1.0 INTRODUCTION

An independent method validation study was conducted at PTRL West, Inc. (625-B Alfred Nobel Dr, Hercules, California) to determine the validity of a procedure to analyze tolfenpyrad in distilled and surface water. See Appendix A for the study Protocol. The study was initiated on April 17, 2012. The independent laboratory validation was conducted from April 20, 2012 through May 25, 2012.

2.0 MATERIALS AND METHODS

2.1 Method

The analytical method for the analysis of tolfenpyrad in water was conducted relative to a method validated at Covance laboratories (Reference 1, See Also Appendix A- Protocol Appendix 1).

The determination of tolfenpyrad was validated by spiking known concentrations of each analyte into distilled and surface water samples. The sample was then transferred to a volumetric flask and brought to the mark with methanol. This sample is then aliquoted for HPLC/MS/MS analyses in the positive mode. The percent recovery was determined relative to an external calibration curve.

2.2 Test System

The test systems were distilled and surface water. The control distilled water sample used for this study had an identification number of 2304W-002 and was purchased from Lucky supermarket, Hercules, CA on April 18, 2012. The control surface water sample used for this study had an identification number of 2304W-001 and was collected at Alvarado park, Richmond, CA on April 15, 2012.

2.3 Reference Substances

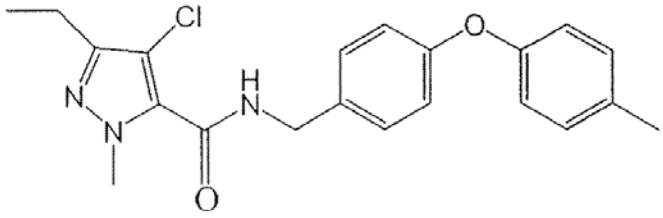
The tolfenpyrad (1540W-004) reference substances were obtained from Nihon Nohyaku Co., Ltd. Stock solutions of the reference substances were prepared at 0.1 mg/mL in acetonitrile and were stored for a maximum of one month and assumed to be stable for that period. Calibrant solutions appeared stable when stored refrigerated for at a maximum of one month, based on the comparison of LC-MS/MS chromatograms during

this study. The certificates of analysis for the reference substance is provided in Appendix B.

Compound: **Tolfenpyrad**

Chemical Name: 4-chloro-3-ethyl-1-methyl-*N*-[[4-(4-methylphenoxy)phenyl]methyl]-1*H*-pyrazole-5-carboxamide

Chemical Structure:



Purity: 100%
Lot Number: 6D-01-1
PTRL West Inventory Number: 1540W-004
Date Received: July 19, 2006
Expiration Date: June 29, 2016
Storage Conditions: Freezer

2.4 Equipment

Balance (various types)
Bottle, amber glass, 120 mL
Graduated cylinder, various sizes
Plastic disposable centrifuge tubes 15 mL
Pasteur pipettes
Sonicator, Branson 2210
Syringes, microliter, various sizes
Volumetric flask, various sizes

2.5 Solvents and Reagents

All solvents and reagents (reagent grade or better) were obtained from Fisher Scientific or VWR. All water used was HPLC grade.

Methanol
Acetonitrile
Water
Formic Acid

2.6 Reference Substance Stock Solution Preparation

Duplicate Stock standard solutions (A and B) of tolfenpyrad (corrected for purity) were prepared at 0.1 mg/mL in acetonitrile. The stock standards were stored refrigerated (< 4ºC) when not in use.

2.7 Preparation of Intermediate/Fortification Solutions

Reference and fortification solutions were prepared from Stock solution A as follows:

1000 µg/L: 0.25 mL of 100 µg/mL stock solution A was transferred into a 25 mL volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

40 µg/L: 1.0 mL of 1000 µg/L solution was transferred into a 25 mL volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

4 µg/L: 0.1 mL of 1000 µg/L solution was transferred into a 25 mL volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

An intermediate standard solutions were prepared as follows:

10 µg/L: 0.25 mL of the 1000 µg/L reference solution was transferred into a single 25 mL volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

Reference, Fortification and Intermediate solutions were transferred to the amber glass bottles and stored refrigerated when not in use.

2.8 Preparation of Calibrants

Calibration standard solutions were prepared as follows:

- 100 ng/L: 250 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 80 ng/L: 200 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 60 ng/L: 150 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 40 ng/L: 100 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 20 ng/L: 50 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 10 ng/L: 25 µL of the 10µg/L intermediate solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 4 ng/L: 1000 µL of the 0.1µg/L calibration solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 2 ng/L: 400 µL of the 0.1µg/L calibration solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 1 ng/L: 200 µL of the 0.1µg/L calibration solution was transferred into a 25 mL volumetric flask. Diluted to the mark with Acetonitrile and mixed well

Standards were mixed well in volumetric flasks, then transferred to amber glass bottles. All calibration solutions were stored in refrigerator when not in use.

2.9 Sample Preparation

Aliquots of the water (10 mL) were measured into 15 mL plastic centrifuge tube prior to fortification (if necessary) and extraction.

2.10 Sample Fortification

Fortification of control distilled and surface water was performed to analyze method percent recoveries for validation. A 10 mL portion of water was fortified with 25 µL of the listed fortification solution as follows:

Fortification Designation	Fortification Level (µg/L)	Concentration of Fortification solution used
F1A	0.01	0.004 µg/mL
F1B	0.01	0.004 µg/mL
F1C	0.01	0.004 µg/mL
F1D	0.01	0.004 µg/mL
F1E	0.01	0.004 µg/mL
F2A	0.1	0.04 µg/mL
F2B	0.1	0.04 µg/mL
F2C	0.1	0.04 µg/mL
F2D	0.1	0.04 µg/mL
F2E	0.1	0.04 µg/mL

2.11 Extraction Method

1. Measure 10 mL of a well- mixed sample into a 15 mL plastic centrifuge tube. Fortify as necessary as per fortification scheme, Section 2.10.

2. Transfer the whole sample to a 25 mL Class A volumetric flask.

3. Add 4 mL of Methanol to the 15 mL centrifuge tube and ultrasonicate briefly before transferring to the 25 mL volumetric flask.

4. Repeat Step 3 and bring the volumetric flask to the mark with Methanol.

5. Mix well. Submit to LC-MS/MS analysis.

2.12.1 LC-MS/MS Analysis of Tolfenpyrad

An Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system with electrospray ionization and a Dionex Ultimate 3000 LC system were used.

2.12.1 LC System Components

SCIEX 4000 (HPLC/Turbo Ion Spray Mode)

Pump: Dionex Ultimate 3000 HPLC pump

Autosampler: Dionex Ultimate 3000 Autosampler

Micro-Degasser: Dionex Ultimate 3000 on line vacuum degasser

Column Compartment: Dionex Ultimate 3000

2.12.2 LC Parameters

Column: Phenomenex Synergi Fusion RP, 100A (100 mm x 2.0 mm I.D.), plus a 4 x 2 mm Phenomenex Fusion security guard pre column cartridge.
Flow Rate: 200 µL/minute
Injection Volume: 50 µL
Temperature: 40°C
Solvent System: Solvent A = Water (0.1% formic acid)
Solvent B = Acetonitrile

Solvent Program:

Minutes	Solvent A	Solvent B
0	75	25
0.5	75	25
10.25	0	100
16.26	0	100
20.25	75	25

Divert Valve: Divert LC flow from column to waste (bypassing MS) from 0 to 3.0 minutes and again after 16 minutes.

Approximate Retention Times: tolfenpyrad: 11.5 minutes,

2.12.3 Mass Spectrometer Parameters

An Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system was used with electrospray ionization in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM):

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential	Collision Energy	Collision Activation Dissociation Gas	Collision Cell Exit Potential
384.2	197.3	150	90	37	10	14
384.2	153.2	150	90	83	10	14

Source Dependent Settings:

Nebulizer Temperature (TEM):	550°C
Nebulizer Gas (GS1):	50
Turbo Gas (GS2):	70
Ion Spray Voltage (IS):	5500
Curtain Gas (CUR):	20
Interface Heater (ihe):	on
Entrance potential:	10

Separation of the analytes was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of it retention time with that of the respective reference standards as well as by monitoring specific ion transitions.

3.0 METHODS OF CALCULATION

3.1 Preparation of Stock Standards

$$\text{Final Concentration (mg/mL)} = \frac{(W) \times (P)}{(VS)}$$

where W = Milligrams of neat standard
P = Chemical purity of neat standard
VS = Volume of Solvent (mL)

3.2 Residue in Matrices

Each analyte was quantified by peak area relative to an external calibration curve. A calibrant peak area (y) from the quantitation ion transition relative to the concentration of the calibrant in ng/L (x) yielded a linearity curve, where y = mx + b was plotted using a least squares fit with 1/x weighting. Curves are determined by Applied Biosystems/MDS SCIEX Instruments Analyst Software version 1.5.1 or the equivalent

The residue of tolfenpyrad in water was calculated as follows:

$$\mu g/L = \frac{\text{ng/L analyte x Final vol. (25 mL) x 0.001 } \mu g/ng \text{ x Dil factor (1.0)}}{\text{Sample volume (10 ml)}}$$

Where:

ng/L analyte	=	ng/L of analyte found from standard curve
Final vol. (mL)	=	Volume of final HPLC ready extract (25 mL)
Sample volume	=	Volume of sample (10 mL)
Dil. Factor	=	1.0
0.001 µg/ng	=	Unit conversion factor

And:

ng/L analyte	=	[(PA – b) ÷ m]
Where:	=	
PA	=	Peak area analyte
B	=	y-intercept of calibration curve
m	=	slope of calibration curve

$$\% \text{ Recovery} = \frac{\text{Analyte Residue Detected } (\mu g/L) - \text{ Avg. Control Residue}}{\text{Analyte Fortification Level } (\mu g/L)} \text{ x } 100$$

An example calculation for the tolfenpyrad residue in distilled water (Fort 1A at 0.01 µg/L) is shown below:

Linear regression analysis (with 1/x weighting) of the tolfenpyrad standards gave a curve as calculated by the Analyst Software version for the quantitation ion transition 384.2/197.3 with the equation

$y = 852.62758242\ x + 846.02876267$ ($r^2 = 0.997962036059$)

The ng/L tolfenpyrad injected determined by this curve was:

ng/L tolfenpyrad = [((3720.422021 – 846.02876267) ÷ 852.62758242 = 3.371 ng/L

Where:

3720.422021 = peak area tolfenpyrad

846.02876267= y-intercept of calibration curve

852.62758242 = slope

The tolfenpyrad residue (µg/L)

= (3.371 ng/L x 25 mL x 1 x 0.001 pg/ng) ÷ (10 mL)

= 0.0084 µg/L

$$\text{Percent Recovery} = \frac{0.0084\,\mu g/L - 0.00000\ \mu g/L}{0.01\,\mu g/L} \times 100 = 84\%$$

4.0 LIMIT OF QUANTITATION AND DETECTION

The limit of detection (LOD) is defined as the concentration of the lowest linearity calibrant injected – 1 ng/L tolfenpyrad. Using the current methodology this is equivalent to an LOD of 0.0025 µg/L. The limit of quantitation (LOQ) is defined as the lowest concentration validated which is 0.01 µg/L for tolfenpyrad.

5.0 STATISTICAL METHODS

The residue data included the following statistical calculations: averages, standard deviations, relative standard deviation and linear regression analysis with 1/x weighting.

6.0 TIME REQUIRED FOR ANALYSIS

A sample set consisting of 12 samples and a reagent blank sample can be completed by one analyst in the following amount of time:

Sample preparation: 4 hrs.

Analysis: 13 hrs for analysis (includes 4 hours for instrument tuning).

Total person-hours = 4 (does not include 7 hrs of unattended LC-MS/MS analysis or 4 hrs for tuning)

Days Required for the Sample Set = 1.5 Days

8.3 Communication, Notes and Minor Modification

There was no communication between the Sponsor Representative and the Study Director with regards to modifications of the method. All contact with the Sponsor Representative was in the form of e-mail from the Study Director and was for routine study updates or routine project management purposes. No contact was made with the developers of the method

9.0 CONCLUSIONS

An analytical method for the analysis of tolfenpyrad has been independently validated at 0.01 µg/L and 0.1 µg/L in distilled and surface water. The limit of quantitation (LOQ) of the residue method was determined as the lowest level validated or 0.01 µg/L for tolfenpyrad. The limit of detection (LOD) for the samples was estimated as 0.0025 µg/L. The recovery data at the LOQ and 10x LOQ levels demonstrated acceptable precision and accuracy of the analytical method, and this study fulfills registration requirements as outlined in EPA guidelines OPPTS 860.1340 and in compliance with Good Laboratory Practices (GLP) as stated in FIFRA 40 CFR Part 160.

10.0 REFERENCES

1. "Determination of Tolfenpyrad Residues in Surface and Distilled Water by UPLC-MS/MS" A. Cashmore, D.R Wright, Covance Laboratories, Inc., Procedure Code CLE 8245058-01V, June 09, 2011.