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VALENT TECHNICAL CENTER
DUBLIN, CALIFORNIA

DETERMINATION OF ETOXAZOLE METABOLITES R4, R7, R8, AND R11 IN SOIL METHOD RM-37SM

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INTRODUCTION

This method determines residues of etoxazole degradates R4, R7, R8, and R11 in soil.

Briefly, R4, R7, R8, and R11 residues are extracted from soil using acetone and acetone:0.05% acetic acid (9:1). The sample is brought up to a known volume and two aliquots are taken. The acetone is evaporated from one aliquot and the sample is diluted in methanol:0.05% acetic acid (1:1) for R4, R7, and R8 analysis. The acetone is evaporated from the second aliquot and the sample is diluted in 0.05% acetic acid for R11 analysis. Resides are quantitated by ion trap HPLC/MS/MS.

REAGENTS

Acetic acid – glacial, reagent grade or equivalent.

Acetone – pesticide quality or equivalent.

Methanol - pesticide quality or equivalent.

Water – HPLC grade.

REAGENT SOLUTIONS

0.05% acetic acid– Add 0.5 mL of acetic acid, glacial to 1 liter of HPLC grade water. Store at room temperature.

Acetone:0.05% acetic acid (9:1, v/v) - Combine 9 parts acetone with 1 part 0.05% acetic acid. For example, add 900 mL acetone and 100 mL 0.05% acetic acid sequentially to a reagent bottle. Store at room temperature.

Methanol:0.05% acetic acid (1:1, v/v) – Combine 1 part methanol with 1 part 0.05% acetic acid. For example, add 500 mL methanol and 500 mL 0.05% acetic acid sequentially to a reagent bottle. Store at room temperature.

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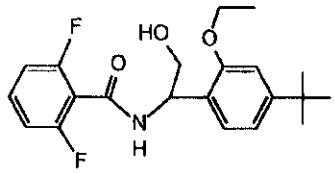
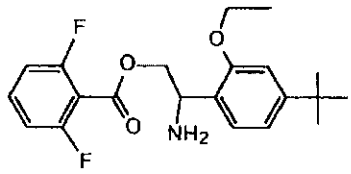
REFERENCE STANDARDS

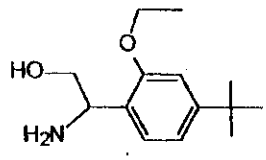
R4, *N*-[1-(4-*tert*-butyl-2-ethoxyphenyl)-2-hydroxyethyl]-2,6-difluorobenzamide - analytical standard of a known purity. Prepare a stock solution containing 1.0 mg/mL in methanol:0.05% acetic acid (1:1). All solutions should be kept refrigerated when not in use.



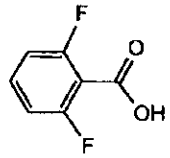
R7, 2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethyl 2',6'-difluorobenzoate - analytical standard of a known purity. Prepare a stock solution containing 1.0 mg/mL in methanol:0.05% acetic acid (1:1). All solutions should be kept refrigerated when not in use.



R8, 2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethanol - analytical standard of a known purity. Prepare a stock solution containing 1.0 mg/mL in methanol:0.05% acetic acid (1:1). All solutions should be kept refrigerated when not in use.



R11, 2,6-difluorobenzoic acid - analytical standard of a known purity. Prepare a stock solution containing 1.0 mg/mL in 0.05% acetic acid. Standard may need to be sonicated to completely dissolve. All solutions should be kept refrigerated when not in use.



STANDARD SOLUTIONS

Fortifying Solution (R4 + R7 + R8)- 10 µg/mL – Transfer 1.0 mL of each 1.0 mg/mL stock solution in to a single 100 mL volumetric flask and dilute to volume with methanol:0.05% acetic acid (1:1). All solutions should be kept refrigerated when not in use.

Fortifying Solution (R11)- 10 µg/mL – Transfer 1.0 mL of 1.0 mg/mL stock solution in to a 100 mL volumetric flask and dilute to volume with 0.05% acetic acid. All solutions should be kept refrigerated when not in use.

Linearity Standard Solutions (R4 + R7 + R8) – Prepare a minimum of six linearity standards by diluting the fortifying stock standard solution with methanol:0.05% acetic acid (1:1) to concentrations of 0.005, 0.01, 0.02, 0.05, 0.075, and 0.1 µg/mL. All solutions should be kept refrigerated when not in use.

Linearity Standard Solutions (R11) – Prepare a minimum of six linearity standards by diluting the fortifying stock standard solution with 0.05% acetic acid to concentrations of 0.005, 0.01, 0.02, 0.05, 0.075, and 0.1 µg/mL. All solutions should be kept refrigerated when not in use.

EQUIPMENT

Büchner funnels - 9 cm diameter.

Filter flasks - 500 mL.

Filter paper - Whatman GF/A glass fiber or equivalent, 9 cm diameter.

Graduated cylinders – 250 mL, with stoppers.

High Performance Liquid Chromatograph with MS/MS detector – Hewlett Packard 1100 Quatenary Pump HPLC system with an autosampler coupled to a Finnagan LCQ MS/MS ion trap with an electrospray ionization interface or equivalent system.

Mason jars - 1 pint with plastic screw cap lids or equivalent.

Reciprocating shaker – Eberbach or equivalent.

Rotary evaporator - Büchi or equivalent, equipped with a water bath.

Round-bottom flasks - 250 mL with ⅀ 24/40 ground glass joints.

Ultrasonic bath – Branson 3200 or equivalent.
Volumetric flasks – 10 mL.

ANALYTICAL PROCEDURE

1. Extraction

Weigh 20 grams (± 0.1 grams) of soil into a one pint Mason jar. At this point, if required by the testing facility, fortify control samples for method recovery with R4, R7, R8, and R11 (See Note 1). Add 60 mL of acetone to the sample and shake for 10 - 15 minutes.

Filter the sample into a 500 mL filter flask using a Büchner funnel containing Whatman GF/A glass fiber filter paper (premoistened with acetone). Transfer the filter cake back to the Mason jar. Add 60 mL of acetone:0.05% acetic acid (9:1, v/v) to the sample and shake for 10-15 minutes. Filter as decribed above into the 500 mL filter flask, combining this extract with the first. Repeat this extraction and filtration procedure with an additional 60 mL of acetone:0.05% acetic acid (9:1, v/v). Rinse the Mason jar with two 10 mL portions of acetone and add each portion to the Büchner funnel. All filtrations are collected in the same filter flask.

Transfer the combined filtrates to a 250 mL graduated cylinder and dilute to 200 mL with acetone. Stopper and invert the cylinder several times to thoroughly mix the extract. Transfer 50 mL of the sample extract (equivalent to 5 grams of sample) to a 250 mL round-bottom flask and reserve for Step 2, Final Volume for R4, R7, and R8. Transfer an additional 50 mL of the sample extract to another 250 mL round-bottom flask and reserve for Step 3, Final Volume for R11.

2. Final volume for R4, R7, and R8

Evaporate the acetone from the extract using a rotary-evaporator and a water bath set at ≤ 25°C. There will be approximately 1.5 – 2 mL of aqueous extract left in the flask.

Transfer the aqueous extract to a 10 mL volumetric flask. **The water extract MUST be transferred to the volumetric flask before addition of methanol to the round-bottom flask.** Add 5 mL of methanol to the 250 mL round-bottom flask, sonicate for approximately 15 seconds, and transfer to the 10 mL volumetric flask. Bring the sample extract up to volume by adding 0.05% acetic acid. Reserve this extract in the refrigerator for up to a week for Step 4, LC/MS/MS Conditions for R4, R7, and R8.

3. Final Volume for R11

Evaporate the acetone from the extract using a rotary-evaporator and a water bath set at ≤ 25°C. There will be approximately 1.5 – 2 mL of aqueous extract left in the flask.

Transfer the aqueous extract to a 10 mL volumetric flask. Add 5 mL of 0.05% acetic acid to the 250 mL round-bottom flask, sonicate for approximately 15 seconds, and transfer to the 10 mL volumetric flask. Bring the sample extract up to volume by adding 0.05% acetic acid. Reserve this extract in the refrigerator for up to a week for Step 5, LC/MS/MS Conditions for R11.

4. LC/MS/MS Conditions for R4, R7, and R8

Condition the instrument with at least three injections of sample extract. Analyze a range of linearity standards with the analytical sequence. The linearity standards should be interspersed with the samples in the run sequence. Each sequence must begin and end with a linearity standard.

Transfer a portion of the sample extract to an autosampler vial and analyze, along with the linearity standard solutions, using the following operating conditions:

HPLC Conditions:

Column:	YMC ODS-AM, 3µm, 100mm x 3.0mm (Waters part # AM 125031003 WT)
Column Oven Temperature:	35°C
Mobile Phase:	A = 0.05% acetic acid in HPLC water B= Methanol
Gradient Program:	T = 0 min, 70% A + 30% B T = 9.0 min, 30% A + 70% B T = 16.0 min, 30% A + 70% B T = 16.01 min, 100% B T = 19.0 min, 100% B Post Run: 3 minutes
Flow Rate:	0.5 mL/minute
Injection:	
Drawing Speed:	200 µL/minute

Injection Volume:	25µL
Ejecting Speed:	200 µL/minute

LC/MS Interface Conditions:

Interface:	Electrospray Ionization
Typical Values Depending Upon Instrument Tune:	
Source Voltage:	5 kV
Capillary Temperature:	200°C
Capillary Voltage:	33 V
Sheath Gas (N_2):	69 units (ca. 1.0 L/minute)
Auxillary Gas (N_2):	6 units (ca. 1.8 L/minute)

MS/MS Conditions:

Scan type: Selected Reaction Monitoring (SRM)

	R8	R7	R4
Percusor Ion Mass:	237.9	377.8	377.8
Isolation Width Window:	3.0 amu	3.0 amu	3.0 amu
Quantitation Ion Mass:	220.9	360.9	220.9
Quantitation Ion Mass Range:	220.4-221.4	359.9-361.9	220.4-221.4
Polarity:	Positive	Positive	Positive
Collision Energy:	24%	26%	25%
Ion Injection Time:	1 second	1 second	1 second
Scan Rate:	1 per second	1 per second	1 per second

The instrument parameters shown above are given only as a guide. They may be modified as needed to optimize the chromatography, to resolve matrix interferences, or to utilize other types of LC/MS instruments. Each set of chromatograms must be clearly labeled with the LC/MS/MS parameters used.

5. LC/MS/MS Conditions for R11

Condition the instrument with at least three injections of sample extract. Analyze a range of linearity standards with the analytical sequence. The linearity standards should be interspersed with the samples in the run sequence. Each sequence must begin and end with a linearity standard.

Transfer a portion of the sample extract to an autosampler vial and analyze, along with the linearity standard solutions, using the following operating conditions:

HPLC Conditions:

Column:	Luna (C18), 3µm, 50mm x 3.0mm (Phenomenex part # 00B-4251-YO)
Column Oven Temperature:	35°C
Mobile Phase:	A = 0.05% acetic acid in HPLC water B = Methanol
Gradient Program:	T = 0 min, 70% A + 30% B T = 9.0 min, 30% A + 70% B T = 9.01 min, 70% A + 30% B T = 12.0 min, 70% A + 30% B Post Run: 3 minutes
Flow Rate:	0.5 mL/minute
Injection:	
Drawing Speed:	200 µL/minute
Injection Volume:	50µL
Ejecting Speed:	200 µL/minute

LC/MS Interface Conditions:

Interface:	Electrospray Ionization
Typical Values Depending Upon Instrument Tune:	
Source Voltage:	5 kV
Capillary Temperature:	220°C
Capillary Voltage:	8 V
Sheath Gas (N_2):	96 units (ca. 1.4 L/minute)
Auxillary Gas (N_2):	3 units (ca. 0.9 L/minute)

MS/MS Conditions:

Scan type:	Selected Reaction Monitoring (SRM)
Percusor Ion Mass:	156.9
Isolation Width Window:	3.0 amu
Quantitation Ion Mass:	113.1
Quantitation Ion Mass Range:	112.6-113.6
Polarity:	Negative
Collision Energy:	28%
Ion Injection Time:	1 second
Scan Rate:	1 per second

The instrument parameters shown above are given only as a guide. They may be modified as needed to optimize the chromatography, to resolve matrix interferences, or to utilize other types

of LC/MS instruments. Each set of chromatograms must be clearly labeled with the LC/MS/MS parameters used.

6. Calculations

The concentration of R4, R7, R8, or R11 in each sample extract is calculated on the basis of peak area using a second order polynomial equation. The equation is automatically generated through the use of the graphing functions of an Excel spreadsheet.(See Note2). The data is presented graphically as concentration of the linearity standards verses the peak areas of the linearity standards which results in the following equation:

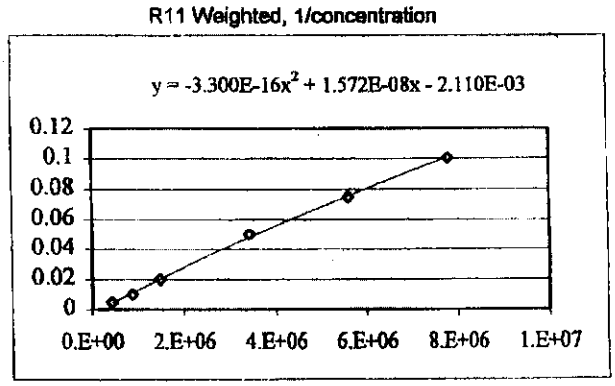
$$Y=Ax^2 + Bx + C$$

The data is weighted inversely proportional to the concentration of each standard. The weighting is accomplished by incorporating each data point into the graph with a frequency equal to (1/concentration). For example, a data point for a linearity standard with a concentration of 0.005 µg/mL would be entered into the graph 200 times while a data point for a linearity standard with a concentration of 0.1 µg/mL would be entered into the same graph 10 times.
Example:

For a linearity area response of:

µg/mL	Area	µg/mL	Area
0.1	7,790,251	0.02	1,472,684
0.075	5,597,524	0.01	865,960
0.05	3,423,204	0.005	421,805

The resulting graph from the Excel spreadsheet is as follows:



$$Y = Ax^2 + Bx + C$$
$$A = -3.300E\text{-}16$$
$$B = 1.572E\text{-}08$$
$$C = -2.110E\text{-}03$$

To ensure that the equation is appropriate, the areas of the linearity standards are entered into the the equation of the line and calculated as samples. The standards must calculate within 20% of their known concentration. An example of this from the above data is the 0.02 µg/mL standard which has an area of 1,472,684. The calculated concentration would be 0.021µg/mL which is 103% of the known concentration.

A sample extract with an area response of 3,377,633 would have a concentration as follows:

$$\mu g/mL = Ax^2 + Bx + C$$
$$\mu g/mL = (-3.300E\text{-}16 \times 3{,}377{,}633 \times 3{,}377{,}633) + (1.572E\text{-}08 \times 3{,}377{,}633) - 2.110E\text{-}03$$
$$\mu g/mL = 0.0472$$

The amount of R4, R7, R8, or R11 found in each sample is calculated using the following formula:

$$ppm = \frac{CxFVxEVxDF}{WxAV}$$

Where:

C = concentration of extract.(µg/mL from equation)

FV = final volume of extract.(10 mL)

EV = total extraction volume.(200 mL)

DF = dilution factor, used if the sample extract is diluted prior to analysis.

W = sample weight analyzed.(20 g)

AV = aliquot of the extraction volume carried through the procedure.(50 mL)

Example: From the above example, a sample with a calculated concentration of 0.0472µg/mL would be calculated as follows:

$$ppm = \frac{(0.0472ug/mL)x(10mL)x(200mL)}{(20g)x(50mL)}$$

$$ppm = 0.0944$$

LIMITS OF DETECTION AND QUANTITATION

The validated limit of quantitation (LOQ) of R4,R7, R8, and R11 in soil analyzed by this method is 0.02 ppm. The estimated limit of detection (LOD) is 0.01 ppm. This LOD is calculated by dividing the lowest analyte concentration from the validated linear range (0.005 µg/mL) of the measurement system by matrix concentration in the sample extracts (0.5 g/mL):

$$LOD = [0.005\ \mu g/mL] \div [0.5\ g/mL] = 0.01\ \mu g/mL$$

ANALYSIS TIME

A trained analyst, familiar with this method, can complete the extraction of a set of twelve samples for R4, R7, R8, and R11 in less than 8 hours. Quantitation will take an additional 8 hours for R4, R7, and R8. Changing the HPLC column and quantitation of R11 will take an additional 8 hours if a second instrument is not available.

NOTES

1. At Valent, a standard operating procedure requires that a fortified control sample be analyzed with each set of samples. If the testing facility does not require concurrent analysis of fortified control samples, or if a UTC sample is not available, this method requirement may be waived.

   The level of fortification is generally at the LOQ of the method and/or five times the LOQ. Method recoveries must be 70% to 120% to be acceptable unless approved by the chemist responsible for the analysis.

2. There are other programs that can calculate a weighted regression graph such as Curve Expert 1.3 (Hyams Development, Starkville,MS).