume of Fort Std. Added
0.01	5.0	500 µL of 0.1 µg/mL
0.10	5.0	500 µL of 1.0 µg/mL

### Preparation of Linearity Standards

The following dilutions of the 0.1 µg/mL and 1.0 µg/mL fortification standards containing Etofenprox, 4'-OH and α-CO were made with Acetonitrile:Water:Formic Acid (50:50:0.1,v/v/v) to yield the required linearity standards:

Standard Conc. (µg/L)	mL of 0.10 µg/mL added	mL of 1.0 µg/mL added	Total Volume (mL)
0.100	0.050	NA	50
0.250	0.125	NA	50
0.500	0.250	NA	50
0.750	0.375	NA	50
1.00	0.500	NA	50
5.00	NA	0.250	50
10.00	NA	0.500	50

### Water Extraction Method:

1. Place 20.0 mL of water into a 125 mL separatory funnel.
2. Fortify, as necessary.
3. Add 25 ml dichloromethane (DCM) and shake manually for 1 minute.
4. Allow phases to separate and drain lower DCM layer into 125 mL round bottom flask.
5. Repeat Step 3, combining DCM layers into the 125 mL round bottom flask.
6. Add 10 mL acetone to DCM. Rotary evaporate extract to near dryness.
7. Dry sample under gentle stream of nitrogen.

8. Reconstitute residue in 2.0 mL of acetonitrile, mix.
9. Add 2.0 mL of 0.2% formic acid in water, mix well.
10. Aliquot sample to GC Vial.

**Soil Extraction Method:**

1. Weigh out 5.0 g of soil into a glass screw cap centrifuge bottle.
2. Fortify, as necessary.
3. Add 50.0 mL of acetone and extract by sonication for ~ 5 minutes.
4. Centrifuge sample at 1,500 rpm for ~ 5 minutes.
5. Remove supernatant, store in clean flask.

**SPE Clean-up**

1. Condition Varian Bond Elut®-Si cartridges (500 mg, 3 mL) with 6.0 mL of acetone discarding all acetone except enough to cover the packing frit in the cartridge.
2. Quantitatively add 1.0 mL of acetone extract from step 5 above to the cartridge.
3. Slowly elute the sample extract at a rate of 1-2 drops per second into a 15 mL disposable glass tube, leaving some acetone in the cartridge covering the packing frit.
4. Elute each cartridge with an additional 2.0 mL of acetone, collecting eluate in same 15.0 mL disposable tube.
5. Evaporate eluant to dryness under gentle stream of nitrogen at 40-50°C.
6. Reconstitute residue in 4.0 mL of acetonitrile with mixing. Then add 4.0 mL of 0.2% formic solution in water, mixing well.
7. Aliquot final sample to GC vials.

**Note:** The separatory funnel for the water extraction and the glass centrifuge bottle for the soil extraction must be pre-rinsed with 5% potassium hydroxide:methanol (1:1, v/v), water, 10% HCl, water and finally acetone. The glassware was then oven dried prior to use.

## LC/MS/MS ANALYSIS

Instrumentation: SCIEX 3000 Components (HPLC/Turbo Ion Spray Mode):

LC Pump	Agilent 1100 Series Binary Pump, Model G1312A
Autosampler	Agilent 1100 Series Autosampler, Model G1313A
Controller	Agilent 1100 Series Handheld Control Module G1323B

Column: Phenomenex Luna C18 (150 x 3.0 mm) 5  $\mu$ m Particle Size

C Flow Rate: 0.250 mL/minute

Injection Volume: 100  $\mu$ L

Solvent System:

Solvent A = Water (0.1% formic acid)

Solvent B = Acetonitrile

Solvent Program:	<u>Minutes</u>	<u>Solvent A</u>	<u>Solvent B</u>
	0	20	80
	0.01	20	80
	1.0	20	80
	6.0	2	98
	10.0	2	98
	10.1	20	80
	16.0	20	80

Retention Time: Etofenprox at ~14.2 minutes for m/z183, 4'-OH at ~10.2 minutes for m/z 108 and  $\alpha$ -CO at ~13.6 minutes for m/z107

Separation of Etofenprox, 4'-OH and  $\alpha$ -CO was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of its retention time with that of a reference standard.

A typical injection sequence was: conditioning standard, solvent blank, 0.1  $\mu$ g/L calibrant, 0.25  $\mu$ g/L calibrant, control 1, control 2, fortified sample, 0.5  $\mu$ g/L calibrant, fortified sample, fortified sample, fortified sample, 0.75  $\mu$ g/L calibrant, fortified sample, fortified

sample, fortified sample, 1.0 µg/L calibrant, 5.0 µg/L calibrant, fortified sample, fortified sample, fortified sample, 10.0 µg/L calibrant, solvent blank, quality control standard.

## VALIDATION SETS

To demonstrate the validity of the analytical method for Etofenprox, 4'-OH and  $\alpha$ -CO in control water and soil, the independent laboratory validation was conducted on the same matrices by fortifying with a standard containing Etofenprox, 4'-OH and  $\alpha$ -CO at two fortification levels with five replicates at each level:

- 1) 0.05 µg/L Etofenprox, 4'-OH and  $\alpha$ -CO in water
- 2) 0.50 µg/L Etofenprox, 4'-OH and  $\alpha$ -CO in water
- 3) 0.01 mg/kg Etofenprox, 4'-OH and  $\alpha$ -CO in soil
- 4) 0.10 mg/kg Etofenprox, 4'-OH and  $\alpha$ -CO in soil

After fortifying the samples at the desired concentration, the samples were extracted, and analyzed by the residue method as described in the Analytical Method section of this report. Calibrant standards were prepared and then analyzed by LC/MS/M.S. A calibration curve (ranging from 0.1 µg/L to 10.0 µg/L Etofenprox, 4'-OH and  $\alpha$ -CO) was generated with each set. Representative calibration curves are provided in Figure 1. Representative solvent blank and calibrant chromatograms for Etofenprox, 4'-OH and  $\alpha$ -CO are presented in Figure 2-9. Representative matrix chromatograms are provided in Figures 10-15. The sample spreadsheets for Etofenprox, 4'-OH and  $\alpha$ -CO fortified water and soil are provided in Appendix C. On March 7, 2006, PTRL West contacted Wildlife International regarding the failure of our first attempt. The originator of the method clarified that the glassware must be carefully pre-rinsed with 5% potassium hydroxide in methanol, followed by rinses with water, 10% HCl in water and acetone. They also clarified that a dilution of the 10X LOQ fortification samples might yield better results, however enhancement of the  $\alpha$ -CO analyte was at times observed in water samples.

## METHODS OF CALCULATION

### *Preparation of Stock Standard*

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard

P = Purity of neat standard

FC = Final Concentration (mg/mL)

The recoveries of Etofenprox, 4'-OH and  $\alpha$ -CO from fortified soil samples were calculated as follows:

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

$$\text{Sample concentration (ng/mL Etofenprox)} = \frac{y - b}{m}$$

where y = Sample peak area

b = Calibration intercept

m = Slope

mg/kg Etofenprox =

$$\frac{\text{Sample Conc. (ng/mL)} \times \text{Final Vol. (mL)} \times 50 \text{ mL acetone} \times 0.001 \mu\text{g/ng}}{5.0 \text{ grams of soil}}$$

Percent Recovery =

$$\frac{\text{Concentration of Etofenprox Fortified Sample (mg/kg)} - \text{Concentration of Control (mg/kg)}}{\text{Etofenprox Fortification Level (mg/kg)}}$$

x 100

### *Sample Calculation*

An example calculation for Etofenprox from sample F1A of the soil method validation set as follows:

Calibration curve:  $y = 11,381 x + 145$  ( $r^2 = 0.9990$ )

Peak area detected for F1A soil = 1,589

Etofenprox detected (ng) =  $(1,589-145) \div 11,381 = 0.127$  ng/mL

Etofenprox (mg/kg) =

$[0.127 \text{ ng/mL Etofenprox} \times 8.0 \text{ mL} \times 50.0 \times 0.001] \div 5.0\text{g} = 0.0102$  mg/kg

% Recovery =  $(0.0102 \text{ mg/kg detected} - 0 \text{ mg/kg}) \div 0.01 \text{ mg/kg fortified} \times 100 = 102.0\%$

The recoveries of Etofenprox, 4'-OH and  $\alpha$ -CO from fortified water samples were similarly calculated, with units in  $\mu\text{g/L}$ .

### **Statistical Analysis**

The residue data included the following statistical calculations: means, averages, standard deviations, relative standard deviations and linear regression analyses.

### **Time Required for Analysis**

Time required per sample set, where a sample set consists of twelve (12) matrix samples and 7 standards:

Extraction and Clean-up take approximately 8 hours

LC-MS/MS analysis takes approximately 6 hours

TOTAL = approximately 14.0 hours (~2 calendar days)