SUMMARY

A method for quantitation of Fluensulfone and its metabolites in soil was successfully validated. Control soil from four field sites was fortified at 0.01 mg/kg (the LOQ) and 2.0 mg/kg with Fluensulfone and 0.01 mg/kg (LOQ) and 0.10 mg/kg (10x LOQ) for its metabolites methyl sulfone (M-3626), butene sulfonic acid (M-3627) and thiazole sulfonic acid (M-3625). The soils were then extracted with acetonitrile:water. The extract was subjected to further cleanup involving an SPE cartridge cleanup. The final samples were analyzed by LC-MS/MS for quantitation of MCW-2, methyl sulfone, butene sulfonic acid and thiazole sulfonic acid. The limit of quantitation for all four analytes in soil is 0.01 ppm. The limit of detection for Fluensulfone and its metabolites was approximately 0.25 ng/mL in solution.

INTRODUCTION

Makhteshim-Agan of North America, Inc. contracted with PTRL West, Inc. to conduct method development and validation of an analytical method for determination of Fluensulfone (MCW-2) and its metabolites commonly named methyl sulfone, butene sulfonic acid and thiazole sulfonic acid in soil

The study is designed to comply with OPPTS 860.1340 and OPPTS 850.1700 guidelines. The study was initiated on June 17, 2010. This study was conducted from June 17 to July 7, 2010. The study was conducted at PTRL West, Inc., 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160 (Appendix A).

MATERIALS AND METHODS

Reference Substances

The reference substance of Fluensulfone (lot number 326-115-01, PTRL Sample No. 1785W-003D) was supplied by LABService with a stated purity of 99.12%. The expiration date on the certificate of analysis is November, 2010 and it was stored refrigerated upon receipt. The reference substance methyl sulfone (M-3626, lot number 381-173-00, PTRL Sample No. 1788W-002) was supplied by Makhteshim Chemical Works with a stated purity of 97%. The expiration date on the certificate of analysis is December 31, 2011 and it was stored refrigerated upon receipt. The reference standard of thiazole sulfonic acid sodium salt (M-3625, lot number 381-174-00, PTRL Sample No. 1788W-003) was supplied by Makhteshim Chemical Works with a stated purity of 90.9%. The expiration date on the certificate of analysis is December 31, 2011 and it was stored refrigerated upon receipt. The reference standard of butene sulfonic acid sodium salt (M-3627, lot number 215PAL2, PTRL Sample No. 1785W-021) was supplied by PharmAgra Labs, Inc. with a stated purity of 99.1%. The expiration date on the certificate of analysis is September 10, 2010 and it was stored at room temperature. See Appendix B for the certificates of analysis.

All stock and working solutions were stored at < 0°C in amber bottles with Teflon®-lined screw-top caps. All solutions were prepared using pipettmen with diposable plastic tips, avoiding reuse of any glassware as carryover of the metabolites was suspected. All reference substances were concluded to be stable in solution throughout the study period based on comparison of chromatograms generated over the study period.

Reagents and Solvents

All solvents were HPLC grade unless noted:

Acetonitrile (ACN)
Formic Acid
Water

Glassware and Miscellaneous Equipment

Balance
Bottle, amber, with Teflon®-lined cap, various sizes

Bottle, plastic, 250 mL centrifuge
Centrifuges, Mistral 3000E and Eppendorf 5415C
Centrifuge tube, plastic 15 mL graduated
Graduated Cylinder, various sizes
Microfilterfuge tube, Rainin 0.45 UM Nylon-66
Pipettemen, assorted sizes with disposable tips
C18 SPE Cartridges, BondElut 0.5 g/6 mL
Turbo-Vap nitrogen evaporator
Vials, glass snap-cap for LCMS analysis
Volumetric pipette, various sizes
Wrist action shaker

ANALYTICAL PROCEDURES

Preparation of Samples

Untreated soil was provided from four field sites (i.e. California, Florida, Texas and Ontario, Canada) by ARCADIS. These soil samples represent the 0-6" soil from test sites to be used for the terrestrial field dissipation study (Reference 1). The soil was characterized under Reference 2. The California soil (PTRL Sample No. 2049W-002) was received at room temperature on May 4, 2010. The Florida soil (PTRL Sample No. 2049W-004) was received at room temperature on May 18, 2010. The Texas soil (PTRL Sample No. 2049W-003) was received at room temperature on May 4, 2010. The Ontario, Canada soil (PTRL West Sample No. 2049W-001) was received at room temperature on May 3, 2010. The soil characterization is provided in Appendix C. The samples were stored at ambient temperature.

Preparation of Stock Standards

A stock standard of Fluensulfone was prepared by dissolving 10.14 mg MCW-2 in 10 mL of acetonitrile, using a volumetric flask. An additional volume (0.04 mL) was added to yield a final concentration of 1.000 mg/mL Fluensulfone. A stock standard of methyl sulfone (M-3626) was prepared by dissolving 10.32 mg in 10 mL acetonitrile:water (1:1, v/v), using a 10 mL volumetric flask. An additional volume (0.01 mL) was added to yield

a final concentration of 1.000 mg/mL. A stock standard of butene sulfonic acid sodium salt was prepared by dissolving 10.15 mg M-3627 in 10 mL of acetonitrile:water (1:1, v/v), using a 10 mL volumetric flask. An additional volume (0.05 mL) of acetonitrile:water (1:1, v/v) was added to yield a final concentration of 1.001 mg/mL. Similarly, a stock standard of thiazole sulfonic acid sodium salt was prepared by dissolving 11.01 mg of M-3625 in 10 mL of acetonitrile:water (1:1, v/v). When adjusted for purity, the concentration of the thiazole sulfonic acid stock solution was 1.001 mg/mL. All stock solutions were transferred to amber bottles and stored frozen when not in use. A working solution of Fluensulfone was made by dilution with acetonitrile (0.50 mL of 1.00 mg/mL diluted to 10 mL) to yield 50 µg/mL Fluensulfone. A mixed intermediate working solution of metabolites was prepared for M-3626, M-3625 and M-3627 at 50 µg/mL by dilution with acetonitrile:water (1:1, v/v) for methyl sulfone, butene sulfonic acid sodium salt and thiazole sulfonic acid sodium salt. All stock standard and working solutions were stored frozen when not in use.

Preparation of Fortification Standards

Mixed fortification solutions were prepared by dilution of the stock standards. A 10 µg/mL mixed Fluensulfone, methyl sulfone (M-3626), butene sulfonic acid sodium salt (M-3627) and thiazole sulfonic acid sodium salt (M-3625) fortification solution was prepared by diluting 2 mL each of the respective 50 µg/mL working solutions to 10 mL in a 10 mL volumetric flask, where the solution was brought to the mark with acetonitrile:water (1:1). A 1.0 µg/mL mixed fortification solution was prepared by diluting 1.0 mL of the mixed 10 µg/mL fortification solution to 10 mL in a 10 mL volumetric flask, where the solution was brought to the mark with acetonitrile:water (1:1). Mixed metabolite fortification stocks were prepared in the same manner, where no Fluensulfone was added. All fortification intermediate stocks were stored refrigerated when not in use.

Sample Fortification

Portions of control soil (50 g) were fortified with Fluensulfone, methyl sulfone (M-3616), butene sulfonic acid sodium salt (M-3627) and thiazole sulfonic acid sodium salt (M-3625) in replicates (5), as shown below, for determination of method recovery.

Fort. Level (ppm)	Volume of Mixed Fortification Solution	Volume of Fluensulfone 1 mg/mL Stock Soln.
0.01	0.05 mL of 10 µg/mL Mixed Fort. Soln.	NA
0.10	0.50 mL of 10 µg/mL Mixed Fort. Soln. – Metabolites only	NA
2.0	NA	0.100 mL

Preparation of Calibrants

All calibrant dilutions were prepared in amber bottles using appropriate pipettemen and disposable tips to dispense mixed standard solutions and solvents. The following dilutions were prepared in 10 mL volumetric flasks and diluted with acetonitrile:water (1:1, v/v).

Calibrant Conc. (ng/mL)	Conc. Stock Std.	Volume (mL)	Final Volume (mL)
100	10 µg/mL	0.10	10
50	10 µg/mL	0.05	10
25	10 µg/mL	0.025	10
10	100 ng/mL	1.0	10
5	100 ng/mL	0.50	10
2.5	100 ng/mL	0.25	10
1.0	100 ng/mL	0.10	10
0.75	50 ng/mL	0.15	10
0.5	50 ng/mL	0.10	10
0.25	50 ng/mL	0.05	10

Note: Actual concentrations of M-3625 and M-3627 were corrected by molecular weight conversion to account for the analysis of the free acid, rather than the sodium salt. The

corrected concentrations for the M-3625 and M-3627 calibrants are presented below, where the molecular weight conversion factor is 0.896 for M-3627 and 0.901 for M-3625.

Calibrant Conc. (ng/mL)	Corrected Conc. M-3627 (ng/mL)	Corrected Conc. M-3625 (ng/mL)
100	89.6	90.1
50	44.8	45.05
25	22.4	22.53
10	8.96	9.01
5	4.48	4.51
2.5	2.24	2.25
1.0	0.90	0.90
0.75	0.67	0.68
0.5	0.45	0.45
0.25	0.22	0.23

EXTRACTION METHOD

1. Weigh out 50 g portions of processed soil into 250 ml plastic bottles.
2. Spike as necessary, using a pipetteman and disposable tips.
3. Add 100 ml of ACN:HPLC water (1:1, v/v) to each bottle.
4. Place on a wrist action shaker for 1 hour.
5. Centrifuge at 10,000 rpm for 10 minutes
6. Transfer aliquot of the supernatant to a microfilterfuge tube (0.45 μm filter) and centrifuge the sample. Transfer the filtered extract to a snap-top GC vial for the direct MCW-2 and M-3626 analysis.
7. Transfer 6.0 mL of the unfiltered supernatant from Step 5 to 15 mL graduated plastic centrifuge tube. Concentrate sample to 3 mL in Turbo-vap at ~35ºC.

SPE Clean-up

8. Condition the Bond Elut 500mg, 6cc SPE cartridge with 5 mL of ACN, then 5 mL of water.

9. Apply concentrated extract from step 7 and collect the eluate into a clean 15 mL plastic graduated tube.
10. Rinse the sample tube from Step 7 with 5 mL of water, apply to the same cartridge and collect the eluate into the same graduated tube. Record the volume. Total volume should be about 8 mL. Mix the sample by vortexing.
11. Aliquot in snap-top vials and analyze on LC-MS/MS for M-3627 and M-3625.

LC-MS/MS CHROMATOGRAPHY

SCIEX API4000 Components (HPLC/Turbo Ion Spray Mode) or equivalent:

LC Pump	Agilent 1100 Series Binary Pump, Model G1312A
Autosampler	Agilent 1100 Series Autosampler, Model G1329A
Vacuum Degasser	Agilent 1100 Series Vacuum Degasser, Model G1379A

A. MCW-2 and M-3626 Analysis Method

Mass Spectrometer Settings

Scan Type:	MRM
Polarity:	Positive
Ion Source:	APCI (+)
CUR:	40.0
GS1:	70.0
GS2:	0.0
TEMP (°C):	500.0
CAD:	9.0
NC	3

Compound Dependent Settings:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Entrance Potential	Declusterin g Potential	Collision Energy	Collision Cell Exit Potential
MCW-2						
292.2	166.4	200	10	56	26	13.8
292.2	89.2	200	10	56	40	7.0
292.2	59.2	300	10	56	60	9.4
M-3626						
198.2	135.3	200	10	56	25	10.0
198.2	93.3	200	10	56	45	15.0
198.2	120.4	100	10	56	30	10.0

Column: Synergi Fusion-RP 80A, 4µm particle size (75 mm x 2.0 mm)

Column Temperature: 30°C

Injection Volume: 20 µL

Solvent System:

Solvent A = Water (0.1% formic acid)
Solvent B = Acetonitrile (0.1% formic acid)

Solvent Program:

Minutes	Flow Rate	Solvent A	Solvent B
0	0.20 mL/min	95	5
1.0	0.20 mL/min	95	5
13.5	0.20 mL/min	10	90
14.0	0.40 mL/min	0	100
16.0	0.50 mL/min	0	100
16.3	0.40 mL/min	95	5
18.0	0.20 mL/min	95	5
18.5	0.20 mL/min	95	5

Retention Times: Fluensulfone was ~13.2 minutes, M-3626 was 9.8 minutes

B. Butene Sulfonic Acid (M-3627) and Thiazole Sulfonic Acid (M-3625) Analysis Method

Mass Spectrometer Settings

	Period 1
Scan Type:	MRM
Polarity:	Negative
Ion Source:	ESI (-)
CUR:	35.0
GS1:	40.0
GS2:	40.0
TEMP (°C):	500.0
CAD:	8.0
IS	-4,500

Period 1 settings (Butene sulfonic acid):

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Entrance Potential	Declustering Potential	Collision Energy	Collision Cell Entrance Potential	Collision Cell Exit Potential
M-3627							
188.9	81.1	150	-4	-40	-23	-8.0	-8.6
188.9	80.1	200	-4	-40	-40	-8.0	-9.0
M-3625							
197.9	82.1	150	-4	-40	-27	-7.0	-8.7
197.9	118.1	200	-4	-40	-20	-7.0	-10.9
197.9	162.0	200	-4	-40	-23	-7.0	-14.1

Column: Synergi 2.5µ Fusion-RP 100A (100 mm x 2.00 mm)

Column Temperature: 30°C

Injection Volume: 20 µL

Solvent System:

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

Solvent Program:

Minutes	Flow Rate	Solvent A	Solvent B
0	0.25 mL/min	95	5
9.0	0.25 mL/min	36	65
9.5	0.40 mL/min	0	100
11.5	0.50 mL/min	0	100
12.0	0.35 mL/min	95	5
15.0	0.25 mL/min	95	5
16.0	0.25 mL/min	95	5

Retention Times: Butene sulfonic acid (M-3627) was ~5.4 minutes
Thiazole sulfonic acid (M-3625) was ~6.5 minutes

Separation of the analytes was achieved by HPLC. The analytes were identified by the coincidence of their retention times with the reference standards and MS characteristics, and quantitated by integration of the peak areas.

A typical injection sequence for Fluensulfone and M-3626 in a method validation set as analyzed by LC-MS/MS was: solvent blank, 0.25 ng/mL calibrant, 0.5 ng/mL calibrant, reagent blank, control sample, control sample, 0.75 ng/mL calibrant, fortified control, fortified control, 1.0 ng/mL calibrant, fortified control, fortified control, 2.5 ng/mL calibrant, fortified control, fortified control, 5.0 ng/mL calibrant, fortified control, fortified control, 10 ng/mL calibrant, fortified control, fortified control, 25 ng/mL calibrant, 50 ng/mL calibrant, 100 ng/mL calibrant, QC standard. A similar injection sequence was used for M-3627 and M-3625 analysis.

Statistical Methods

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

Limit of Quantitation

The limit of quantitation was assigned as the lowest fortification level of analyte validated by the residue method. The LOQ for Fluensulfone, methyl sulfone (M-3626), butene sulfonic acid sodium salt (M-3627) and thiazole sulfonic acid sodium salt (M-3625) in soil was 0.01 ppm. The limit of detection (LOD) was assigned as the lowest calibrant used in the analysis or 0.25 ng/mL Fluensulfone and M-3626. The LOD was 0.25 ng/mL butene sulfonic acid and thiazole sulfonic acid, as sodium salt equivalents.

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 in Soil

The Fluensulfone, methyl sulfone (M-3626), butene sulfonic acid (M-3627) and thiazole sulfonic acid (M-3625) quantitation was conducted by peak area relative to an external calibration curve.

Linear regression formula for analyte peak area, calibration curve $y = mx + b$

where y = peak area
x = µg/mL analyte injected
m = Slope
b = Calibration intercept

For butene sulfonic acid and thiazole sulfonic acid, the linear regression analysis was prepared using concentrations that had been adjusted for the molecular weight conversion of the salt to the free acid forms of these metabolites. The molecular weight of butene sulfonic acid sodium salt is 212.12 g/mole, while the free acid molecular weight is 190.14 g/mole. The molecular weight conversion factor was 190.14 divided by 212.12 or 0.896 for butene sulfonic acid. The molecular weight of thiazole sulfonic acid sodium salt is

221.62 g/mole, while the free acid molecular weight is 199.64 g/mole. The molecular weight conversion factor for the thiazole sulfonic acid was 0.901.

µg/mL butene sulfonic acid = 0.896 x µg/mL butene sulfonic acid sodium salt

µg/mL thiazole sulfonic acid = 0.901 x µg/mL thiazole sulfonic acid sodium salt

The linear regression generated by the LCMS program (Analyst 1.4.2) was used to calculate the concentration (ng/mL) of the Fluensulfone and methyl sulfone present in the sample for soil method validations.

The residue on fortified soil was calculated as follows:

$$\text{ppm Fluensulfone (mg/kg)} = \frac{\text{ng/mL MCW - 2} \times \text{Extract vol. (mL)} \times \text{Dil. Factor} \times 0.001\ \mu\text{g/ng}}{\text{Sample Wt. (g)}}$$

ppm butene sulfonic acid or thiazole sulfonic acid =

$$\frac{\text{ng/mL acid} \times \text{Eluate vol. (mL)} \times \text{dilution factor} \times \text{Init. Extract Vol. (mL)} \times 0.001\ \mu\text{g/ng}}{\text{Aliquot vol. (mL)} \times \text{Sample Wt. (g)}}$$

Percent recovery for each analyte was calculated as follows:

$$\text{\% MCW-2 Recovery} = \frac{\text{Residue Detected (ppm) - Average (ppm) Control}}{\text{Fortification Level (ppm)}} \times 100$$

% BSA or %TSA Recovery =

$$\frac{\text{Residue Detected (ppm) - Average (ppm) Control}}{\text{Fortification Level (ppm)} \times \text{Mol. Wt. Conversion Factor}} \times 100$$

Validity of the Fluensulfone, methyl sulfone, butene sulfonic acid and thiazole sulfonic acid residue analytical methods was established by acceptable average recovery (70-120%) from fortified untreated control samples.

An example calculation for the Fluensulfone recovery in the CA soil method validation (0.01 ppm) is shown below:

$$\text{Fluensulfone ppm} = \frac{5.358\ \text{ng/mL x}\ \ 100\ \text{mL x}\ 0.001}{50\ \text{g}} = 0.011\ \text{ppm}$$

$$\text{Percent Recovery MCW-2} = \frac{0.011 - 0.000\ \ \text{ppm}}{0.01\ \text{ppm}}\ \text{x}\ 100 = 110\%$$

An example calculation for the butene sulfonic acid recovery in cucumber method validation is shown below, where a similar calculation was conducted for thiazole sulfonic acid:

$$\text{ppm butene sulfonic acid} = \frac{3.31\ \text{ng/mL}\ \ \text{x}\ 7.5\ \text{mL x}\ 1\ \text{x}\ 100\ \text{mL}}{6.0\ \text{mL x}\ 50\ \text{g}} = 0.008$$

$$\text{Percent Recovery} = \frac{0.008 - 0.000\ \ \text{ppm}}{0.01\ \text{ppm x}\ 0.896} = 89\%$$

Time Required for Analysis

Time required per sample set, where a sample set consists of twelve (12) matrix samples, 8 standards and 1 reagent blank:

Extraction and Clean-up take approximately 8 hours for one analyst

LC-MS/MS analysis and data processing takes approximately 17 hours

TOTAL = approximately 25 hours for one analysis (2 calendar days)