INTRODUCTION

Valent USA Corporation has developed a method for determining residues of etoxazole metabolites R4, R7, R8 and R11 in soils treated with etoxazole. As a part of the registration package, an independent laboratory validation of this method, RM-37SM, "Determination of Etoxazole Metabolites R4, R7, R8 and R11 in Soil" is required.

Valent USA Corporation conducted this independent laboratory validation using personnel and laboratories separate from those involved in the development and use of this method.

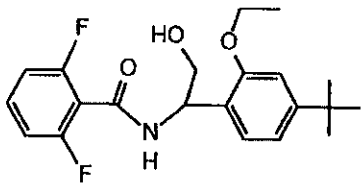
EXPERIMENTAL

The protocol, with the analytical method RM-37SM, can be found in Appendix I.

Test and Reference Substances: The test substances for this study were laboratory dilutions of standards the R4, R7, R8 and R11 metabolites, obtained from Valent USA Corporation. The chemical names, structures, lot numbers, purity and certification dates are shown below:

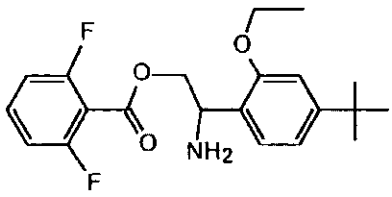
R4: *N*-[-1-(4-*tert*-butyl-2-ethoxypheny;)-2-hydroxyethyl]-2,6 difluorobenzamide

Lot Number:	AS 1856b
Purity:	98.8%
Certification Date:	May 5, 2000

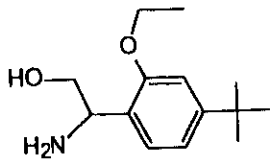


R7: 2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethyl-2',6'-difluorobenzoate

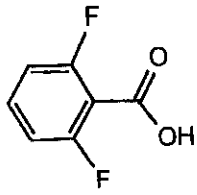
Lot Number:	AS 1857c
Purity:	97.0%
Certification Date:	May 8, 2000



R8: 2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethanol
Lot Number: AS 1858a
Purity: 97.9%
Certification Date: August 23, 2001



R11: 2,6-difluorobenzoic acid
Lot Number: AS 1859c
Purity: 100%
Certification Date: August 6, 2001



Certificates of Analysis for these standards can be found in Appendix II.

Individual 1.0 mg/mL solutions of the R4, R7 and R8 metabolites were made in methanol:0.05% aqueous acetic acid (1:1, v:v). A 1.0 mg/mL solution of R11 was made in 0.05% aqueous acetic acid.

A 10 µg/mL test substance solution containing the R4, R7 and R8 metabolites was prepared by diluting the three individual analyte solutions in methanol:0.05% aqueous acetic acid (1:1, v:v). This solution was further diluted in methanol:0.05% aqueous acetic acid (1:1, v:v) to make analytical solutions of R4, R7 and R8 in concentrations ranging from 0.005 µg/mL to 0.1 µg/mL.

A separate 10 µg/mL test substance solution containing R11 was prepared by diluting the R11 metabolite in 0.05% aqueous acetic acid. This solution was further diluted in 0.05% aqueous acetic acid to make analytical solutions of R11 in concentrations ranging from 0.005 µg/mL to 0.1 µg/mL.

Test System: The test system for this study is soil. The soil used for the study was obtained from terrestrial field dissipation studies for etoxazole, conducted in California (V-20271, 1999-

2000) and Oregon (V-22154, 2000-2001). Untreated soil samples, described below, were combined and homogenized to create a sample of adequate size.

Sample ID	Soil Depth (cm)	Amount Used (g)
V-20271-7U-1	0-7.5 cm	450 g
V-20271-7U-2	7.5-15 cm	250 g
V-20271-4U-1	0-7.5 cm	250 g
V-20271-4U-5	45-60 cm	250 g
V-20271-13U-3	15-45 cm	300 g
V-20271-15U-1	0-7.5 cm	550 g
V-20271-3U-1	0-7.5 cm	400 g
V-20271-3U-3	15-45 cm	500 g
V-20271-17U-1	0-7.5 cm	600 g
V-22154-7U-1	0-7.5 cm	600 g
V-22154-11U-1	0-7.5 cm	750 g

The method trial consisted of a reagent blank, two control soil samples, five soil sample spiked at 0.02 ppm and five samples spiked at 0.20 ppm. All fortified samples were spiked with both test solutions (R4 + R7 + R8 and R11).

Reagents and Equipment: See Appendix I – Study Protocol and Analytical Method for a complete list of reagents and equipment used for this study. No substitutions were made to the reagents or equipment described.

Analytical Method: A complete description of the analytical method can be found in Appendix I - Study Protocol and Analytical Method.

Briefly, 20 g of soil was extracted with 60 mL of acetone by shaking for ca. 10 minutes followed by two 10 minute extractions using 60 mL of acetone:0.05% aqueous acetic acid (9:1, v:v). The sample extracts were suction filtered through a GF/A filter paper and combined. The filtrate was transferred to a graduated cylinder and the volume was adjusted to 200 mL with acetone.

For the R4, R7 and R8 analyses, a 50 mL (5 g soil equivalent) aliquot of the initial extract was transferred to a 250 mL round bottom flask, and concentrated using rotary evaporation to remove all of the acetone. The remaining aqueous sample (ca. 2 mL) was transferred to a 10 mL volumetric flask using 5 mL of methanol, and brought to volume with 0.05% aqueous acetic acid. Analyses for R4, R7 and R8 were conducted by LC/MS/MS using the conditions described below.

For the R11 analysis, a second 50 mL (5 g soil equivalent) aliquot of the initial extract was transferred to a 250 mL round bottom flask, and concentrated using rotary evaporation to remove all of the acetone. The remaining aqueous sample (ca. 2 mL) was transferred to a 10 mL volumetric flask using 5 mL of 0.05% aqueous acetic acid, and brought to volume with 0.05% aqueous acetic acid. Analyses for R11 were conducted by LC/MS/MS using the conditions described below.

Instrument Conditions – R4, R7 and R8: The following instruments and conditions were used for the analysis of the R4, R7 and R8 portion of the sample:

Instrument:	HPLC:	Hewlett Packard 1100 Quatenary Pump HPLC system with autosampler and column heater.
	MS/MS Detector:	Finnigan LCQ MS/MS ion trap
	Interface:	Electrospray ionization
	Data System:	Finnigan Xcaliber software
HPLC Column:	YMC ODS-AM, 3 µm, 100 mm x 3.0 mm (Waters)	
HPLC Conditions:	Column Temp.:	35°C
	Mobile Phase:	A: 0.05% aqueous Acetic Acid; B: Methanol
	0 minutes:	70% A / 30% B
	9 minutes:	70% A / 30% B
	16 minutes:	30% A / 70% B
	16.01 minutes:	0% A / 100% B
	19 minutes:	0% A / 100% B
	19.01 minutes:	70% A / 30% B
	Post run:	3 minutes
	Flow Rate:	0.5 µL/min
Injection:	Draw Speed:	200 µL/min
	Volume:	25 µL
	Eject Speed:	200 µL/min
Ionization Parameters:	(controlled by LCQ Tune File)	
	Source Voltage:	5 kV
	Capillary Temp:	200°C
	Capillary Voltage:	8 V
	Sheath Gas (N_2):	69 units (ca. 1.0 L/min)
	Auxiliary Gas (N_2):	6 units (ca. 1.8 L/min)
	Ion Injection Time:	1000 msec
	Multipole 1 Offset:	-5.50 V
	Multipole 2 Offset:	-10.5 V
	InterMultipole Lens:	-82.00 V

MS/MS Conditions: (controlled by LCQ Method File)

Divert Valve:	0 minutes:	To Waste
	2.5 minutes:	To Source
	16.8 minutes:	To Waste

	R8	R7	R4
Precursor Ion:	237.9	377.8	377.8
Isolation Width:	3.0 amu	3.0 amu	3.0 amu
Polarity:	Positive	Positive	Positive
Collision Energy:	24.0%	26.0%	25.0%
Quantitation Ion:	220.9	360.9	220.9
Mass Range	1 amu	1 amu	1 amu
Peak Retention Times:	7.7 minutes	11.1 minutes	15.2 minutes

Instrument Conditions – R11: The following instruments and conditions were used for the analysis of the R11 portion of the sample:

Instrument:

HPLC:	Hewlett Packard 1100 Quatenary Pump HPLC system with autosampler and column heater.
MS/MS Detector:	Finnigan LCQ MS/MS ion trap
Interface:	Electrospray ionization
Data System:	Finnigan Xcaliber software

HPLC Column: Luna (C18), 3 µm, 50 mm x 3.0 mm (Phenomenex)

HPLC Conditions:

Column Temp.:	35°C	
Mobile Phase:	A 0.05% aqueous Acetic Acid	B Methanol
0 minutes:	70%	30%
9 minutes:	30%	70%
9.01 minutes:	70%	30%
12 minutes:	70%	30%
Post run:	3 minutes	
Flow Rate:	0.5 µL/min	

Injection:

Draw Speed:	200 µL/min
Volume:	50 µL
Eject Speed:	200 µL/min

Ionization Parameters: (controlled by LCQ Tune File)

Source Voltage:	5 kV
Capillary Temp:	220°C
Capillary Voltage:	8 V

Sheath Gas (N_2):	96 units (ca. 1.4 L/min)
Auxiliary Gas (N_2):	3 units (ca. 0.9 L/min)
Ion Injection Time:	1000 msec
Multipole 1 Offset:	4.75 V
Multipole 2 Offset:	8.00 V
InterMultipole Lens:	14.00 V

MS/MS Conditions: (controlled by LCQ Method File)

Divert Valve:	0 minutes:	To Waste
	5.0 minutes:	To Source
	19.5 minutes:	To Waste

	R11
Precursor Ion:	156.9
Isolation Width:	3.0 amu
Polarity:	Negative
Collision Energy:	28.0%
Quantitation Ion:	113.1
Mass Range	1 amu

Peak Retention Times: 6.3 minutes

Analysis Procedure (R4, R7, R8 and R11 Analyses): Prior to analysis, the 10x LOQ fortified samples (F6, F7, F8, F9 and F10) were diluted by taking 0.5 mL of sample and adding 0.5 mL of the appropriate solvent. The LC system required conditioning by injecting three or four sample extracts prior to the initial standard injection. Analytical standards of different concentrations were injected after every two or three samples and at the end of the analytical sequence.

Calculations: A second order polynomial curve was generated for each anayte based on the results of the analysis of six concentrations of the analytical standards. The curve equation was generated using a Microsoft Excel spreadsheet by plotting the data as the µg/mL concentration of the standard (y) verses the peak area response (x), giving the equation:

$$y = Ax^2 + Bx + C,$$

where y = concentration,
x = area response,
A, B and C = curve constants generated from the standards

The curve was weighted proportional to the concentration of each standard by plotting each standard data point into the graph with a frequency equal to 1/concentration. For example, the 0.005 µg/mL standards was entered 200 times (1/0.005), and the 0.2 µg/mL standard was entered 5 times (1/0.2).

The µg/mL concentrations for each sample were calculated from the curve by entering the area response for the samples as the *x* value and solving for *y*. The amounts, as ppm, were then calculated using the formula:

$$ppm = \frac{C * FV * EV * DF}{W * AV}$$

Where:
C = concentration of sample (µg/mL from curve)
FV = final volume of sample (10 mL)
EV = total extraction volume (200 mL)
DF = dilution factor, used when the sample is diluted prior to analysis
W = initial sample weight (20 g)
AV = aliquot of the sample volume carried through the procedure (50 mL).

The percent recoveries were calculated by:

$$\% \ recovery = \frac{ppm \ in \ sample}{ppm \ fortification \ level} * 100\%$$

An example of these calculations is included in Appendix III, Analytical Data.

RESULTS AND DISCUSSION

Results: The method was successfully validated on the first trial.

Problems Encountered:

During the initial standard solution preparation, difficulties of getting the analytes into solution, especially R4, were encountered. Sonication (as recommended for R11) was used for all the analytes. The R4 analyte required approximately 45 minutes of sonication to become fully dissolved.

Instrument conditioning was critical to successful analyses. Sequences were programmed to run twice in order to make sure the instrument performance was acceptable. For both analyses, the first sequence was not successful, and the second sequence was needed.

Description of Contact:

Contact between the Study Director and the method's author was limited to a request for additional LC/MS/MS tune parameters for optimizing the performance of the instrument.

Printouts of the tune file parameters for the R4, R7, R8 method and the R11 method were received from the method's author.

Time Requirement:

The time required to complete the workup of samples from extraction to the final volume adjustment was approximately 6 hours. The sample workup was conducted over a two day period. The samples were stored refrigerated after the initial extraction prior to taking the aliquots.

The time required for the analysis of the R4, R7 and R8 metabolites was approximately 7.5 hours for the set of thirteen samples and the linearity standards. The instrument was programmed to run the sequence two times, for a total of ca. 15 hours analysis time.

Approximately two hours was required after the R4, R7, R8 analysis to purge and set up the LC/MS/MS system for the R11 analysis.

The analysis time for the set of conditioners, thirteen samples and linearity standards for the R11 metabolite was approximately six hours. The sequence was programmed to run twice, for a total of ca. 12 hours analysis time.

CONCLUSIONS

The analytical method RM-37SM, "Determination of Etoxazole Metabolites R4, R7, R8 and R11 in Soil" was successfully validated within the guidelines of EPA's Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods. The method, as written, provides acceptable recoveries, however, additional comments regarding the solubility of the analytes and the need for instrument conditioning, and additional information for optimizing the LC/MS/MS should be amended to the method.