INTRODUCTION

Kumiai Chemical Industry Co., Ltd. contracted with PTRL West (625-B Alfred Nobel Dr., Hercules, CA 94547) to conduct the magnitude of residues of pyroxasulfone metabolite M-28 in/on soybean seed and processed commodities. The protocol amendments used for this portion of the study is provided in Appendix N.1.

MATERIALS AND METHODS

Equipment

Glassware and Miscellaneous Equipment

Balance
Bottle, amber, with Teflon®-lined cap
Bottle, centrifuge 250 mL
Filter flask, 0.250 L or 0.5 L
Filter paper, Whatman GF/A
Funnel, Büchner
Graduated Cylinder, various sizes
Vials, glass with Teflon®-lined cap
Volumetric flask, various sizes
Wrist action shaker

Solvents/Reagents

All solvents were HPLC grade unless noted:
Acetonitrile
Formic Acid
Methanol
Water

Preparation of Sample

All soybean seed samples were received frozen at PTRL West, Inc. and remained frozen until processed. Soybean seed samples were processed with dry ice in a Waring Blender or other food processor. Processed samples were stored frozen in zipper locked bags,

allowing the dry ice to sublime. The processed samples were stored frozen (<0ºC) until used for fortification and analysis.

Preparation of Standards

Reference substance M-28 was provided by Kumiai Chemical on August 4, 2011. The stated purity (Lot 3, PTRL West no. 1519W-375) was 99.20%, with an expiration date of April 20, 2013. The certificate of analysis is provided in Figure 1.

Stock solutions (1.0 mg/mL) of M-28 reference substances were prepared in acetonitrile:water (1:1, v/v) as described under the "Method of Calculations" section. Dilution of these stocks was prepared (0.10 mL of 1.0 mg/mL M-28 were added to a 10 mL volumetric flask and diluted to the mark with acetonitrile:water (1:1, v/v)) to yield a 10 µg/mL M-28 fortification solution. A 10-fold dilution of the 10 µg/mL fortification solution yielded a 1.0 µg/mL working solution. Calibrants were made by diluting the working solutions as described below. All stock and fortification solutions for M-28 were stored refrigerated.

Fortification Procedure

Fortification of untreated soybean seed and processed commodities was conducted to determine the percent recovery within each sample set for M-28. Fortification of M-28 was conducted in triplicate during method validation and in duplicate within the sample set. The following fortifications were conducted:

Fortification Level (ppm)	M-28
0.01	100 µL of 1.0 µg/mL
0.10	100 µL of 10 µg/mL
0.50	500 µL of 10 µg/mL

Preparation of Calibrant Standards

M-28 dilutions were made with control matrix extract. Intermediate working solutions were prepared at 0.025 µg/mL (0.025 mL of 10 µg/mL diluted to 10 mL) and 0.05 µg/mL (0.05 mL of 10 µg/mL diluted to 10 mL) by dilution to the mark with acetonitrile:water (1;1, v/v).

Calibration Standard (ng/ml)	Concentration of Stock Solution (µg/mL)	Volume of Stock Solution (mL)	Final Volume (mL)
0.5	0.025	0.020	1.00
0.75	0.025	0.030	1.00
1.0	0.05	0.020	1.00
2.5	0.05	0.050	1.00
5.0	0.05	0.100	1.00
10.0	1.0	0.010	1.00
20.0	1.0	0.020	1.00

All dilutions were prepared in volumetric flasks using pipettemen with disposable tips. A calibration curve was generated with each sample set to determine linearity and to quantitate M-28, see “Methods of Calculation” for example.

Seed Extraction Method for M-28

1. Weigh 10.0 grams of sample into 250 mL plastic centrifuge bottle.
2. Spike samples, as needed.
3. Add 40 mL of HPLC grade water, let sample soak for 15 minutes. Add 40 mL of acetonitrile (ACN), mix well and let sample soak for another 15 minutes
4. Place samples on wrist action shaker for 10 minutes.
5. Vacuum filter the mixture through filter paper (Whatman GF/A) in a Büchner funnel into side-arm flask. Rinse the centrifuge bottle with 20 mL of ACN:water (HPLC grade) (1:1, v/v), rinse the filtercake with this solvent and combine all filtrates in side-arm flask. Transfer to 100 mL graduated cylinder.
6. Adjust volume to 90 mL with ACN:water (HPLC grade) (1:1, v/v), rinse side-arm flask prior adding solvent to graduated cylinder.
7. Transfer to amber bottles, aliquot into GC vials and prepare matrix based calibrant for analysis.

LC/MS/MS Analysis of M-28

SCIEX 3200 Components (HPLC/Turbo Ion Spray Mode) or equivalent:

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 Compartment	Agilent 1100 Series Column Compartment, Model G1316A
Vacuum Degasser	Agilent 1100 Series Vacuum Degasser, Model G1379A
Divert Valve	Valco Switching Valve

Column: Luna C8, 150 mm x 2.0 mm, 5µ 100A with pre-column (4 cm x 2 mm)

Injection Volume: 10 µL

Solvent System and Gradient Program:

Solvent A = Water (0.05% formic acid)
Solvent B = Methanol (0.05% formic acid)

Time:	Flow Rate	Solvent A	Solvent B
0.00	230 µL/min	95	5
10.00	230 µL/min	0	100
11.00	230 µL/min	0	100
12.00	400 µL/min	0	100
12.50	400 µL/min	95	5
16.00	230 µL/min	95	5
18.00	230 µL/min	95	5

Period 1 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)
305	206	200
305	174	300
305	132	250

Representative Mass Spectrometer Settings

	Period 1 API 3200
Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
CUR:	35.00
CAD:	5.00
GS1:	60.00
GS2:	70.00
IS:	5500.0
TEMP:	500.0
DP:	35.00

Retention Time: M-28 at ~10.5 minutes

Separation of the analyte was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of their retention time with the reference standard, and quantitated by integration of the peak areas.

A typical injection sequence for M-28 soybean seed samples as analyzed by LC-MS/MS was: conditioning control, 0.5 ng/mL calibrant, 0.75 ng/mL calibrant, 1.0 ng/mL calibrant, control sample, control sample, control sample, 2.5 ng/mL calibrant, fortified control, fortified control, diluted fortified control, diluted fortified control, 5.0 ng/mL calibrant, treated sample, treated sample, treated sample, 10 ng/mL calibrant, treated sample, treated sample, treated sample, 20.0 ng/mL calibrant, QC standard.

Storage Stability of M-28 in Soybean Seeds

The stability of M-28 was determined in a related study (Ref. 1). A ^{14}C-soybean seed sample (1472W-056) generated in a confined rotational crop study and kept in frozen storage at PTRL West, Inc. was extracted and analyzed by the same chromatographic method employed in the original study. The M-28 residue results were compared with the original M-28 residues detected in September 2007.

Statistical Methods

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

Time Required for Analysis

The total time required for analysis of M-28 in soybean seeds was 6 hours for extraction and preparation of matrix based calibrants plus 8 LCMS analysis. The total time required to conduct a sample set of 11 samples was 14 hours or 1 calendar day.

Limit of Quantitation

The limit of quantitation was assigned as the lowest fortification level of analyte validated by the residue method. The M-28 residue analysis methods have limits of quantitation (LOQ) of 0.01 mg/kg (ppm) in soybean seed raw agricultural and processed commodities.

METHODS OF CALCULATION

Preparation of Stock Standards

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

where W = Milligrams of neat standard
P = Chemical purity of neat standard
FC = Final Concentration (mg/mL)

Residue on Soybean Matrices

Linear regression formula for M-28 peak area, calibration curve y = mx + b, as determined by the Analyst software.

where y = peak area
x = ng/mL M-28 injected
m = Slope
b = Calibration intercept

The residue on treated soybean matrix was calculated as follows:

$$\text{ppm M-28 (mg/kg)} = \frac{\text{ng/mL M - 28 x Final vol. (mL) x 0.001 } \mu\text{g/ng}}{\text{Representative Sample Wt. (g)}}$$

Recoveries – M-28

$$\text{\% Recovery} = \frac{\text{M - 28 Residue Detected (ppm) - ppm Control}}{\text{M - 28 Fortification Level (ppm)}} \text{ x } 100$$

Validity of the M-28 residue analytical method was established by acceptable recovery (70-120%) from fortified untreated control samples. Residues of M-28 (ppm) in treated samples were calculated as for the fortified samples, without control subtraction.

An example calculation for the M-28 residue in the soybean seed set 5 (0.01 ppm) is shown below:

Linear regression analysis of the M-28 standards gave a curve with the equation x = (y – 150.90974318) ÷ 2931.1671991 (r^2 = 0.9999). The ng/mL M-28 injected determined by this curve was 9.04 ng/mL.

µg/mL M-28 = ng/mL M-28 x 0.001 µg/ng

$$\text{M-28 ppm (mg/kg)} = \frac{0.817 \text{ ng/mL x 90 mL x 0.001 } \mu\text{g/ng}}{10 \text{ g}} = 0.007 \text{ ppm}$$

$$\text{Percent M-28 Recovery} = \frac{0.007 - 0 \text{ ppm}}{0.10 \text{ ppm}} \text{ x } 100 = 70\%$$