

1. SUMMARY

The purpose of this study was to independently validate the analytical method entitled "Determination of FOE 5043 and Four Metabolites in Soil by High Performance Liquid Chromatography Electrospray Tandem Mass Spectrometry (LC-ESI/MS/MS)", ALTA method AMFOE2.

10 g of soil was shake extracted with 20 mL of an acidified water/acetonitrile solution. After centrifuging, a 10 mL aliquot of the supernatant was removed and added to the internal standard. The aliquot volume was reduced by nitrogen evaporation to a 5 mL final volume and syringe filtered (a concentration factor of 1 g/mL). Chromatographic separation of the analytes was achieved by elution through a base deactivated column in the reverse phase which was connected to an Electrospray Atmospheric Pressure Ionization (API) interface. The sulfonic acid, oxalate, and thiadone metabolites were analyzed by negative ionization MS/MS and the parent and alcohol metabolite were analyzed by positive ionization MS/MS with each ionization mode requiring separate injections. Quantitation was accomplished by average area response factors generated from a four-point calibration curve injected in triplicate. The calibration curve was verified by the injection of continuing calibration standards. The initial QC calibration curves were good for all the compounds except thiadone which exhibited a lower response.

Each method validation trial consisted of 2 unfortified controls, one method blank, 5 controls fortified at approximately 10 ng/g, and 5 controls fortified at approximately 100 ng/g. These nominal values are used on the tables and in the text.

The control samples and method blank were verified to be free of interferences at the retention times of interest. There were no perceived problems with thiadone evaporation losses since there were good recoveries for Trial II. The moisture content of the soil employed in this study was determined to be 8.3%.

1. SUMMARY cont'd

One set (12 samples plus one method blank) required approximately 7 hours to extract and prepare for analysis. Each analysis and/or injection (i.e. negative ion analysis only) required approximately 15 minutes.

This method has been validated on a soil from a particular site in Wisconsin and verification of its applicability to other soil types would be required.

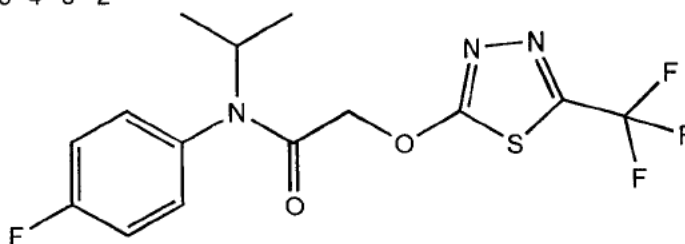
The protocol (including the Analytical Method AMFOE2) applicable to this study are included in Appendix I. There were no Standard Operating Procedure deviations. A summary of all correspondence among Enviro-Test Labs, Alta Analytical Labs and Bayer Corporation is provided in Appendix III.

2. MATERIALS

2.1 Reference Substances and Chemical Structures:

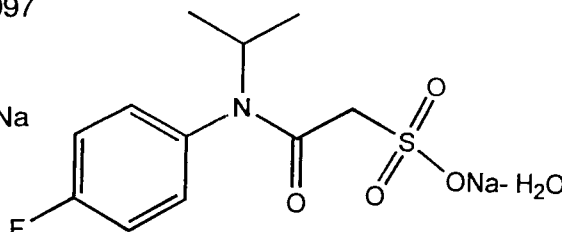
2.1.1 Characterization Information

Standard Name: FOE 5043
Chemical Name: N-(4-fluorophenyl)-N-(1-methylethyl)-2-([5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxyl)acetamide
Date of Receipt: October 22, 1997
Reference No.: 93R-008-88 (AS005)
CAS No.: 142459-58-3
Empirical Formula: $C_{14}H_{13}F_4N_3O_2S$

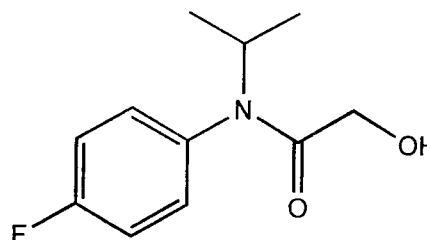


2.1.1 Characterization Information cont'd

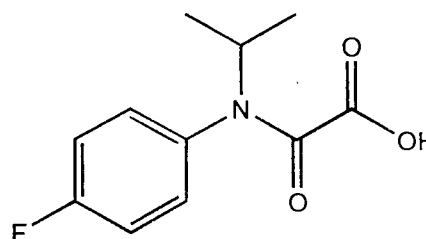
Standard Name: FOE 5043 sulfonic acid, sodium salt, monohydrate
Chemical Name: 4-fluoro-N-methylethylanilinesulfoacetamide, sodium salt, monohydrate
Date of Receipt: October 22, 1997
Reference No.: 95B53-189
CAS No.: Not available
Empirical Formula: $C_{11}H_{15}FNO_5SNa$



Standard Name: FOE 5043 alcohol
Chemical Name: N-(4-fluorophenyl)-2-hydroxy-N-(1-methylethyl)acetamide
Date of Receipt: October 22, 1997
Reference No.: 9112433-141
CAS No.: Not available
Empirical Formula: $C_{11}H_{14}FNO_2$

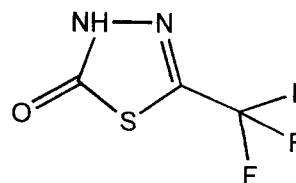


Standard Name: FOE 5043 oxalate
Chemical Name: N-(4-fluorophenyl)-N-methylethyl-aminooxoacetic acid
Date of Receipt: October 22, 1997
Reference No.: 94B175-051
CAS No.: Not available
Empirical Formula: $C_{11}H_{12}FNO_3$

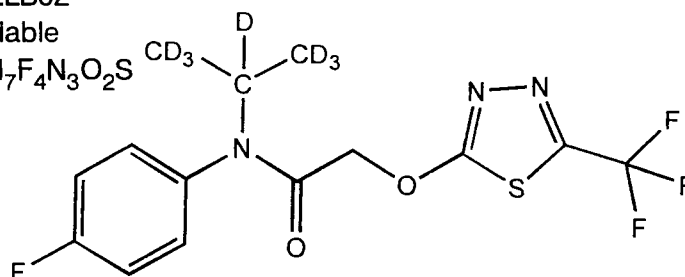


2.1.1 Characterization Information cont'd

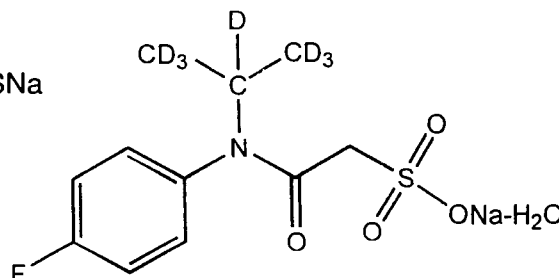
Standard Name: FOE 5043 thiadone
 Chemical Name: 5-(trifluoromethyl)-1,3,4-thiadiazol-2(3o)-one
 Date of Receipt: October 22, 1997
 Reference No.: 90-10-76
 CAS No.: 84352-75-0
 Empirical Formula: $C_3HF_3N_2OS$



Standard Name: d₇-FOE 5043
 Chemical Name: N-(4-fluorophenyl)-N-[1-(methyl-d₃)ethyl-1,2,2,2-d₄]-2-([5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxy)acetamide
 Date of Receipt: October 22, 1997
 Reference No.: 940202ELB02
 CAS No.: Not available
 Empirical Formula: $C_{14}H_6^2H_7F_4N_3O_2S$

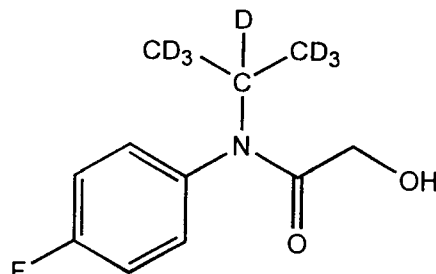


Standard Name: d₇-FOE 5043 sulfonic acid, sodium salt, monohydrate
 Chemical Name: 4-fluoro-N-[1-(methyl-d₃)ethyl-1,2,2,2-d₄]anilinesulfoacetamide, sodium salt, monohydrate
 Date of Receipt: October 22, 1997
 Reference No.: 940422ELB02
 CAS No.: Not available
 Empirical Formula: $C_{11}H_8^2H_7FNO_5SNa$

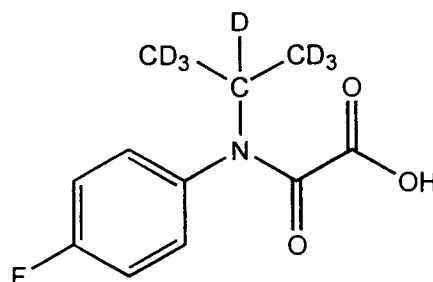


2.1.1 Characterization Information cont'd

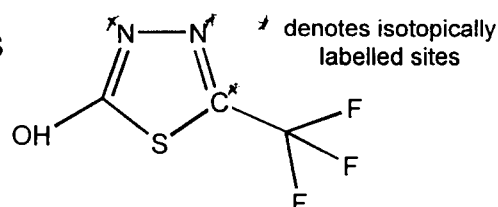
Standard Name: d₇-FOE 5043 alcohol
 Chemical Name: N-(4-fluorophenyl)-2-hydroxy-N-[1-(methyl-d₃)ethyl-1,2,2,2-d₄]acetamide
 Date of Receipt: October 22, 1997
 Reference No.: 960320ELB01
 CAS No.: Not available
 Empirical Formula: C₁₁H₇²H₇FNO₂



Standard Name: d₇-FOE 5043 oxalate
 Chemical Name: 4-(fluorophenyl)-[1-(methyl-d₃)ethyl-1,2,2,2-d₄]amino oxoacetic acid
 Date of Receipt: October 22, 1997
 Reference No.: 960320ELB02
 CAS No.: Not available
 Empirical Formula: C₁₁H₅²H₇FNO₃



Standard Name: FOE 5043 thiadone (¹³C/¹⁵N₂)
 Chemical Name: 5-trifluoromethyl-1,3,4-thiadiazol-2(3H)ol
 Date of Receipt: October 22 and December 23, 1997
 Reference No.: 93B115-191
 CAS No.: Not available
 Empirical Formula: C₂¹³CHF₃¹⁵N₂OS



2.1.2 Solution Preparation:

All standard solutions were stored at approximately -20°C in amber bottles with teflon lined screw caps and the absence of light when possible.

2.1.2.1 Native Stock Solutions

Individual stock solutions (nominally 0.1 mg/mL) of each native analyte were prepared by dissolving approximately 10 mg of reference material in 100 mL of methanol. For the analyte, FOE 5043 sulfonic acid, the reference material, FOE 5043 sulfonic acid, sodium salt, monohydrate involved a conversion factor of 0.8731.

$$\frac{\text{Molecular Weight of FOE 5043 sulfonic acid}}{\text{Molecular Weight of FOE 5043 sulfonic acid salt}} = \frac{275.3 \text{ g/mol.}}{315.3 \text{ g/mol.}}$$

For compound purities of less than 96%, a correction in the weight was made in order to calculate the concentration. None of the native reference materials or their stock solutions expired during the course of the study.

2.1.2.2 Internal Standard Stock Solutions

Individual stock solutions (nominally 0.1 mg/mL) of each internal standard were prepared by dissolving approximately 10 mg of reference material in 100 mL of methanol. As in the preparation of the native sulfonic acid a conversion factor of $282.3/322.3=0.8759$ was applied to the concentration of the salt form. The isotopically labelled thiadone was received as solution and was diluted to obtain a nominally 0.1 mg/mL solution in 100 mL of methanol. Compound purities of less than 96% were weight corrected. None of the internal standard reference materials or their stock solutions expired during the course of the study.

2.1.2.3 Fortification Standard Solutions

A 5 µg/mL mixed intermediate native stock solution was prepared by diluting 1.25 mL of each native stock solution (nominally 0.1 mg/mL) to 25 mL with methanol. A 1 µg/mL mixed intermediate native stock solution was prepared by diluting 5 mL of the 5 µg/mL mixed intermediate native stock solution to 25 mL with methanol.

2.1.2.4 Mixed Intermediate Internal Standard Stock Solution

A 2 $\mu\text{g/mL}$ mixed intermediate internal standard stock solution was prepared by diluting 0.5 mL of each internal standard stock solution (0.1 mg/mL) to 25 mL with methanol.

2.1.2.5 Calibration Curve Standard Solutions (Not Actual)

Volume of 5 $\mu\text{g/mL}$ mixed inter. native stock solution (mL)	Volume of 2 $\mu\text{g/mL}$ mixed inter. internal standard stock solution (mL)	Volume of Methanol (mL)	Volume of 0.1% Formic acid (mL)	Nominal Native Standard Conc. (ng/mL)	Nominal Internal Standard Conc. (ng/mL)
4	2	0	94	200	40
0.8	2	3.2	94	40	40
0.4	2	3.6	94	20	40
0.1	2	3.9	94	5	40

2.2 Reagents

Acetonitrile, HPLC grade, EM Science, OmniSolv®

Argon, Zero Gas, Praxair

Formic acid, 'AnalaR', BDH Inc.

Hydrochloric acid, TraceMetal grade, Fisher Scientific

Methanol, HPLC grade, EM Science, OmniSolv®

Nitrogen, Pre Pure, Praxair

Water, HPLC grade, EM Science, OmniSolv®

0.1% formic acid in water (v/v). Add 4 mL of formic acid to 4 L of water.

1:1 0.1N hydrochloric acid:acetonitrile (v/v). Dilute 8.3 mL of hydrochloric acid to 1L of water (makes 0.1N hydrochloric acid). Add 500 mL of 0.1N hydrochloric acid to 500 mL of acetonitrile.

0.1% formic acid in acetonitrile (v/v). Add 4 mL of formic acid to 4 L of acetonitrile.

2.3 Laboratory Materials and Apparatus:

Balance, analytical, model ER-182A, A N D (accuracy to the nearest 0.00001 g)
Balance, top-loading, model FX-4000 A N D (accuracy to the nearest 0.01 g)
Bottles, sample, amber, 100 mL and 1 L, EPA Certified Clean Ltd.
Centrifuge, table-top, model HN-S, International Equipment Company
Cylinders, graduated, Kimax®, 10, 25 and 500 mL and 1 and 2 L, Kimble Glass Inc.
Evaporator, N-Evap, model 111, Organomation Associates Inc.
Filters, syringe, 0.45 μ m , Acrodisc GHP13, Gelman
Flasks, volumetric, 25 and 100 mL, Kimax®, Kimble Glass Inc.
Oven, Thelco, model 27, Precision Scientific Co.
Pipets, micro, 100 and 200 μ L, Drummond
Pipet, 0.5-5 mL, Socorex, Swiss
Pipets, pasteur, 145 mm, Maple Leaf Brand
Pipet, repeater, Eppendorf
Scoopula, stainless steel, 165 mm, VWR Scientific Products
Shaker, wrist-action, Burrell Corp.
Spatulas, micro, VWR Scientific Products
Syringes, single use, 3cc, Luer Lok®, Becton Dickinson
Tubes, culture, 16 \times 125 mm, Kimax®, Kimble Glass Inc.
Vials, autosampler, clear, National Scientific Co.
Vials, volatile organic analysis (VOA), amber, EPA Certified Clean Ltd.
Vials, VOA, clear, EPA Certified Clean Ltd.
Weighing dish, aluminum, VWR Scientific Products

2.4 Instrumentation, Conditions and Parameters:**2.4.1 Instrumentation:**

PE Sciex Biomolecular Mass Analyzer (API III) equipped with Electrospray Ionization Inlet Source

2.4.2 HPLC Operating Conditions:

Column: Inertsil 5 μ ODS-2, 50 \times 2.0 mm, MetaChem Technologies Inc.
Flow Rate: 0.80 mL/min.
Split: approximately 15:1

2.4.2 HPLC Operating Conditions: cont'd

Negative Ion Analysis:Injection Volume: 20 μ L

Time (min.)	Gradient	Mobile Phase	
		0.1% Formic Acid in Water (%)	0.1% Formic Acid in Acetonitrile (%)
0.00	---	90	10
1.00	---	90	10
8.00	linear	30	70
8.10	step	90	10
13.10	---	90	10

Positive Ion Analysis:Injection Volume: 50 μ L

Time (min.)	Gradient	Mobile Phase	
		0.1% Formic Acid in Water (%)	0.1% Formic Acid in Acetonitrile (%)
0.00	---	90	10
6.00	linear	10	90
7.10	step	90	10
12.10	---	90	10

2.4.3 Mass Spectrometer Operating Parameters:

Nebulizer Gas Pressure: 80 psi (N_2)
 Auxillary Gas Flow Rate: 2.5 L/min. (N_2)
 Curtain Gas Flow Rate: 1.0 L/min. (N_2)
 Collision Gas Thickness: approximately 275 (Ar)

2.4.3 Mass Spectrometer Operating Parameters: cont'd

Negative Ion Analysis:

Ion Spray Voltage: -3500 V

Orifice Potential: -60 V

Collision Offset Voltage: 15 V

Dwell Time: 0.3 s

Analyte	Mass Transition (m/z)	Retention Time (min.)
FOE 5043 thiadone	169→113	5.5
FOE 5043 thiadone (¹³ C/ ¹⁵ N ₂)	172→113	5.5
FOE 5043 oxalate	224→152	6.2
d ₇ -FOE 5043 oxalate	231→159	6.2
FOE 5043 sulfonic acid	274→121	7.1
d ₇ -FOE 5043 sulfonic acid	281→121	7.1

Positive Ion Analysis:

Ion Spray Voltage: 4500 V

Orifice Potential: 40 V

Collision Offset Voltage: -15 V

Dwell Time: 0.4 s

Analyte	Mass Transition (m/z)	Retention Time (min.)
FOE 5043 alcohol	212→170	4.3
d ₇ -FOE 5043 alcohol	219→171	4.3
FOE 5043	364→194	6.3
d ₇ -FOE 5043	371→201	6.3

2.5 Sample Management:

Approximately 1 kg of untreated control soil (from Bayer study no. 3022103 Wisconsin) was received at ETL in Edmonton, Alberta from Bayer Corporation in Stilwell, KS on October 20, 1997. This untreated control sample (Bayer sample no. 93412001, ETL sample no. E7-10-716-01A) arrived at ambient temperature and was stored at -20±5°C until subsampling. The sample was received already processed and did not require any further processing.

2.5 Sample Management: cont'd

On November 5, 1997, the sample was thawed and one 6 g and two 10 g subsamples were weighed out. The 6 g subsample was used for moisture determination and the two 10 g subsamples were extracted the same day as part of the Initial Quality Control Check. The negative ion analysis was performed on November 17, 1997 and the positive ion analysis on November 19, 1997. These extracts and those following were stored at $-20\pm 5^{\circ}\text{C}$ until analysis if they were not analyzed within the same day they were extracted.

For Trial I of the Method Validation, twelve 10 g subsamples were weighed out on December 11, 1997, extracted on December 11, 1997 and analyzed on December 11-12, 1997.

For Trial II, twelve 10 g subsamples were weighed out on January 7, 1998, extracted on January 7, 1998 and analyzed on January 8, 1998.

3. ANALYTICAL PROCEDURES

The analytical method used for the analysis of soil in this study was ALTA method no. AMFOE2.

In the method set-up, the LC/MS/MS system sensitivity was significantly greater than what appeared in the analytical method. In order to obtain similar sensitivities to those in the method, the injection volume was changed from 50 to 20 μL for the negative ion analysis. Also a conversion in the mass spectrometry and interface parameters was required due to the use of a Turbo Ion Spray - API interfaced with a PE Sciex LC/MS/MS system versus a heated capillary - API interfaced with a Finnigan LC/MS/MS system which was employed in the original method. The exact parameter values for the current system are included with each trial's data packet.

APPENDIX V**Statistical Calculations**

- mean of a sample

$$m = \frac{\sum x}{n}$$

m – mean
x – measured value
n – number of measurements

- estimate of the standard deviation of the population from the sample data

$$s = \left[\frac{\sum x^2 - (\sum x)^2}{n - 1} \right]^{1/2}$$

s – standard deviation

- estimate of the standard deviation of a distribution of sample means of a one-sample test

$$s_m = \frac{s}{\sqrt{n}}$$

s_m – standard deviation (population estimate)

- degrees of freedom for a single small sample size

degrees of freedom = $n - 1$

- confidence interval for means based on small samples

$$m \pm t_c s_m$$

t_c – Student's score critical value

APPENDIX V (continued)Sample Calculations

- response factor (RF)

$$RF = \frac{(Area_{nat}) * (Conc_{istd})}{(Area_{istd}) * (Conc_{nat})}$$

Area_{nat} – area response of native analyte
 Conc_{istd} – concentration of internal standard (ng/mL)
 Area_{istd} – area response of internal standard (ng/mL)
 Conc_{nat} – concentration of native analyte

- average response factor (RF_{ave})

$$RF_{ave} = \frac{\sum RF_i}{n}$$

- accuracy

$$Accuracy = \frac{RF_j}{RF_{ave}}$$

i – measurement number

- relative standard deviation (RSD)

$$RSD = \frac{(s) * (100\%)}{RF_{ave}}$$

- aliquot factor (Aliq. Factor)

$$Aliq. Factor = \frac{Vol_{.alq}}{Vol_{.ext}}$$

Vol_{.alq} – aliquot volume (mL)
 Vol_{.ext} – extraction volume (mL)

- analytical result (Wet Wt.)

$$Analytical Result = \frac{(Area_{nat}) * (Conc_{istd}) * (Conc. Factor) * (Final Vol.)}{(RF_{ave}) * (Area_{istd}) * (Samp. Mass) * (Aliq. Factor)}$$

Final Vol. – final extract volume (mL)
 Samp. Mass – Extracted sample weight (g)

- moisture content (M)

$$M = \left[1 - \frac{(Dry Wt.)}{(Wet Wt.)} \right] * (100\%)$$

Dry Wt. – mass of dry subsample (g)

Sample Calculations (continued)

- recovery

$$\text{Recovery} = \frac{(\text{Analytical Result})}{(\text{Fort. Level})} (100\%)$$

Fort. Level – spiking level (ng/g)

Example Calculation

- control sample fortified at 10.9 ng/g (E7-10-716-01A-S15)

FOE 5043 thiadone

$$\text{Aliq. Factor} = \frac{(10.0 \text{ mL})}{(20.0 \text{ mL})} = 0.500 \text{ mL/mL}$$

$$\text{Analytical Result} = \frac{(17135) \cdot (42.8 \text{ ng/mL}) \cdot (5.0 \text{ mL})}{(0.915) \cdot (78094) \cdot (10.0 \text{ g}) \cdot (0.500)} = 10.3 \text{ ng/g}$$

$$\text{Recovery} = \frac{(10.3 \text{ ng/g})}{(10.9 \text{ ng/g})} (100\%) = 94\%$$