

1. SUMMARY

The purpose of this study was to independently validate the analytical method entitled "Determination of FOE 5043 and the Alcohol, Oxalate, Thiadone and Sulfonic Acid Metabolites in Groundwater by High Performance Liquid Chromatography Electrospray Tandem Mass Spectrometry (LC-ESI/MS/MS)", ALTA method AMFOE3.

50 mL of groundwater was acidified and the analytes were retained on an octadecyl bonded sorbent. The analytes and internal standards were eluted with methanol, the volume was reduced by nitrogen evaporation to a 2 mL final volume, and syringe filtered (a concentration factor of 25 mL/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.

The method validation trial consisted of 2 unfortified controls, one method blank, 5 controls fortified at approximately 0.1 ng/mL, and 5 controls fortified at approximately 1 ng/mL. 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.

The low response of the thiadone internal standard from the initial QC check calibration curve was observed. Communication with the Study Monitor revealed that the isotopically labelled thiadone internal standard exhibited a poor response relative to the native response in the calibration curve at ETL compared to the responses observed in the development of the analytical method at Alta Labs. It was discovered that the thiadone internal standard received by ETL was a 4.26% solution in acetonitrile and not a neat standard as the documentation showed at the time of receipt. A new aliquot of this standard at 4.26% was acquired from Bayer and new standard solutions were prepared for use in the second trial. A new calibration curve was shot using these new standards.

1. SUMMARY cont'd

One set (12 samples plus one method blank) required approximately 5 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 groundwater from a site in Janesville, Iowa and verification of its applicability to other groundwater types would be required.

The protocol (including the Analytical Method AMFOE3) applicable to this study is included in Appendix I. There was one protocol deviation issued which dealt with the draft report being sent later than the protocol stated. (See Appendix II) There were no Standard Operating Procedure deviations. Appendix III contains a detailed communication log between ETL and either Bayer or Alta Labs. A summary of all correspondence among Enviro-Test Labs, Alta Analytical Labs and Bayer Corporation is provided in Appendix III. Appendix IV and V contain the spreadsheets for the analysis, example chromatograms and example sample calculations.

Note that some of the original raw data, particularly Sections 2 and 3, have photocopies of the original document. The original documentation, that was shared with the "Independent Laboratory Validation of Analytical Method AMFOE2 for the Analysis of Soil for FOE 5043 and Four Metabolites Residues" (Sponsor study no. F3112106, Protocol no. 97ILV101), is contained in the Analytical Raw Data Package of that report.

2. MATERIALS

2.1 Reference Substances and Chemical Structures:

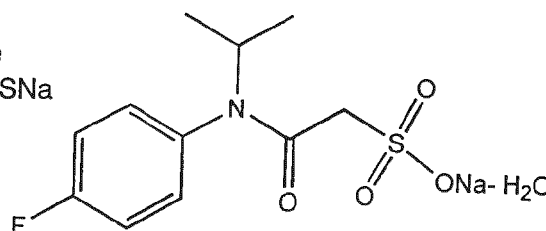
(Storage conditions for reference substances was $-20 \pm 5^\circ\text{C}$.)

2.1.1 Characterization Information

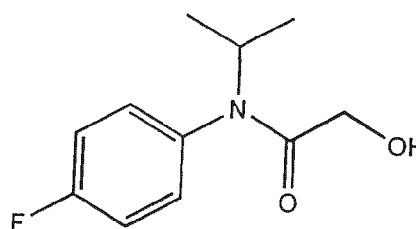
Standard Name:	FOE 5043
Chemical Name:	N-(4-fluorophenyl)-N-(1-methylethyl)-2-([5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxyl)acetamide
Date of Receipt:	October 22, 1997
Reference No.:	93R-008-88 (AS005)
CAS No.:	142459-58-3
Empirical Formula:	$\text{C}_{14}\text{H}_{13}\text{F}_4\text{N}_3\text{O}_2\text{S}$

2.1.1 Characterization Information cont'd

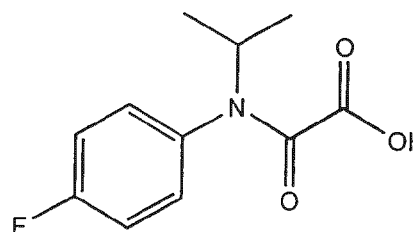
Standard Name: FOE 5043 sulfonic acid, sodium salt, monohydrate
Chemical Name: 4-fluoro-N-methylethylanilinesulfoacetamide, sodium salt, monohydrate
Date of Receipt: October 22, 1997
Reference No.: 95B53-189
CAS No.: Not available
Empirical Formula: $C_{11}H_{15}FNO_5SNa$



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: $C_{11}H_{14}FNO_2$

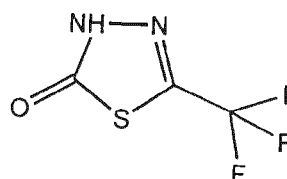


Standard Name: FOE 5043 oxalate
Chemical Name: N-(4-fluorophenyl)-N-methylethyl-aminooxoacetic acid
Date of Receipt: October 22, 1997
Reference No.: 94B175-051
CAS No.: Not available
Empirical Formula: $C_{11}H_{12}FNO_3$

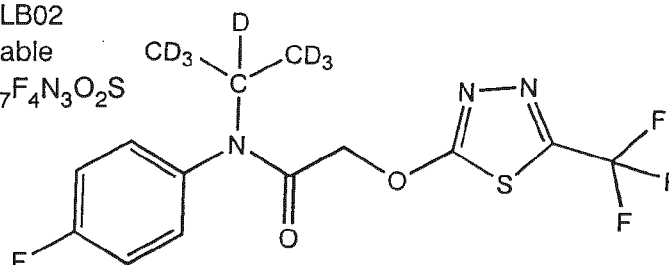


2.1.1 Characterization Information cont'd

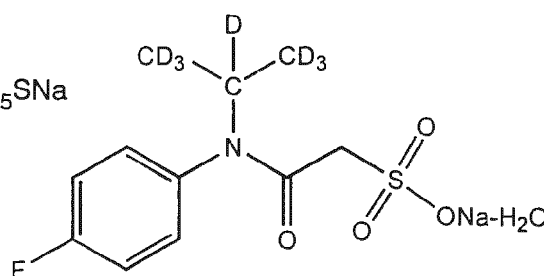
Standard Name: FOE 5043 thiadone
 Chemical Name: 5-(trifluoromethyl)-1,3,4-thiadiazol-2(3o)-one
 Date of Receipt: October 22, 1997
 Reference No.: 90-10-76
 CAS No.: 84352-75-0
 Empirical Formula: $C_3HF_3N_2OS$



Standard Name: d_7 -FOE 5043
 Chemical Name: N-(4-fluorophenyl)-N-[1-(methyl- d_3)ethyl-1,2,2,2- d_4]-2-([5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxyl)acetamide
 Date of Receipt: October 22, 1997
 Reference No.: 940202ELB02
 CAS No.: Not available
 Empirical Formula: $C_{14}H_6^2H_7F_4N_3O_2S$

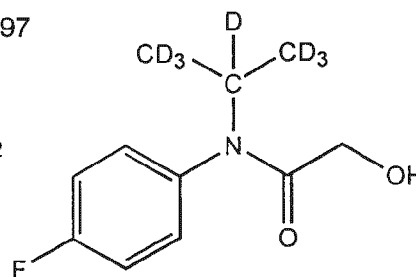


Standard Name: d_7 -FOE 5043 sulfonic acid, sodium salt, monohydrate
 Chemical Name: 4-fluoro-N-[1-(methyl- d_3)ethyl-1,2,2,2- d_4]anilinesulfoacetamide, sodium salt, monohydrate
 Date of Receipt: October 22, 1997
 Reference No.: 940422ELB02
 CAS No.: Not available
 Empirical Formula: $C_{11}H_8^2H_7FNO_5SNa$

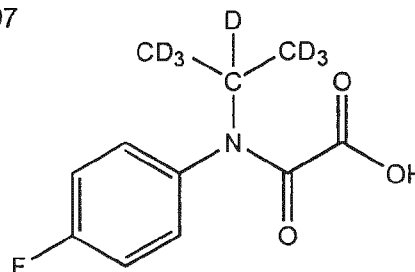


2.1.1 Characterization Information cont'd

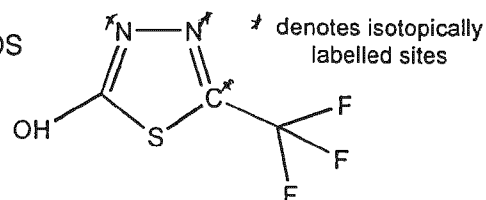
Standard Name: d₇-FOE 5043 alcohol
 Chemical Name: N-(4-fluorophenyl)-2-hydroxy-N-[1-(methyl-d₃)ethyl-1,2,2,2-d₄]acetamide
 Date of Receipt: October 22, 1997
 Reference No.: 960320ELB01
 CAS No.: Not available
 Empirical Formula: C₁₁H₇²H₇FNO₂



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.

2.1.2.3 Fortification Standard Solutions cont'd

A 0.05 $\mu\text{g}/\text{mL}$ mixed intermediate native stock solution was prepared by diluting 0.25 mL of the 5 $\mu\text{g}/\text{mL}$ mixed intermediate native stock solution to 25 mL with methanol.

2.1.2.4 Mixed Intermediate Internal Standard Stock Solution

A 1 $\mu\text{g}/\text{mL}$ mixed intermediate internal standard stock solution was prepared by diluting 0.25 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 1 $\mu\text{g}/\text{mL}$ mixed inter. native stock solution (mL)	Volume of 5 $\mu\text{g}/\text{mL}$ mixed inter. native stock solution (mL)	Volume of 1 $\mu\text{g}/\text{mL}$ 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)
---	2	5	43	50	100	50
---	0.4	5	44.6	50	20	50
0.5	---	5	44.5	50	5	50
0.125	---	5	44.8	50	1.25	50

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®

1N hydrochloric acid in water. Dilute 83 mL of hydrochloric acid to 1L of water.

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

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.
Cylinders, graduated, Kimax®, 50 and 100 mL, Kimble Glass Inc.
Evaporator, N-Evap, model 111, Organomation Associates Inc.
Extraction cartridges, Solid Phase, Mega Bond Elut® C18, 1 g, Varian Canada, Inc.
Filters, syringe, 0.45 μ m, Acrodisc GHP13, Gelman
Flasks, Erlenmeyer, 125 mL, Kimax®, Kimble Glass Inc.
Flasks, volumetric, 25 and 100 mL, Kimax®, Kimble Glass Inc.
Pipets, micro, 100 μ L, Drummond
Pipet, 0.5-5 mL, Socorex, Swiss
Pipets, pasteur, 145 mm, Maple Leaf Brand
Pipet, repeater, Eppendorf
Sample reservoir, 50 mL
Spatulas, micro, VWR Scientific Products
Syringes, single use, 3cc, Luer Lok®, Becton Dickinson
Tubes, culture, 16 \times 125 mm, Kimax®, Kimble Glass Inc.
Vacuum manifold, Solid Phase Extraction, Visiprep 12-port model, Supelco
Vacuum pump, Magna Force, 2 HP, Sanborn Manufacturing Co.
Vials, autosampler, clear, National Scientific Co.
Vials, volatile organic analysis (VOA), amber, EPA Certified Clean Ltd.

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 3.0 mm, MetaChem Technologies Inc.
Flow Rate: 0.40 mL/min.
Split: approximately 10:1

2.4.2 HPLC Operating Conditions: cont'd

Negative Ion Analysis Injection Volume: 20 μ LPositive Ion Analysis Injection Volume: 40 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	---	10	90
7.00	linear	10	90
7.10	step	90	10
17.10	---	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)

Negative Ion Analysis:

Ion Spray Voltage: -3500 V

Orifice Potential: -60 V

Collision Offset Voltage: 15 V

Dwell Time: 300 ms

Analyte	Mass Transition (m/z)	Retention Time (min.)
FOE 5043 thiadone	169→113	4.2
FOE 5043 thiadone ($^{13}C/^{15}N_2$)	172→113	4.2
FOE 5043 oxalate	224→152	4.4
d_7 -FOE 5043 oxalate	231→159	4.4
FOE 5043 sulfonic acid	274→121	5.3
d_7 -FOE 5043 sulfonic acid	281→121	5.3

2.4.3 Mass Spectrometer Operating Parameters: cont'd

Positive Ion Analysis:

Ion Spray Voltage: 4500 V

Orifice Potential: 40 V

Collision Offset Voltage: -15 V

Dwell Time: 400 ms

Analyte	Mass Transition (m/z)	Retention Time (min.)
FOE 5043 alcohol	212→170	4.2
d ₇ -FOE 5043 alcohol	219→171	4.2
FOE 5043	364→194	5.1
d ₇ -FOE 5043	371→201	5.1

2.5 Sample Management:

Six groundwater samples, 200-250 mL each, (from Bayer study no. 3212401 Iowa) were received at ETL in Edmonton, Alberta from Bayer Corporation in Stilwell, KS on October 20, 1997. These control samples (Bayer sample no.'s 1MW1D-23, 1MW2D-23, 1MW3D-23, 1MW6S-23, 1MW2D-24, and 1MW3D-24, ETL sample no.'s E7-10-751-01A to -06A, respectively) arrived at ambient temperature and were stored at 5±5°C until analysis.

On November 6, 1997, two 50 mL subsamples were measured and extracted as part of the Initial Quality Control Check. The positive ion analysis was performed on November 6, 1997 and the negative ion analysis on November 12, 1997. These extracts and those following were stored at -20±5°C until analysis if they were not analyzed within the same day they were extracted.

For the Method Validation Trial, twelve 50 mL subsamples were taken on January 13, 1998, extracted on January 13-14, 1998 and analyzed on January 14-15, 1998.

3. ANALYTICAL PROCEDURES

The analytical method used for the analysis of groundwater in this study was ALTA method no. AMFOE3.

In the method set-up, the LC/MS/MS system sensitivity was significantly greater than what appeared in the analytical method.

108265

Protocol No: 97ILV102
ETL Report No.: 98BAY05.REP

3. ANALYTICAL PROCEDURES cont'd

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 and from 50 to 40 μL for the positive ion analysis. The HPLC conditions were changed for both the positive and negative MS/MS analysis to a gradient common to both analyses in order to obtain retention similar to that in the original method. The exact parameter values for the current system are included with each data packet.

APPENDIX VStatistical 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

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

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

$$\sigma = n - 1$$

σ – degrees of freedom

- 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

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

i – measurement number

- relative standard deviation (RSD)

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

- analytical result

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

Final Vol. – final extract volume (mL)

Samp. Vol. – Extracted sample volume (mL)

- recovery

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

Fort. Level – spiking level (ng/g)

APPENDIX V (continued)Example Calculation

- control sample fortified at 0.109 ng/mL (E7-10-751-03A-S4)

FOE 5043 thiadone

$$\text{Analytical Result} = \frac{(7813) * (53.5 \text{ ng/mL}) * (2.0 \text{ mL})}{(0.937) * (177909) * (50.0 \text{ mL})} = 0.100 \text{ ng/mL}$$

$$\text{Recovery} = \frac{(0.100 \text{ ng/mL}) * (100\%)}{(0.109 \text{ ng/mL})} = 92\%$$