1. INTRODUCTION

1.1 Scope of the Method

Valent Analytical Method RM-37S-3 was developed by Valent U.S.A. LLC to determine the residues of etoxazole and its metabolites R8 and R13 using LC-MS/MS. This report represents the method's validation by an independent laboratory, ADPEN Laboratories, Inc. in Jacksonville, Florida.

The independent laboratory validation was conducted at two fortification levels, the limit of quantitation (LOQ), which is 0.01 ppm (mg/kg), and 10xLOQ, which is 0.1 ppm (mg/kg), in sediment and surface water. Each analytical set contained one reagent blank, two unfortified control samples, and seven replicates at each fortification level.

1.2 Principle of the Method

Residues of etoxazole, R8, and R13 are extracted from sediment with an aqueous buffer solution (10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v)) using Omni Beadruptor homogenizer. A 0.2 mL aliquot of the extract was diluted to 10 mL volume with methanol/water (50/50, v/v), vortexed and vialed for LC-MS/MS analysis.

Residues of etoxazole, R8, and R13 are extracted from surface water with an aqueous buffer solution (10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v)) by vortexing. A 0.5 mL aliquot of the extract was diluted to 20 mL volume with methanol/water (50/50, v/v), mixed and vialed for LC-MS/MS analysis.

1.3 Specificity

To demonstrate the specificity of the analytical method, one additional mass transition (confirmatory) were monitored for each of the analytes simultaneous to the primary quantitation transitions as specified below.

Analyte	Mode	Quantitation (*m/z*)	Confirmation (*m/z*)
Etoxazole	Positive	360→141	360→177
R8	Positive	238→165	238→147
R13	Positive	358→141	358→274

The method was able to accurately determine residues of etoxazole and its metabolites, and no interferences were observed at the retention time of the analyte peaks.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test System

The test systems considered in this study were untreated control sediment and surface water. The sediment sample (Lab Code #170110002-018) was collected from an unassociated terrestrial field dissipation study. The surface water sample (Lab Code #170110002-019) was collected locally in Jacksonville, Florida.

The test systems were characterized at AGVISE Laboratories, (604 Highway 15 West, Northwood, ND 58267). Copies of these characterization data reports are provided in Appendix A.

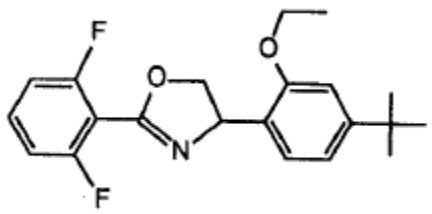
Each analytical set was identified with an analytical set number, which consisted of a unique number (e.g., WO-17121802). The test system samples were assigned unique lab code numbers, and these were recorded in the raw data (e.g., control sediment, PA.CA.T.Bulk.Sediment, was identified as 171218001-001). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

2.2 Test and Reference Substances

The test substances shown below were provided by Valent USA LLC and were kept frozen at an average temperature of -22 ºC until use for the analytical portion of this study. Valent USA LLC has determined the purity and characterization of the test substances being used in this study. Detailed reports are available at Valent in their Dublin, California facility.

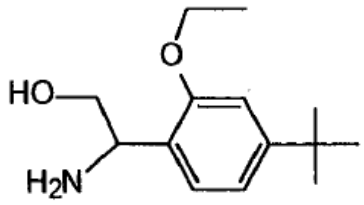
The certificates of analysis are presented in Appendix B. A detailed summary of the reference substances are presented below:

Common Name:	**Etoxazole**
CAS Name:	2-(2,6-difluorophenyl)-4-[4-(1,1-dimethylethyl)-2-ethoxyphenyl]-4,5-dihydrooxazole
Lot Identification:	AS 1800e
Molecular Formula:	$C_{21}H_{23}F_2NO_2$
Molecular Weight:	359.4 g/mol
Purity:	99.3%
Expiration Date:	April 10, 2019
Storage:	Freezer
Source:	Valent USA, LLC
Structural Formula:	

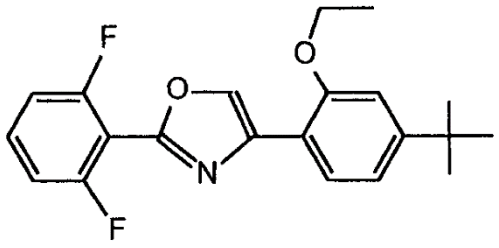


Test and Reference Substance (continued)

Common Name:	**R8 (Etoxazole Metabolite)**
CAS Name:	2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethanol
Lot Identification:	AS 1858e
Molecular Formula:	$C_{14}H_{23}NO_2$
Molecular Weight:	237.3 g/mol
Purity:	98.9%
Expiration Date:	August 21, 2019
Storage:	Freezer
Source:	Valent USA, LLC
Structural Formula:	



Common Name:	**R13 (Etoxazole Metabolite)**
CAS Name:	4-(4-tert-butyl-2-ethoxyphenyl)-2-(2,6-difluorophenyl)oxazol
Lot Identification:	AS 1860e
Molecular Formula:	C21H21F2 NO_2
Molecular Weight:	357.4 g/mol
Purity:	99.7%
Expiration Date:	August 10, 2019
Storage:	Freezer
Source:	Valent USA, LLC
Structural Formula:	



3. ANALYTICAL METHOD

Residues of etoxazole and its metabolites R8 and R13 were determined using the extraction and instrument parameters as described in Valent analytical method RM-37S-3 entitled, "The Determination of Residues of Etoxazole and its Metabolites, R8 and R13 in Sediment and Surface water Matrices Using LC-MS/MS.", which is attached in Appendix C. Instrument parameters used for analyses are described in Table 14. A brief description of the analytical procedures is described below.

Sediment: A 5 gram sample of sediment was mixed with 20 mL of aqueous buffer solution (10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v)). The mixture was sonicated for 5 minutes and mixed using Omni Beadruptor homogenizer twice for 30 second intervals at 4 m/s, centrifuged for 15 minutes at 3500 rpm and the supernatant (extract) was transferred to a separate centrifuge tube. The sample marc was re-extracted following the steps above and combining the supernatants in the same centrifuge tube. A 0.2 mL aliquot of the combined extract was diluted to 15 mL using methanol/water (50/50, v/v), vortexed and vialed for LC-MS/MS analysis.

Surface water: A 15 mL of surface water sample was mixed with 15 mL of aqueous buffer solution (10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v)) and centrifuged for 10 minutes at 3500 rpm. A 0.5 mL aliquot of the extract was diluted to 20 mL using methanol/water (50/50, v/v), vortexed and vialed for LC-MS/MS analysis.

Figure 30 Typical Reside Calculations for Etoxazole, R8 ad R13

Calibration standards and samples were analyzed using LC-MS/MS. Calibration curves and residues (ppm) were calculated using Analyst 1.6.2 data handling software via linear regression with 1/x weighting (for all analytes with the exception of etoxazole metabolite R8 in sediment, where a quadratic regression with 1/x weighing was used to calculate the recoveries) and transcribed into the detailed analytical data report sheets (Appendix E). The recoveries calculated using following formulas:

a) Calibration curve: $y = mx + b$ Solving for x: $x = \frac{y - b}{m}$

Where, m = slope
b = y intercept
x = Amount found (ng)
y = Peak Area

The following equations were used within LIMS for residue and recovery calculations:

b) Amount of sample injected (mg) = (sample weight × injection size) / final sample volume or sample injected (µL) = (sample amount mL × injection size) / final sample volume

Where,

Final sample volume = [(extract volume ÷ aliquot volume) × final extract volume × DF]

c) Amount Found (ppm) = (ng found / mg of sample injected.)

d) $\text{Percent recovery}\,(\%) = \frac{\text{Amount found (ppm)}}{\text{Amount fortified (ppm)}} \times 100$

Example: Recovery calculations of etoxazole (m/z 360.2 → 141.0) in surface water. All metabolite residue calculations were performed similarly.

Set ID: WO-17121802 Lab Code: 17121802-Recovery1-1
Analyte: Etoxazole Sample Name: Control + 0.01 ppm

a) Calibration curve: y = (5.40313e+008)x + 35480.9

Solving for x: 1612413 = (5.40313e+008)x + 35480.9
x =(1612413 - 35480.9)/(5.40313e+008) = 0.0029186 ng

b) Amount of sample injected $(\text{mL}) = \frac{25\ \mu\text{L}}{1200\ \text{mL}} \times 15\ \text{mL} = 0.3125\ \mu\text{L}$

c) Amount found $(\text{ppm}) = \frac{0.0029186\ \text{ng}}{0.3125\ \mu\text{L}} = 0.00933952\ \text{ppm}$

d) Recovery $(\%) = \frac{0.00933952\ \text{ppm}}{0.01\ \text{ppm}} \times 100 =$ **93.4%**

1.0 INTRODUCTION

1.1 Scope of the Method

The objective of this study was to develop analytical method for the determination of Etoxazole and its metabolite R-8, and R-13 (Figure 1, 2, and 3) in surface water and sediment. The limit of quantitation (LOQ) of each analyte of the method has been established at 0.01 µg/mL (0.01 ppm) for surface water, and at 0.01 µg/g (0.01 ppm) for sediment.

Residues of etoxazole, R-8 etoxazole metabolite, and R-13 etoxazole metabolite are extracted from surface water and sediment using aqueous buffer solution containing 10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v) by shaking (vortexing) method for surface water sample and by high speed extraction method using beadruptor homogenizer at speed setting of 4m/s (addition of beads is not required) for sediment sample. The extracts of surface water and sediment are centrifuged for 10 minutes at 3500 rpm. The supernatant is diluted with methanol/water, 50/50 (v/v) and the residues of compounds are analysed by LC/MS/MS with quantitation based on a comparison of peak areas with those of known standards.

This method satisfies US EPA guideline EPA OCSPP 850.6100

1.2 Method Summary

Residues of etoxazole and its metabolites R-8 and R-13 in surface water and sediment matrices are analyzed by extracting with basified aqueous buffer solution in methanol/water, 90/10 (v/v) using shaking extraction method for surface water and high speed beadruptor extraction method (addition of beads is not required) and analysed by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

The LOQ of this method for both surface water and sediment is 0.01 ppm.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

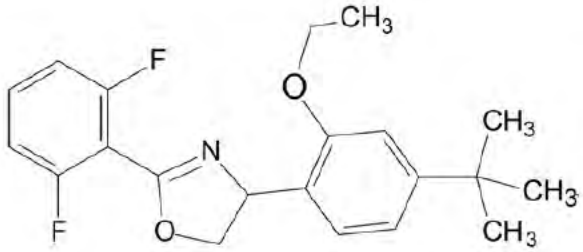
The recommended equipment and apparatus are listed in. Equipment with equivalent performance specifications may be substituted.

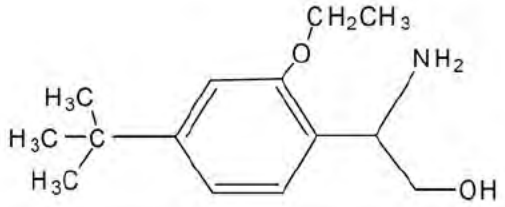
Bead Ruptor Homogenizer, OMNI
Balance, analytical or equivalent.
Cylinder, graduated, 10-mL, 25-mL, 50-mL, 100-mL, 500 mL, and 1000-mL or equivalent.

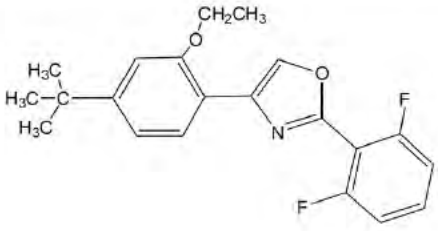
Pipets, glass, Class A certified, assorted volumes. These pipets are used when an exact addition of liquid is required (i.e., sample fortification, standard solution preparation and dilutions).
Pipetter, Eppendorf Repeater, 100 – 1000 µL variable volume range and 500-5000 µL variable volume range, or equivalent.
Vials, clear or amber, 1.5-mL with Teflon/Silicone plug with slit, or equivalent.
Centrifuge, IEC Centra GP8R, or equivalent.
Tube, centrifuge, polypropylene, 50-mL graduated with plastic screw cap, or equivalent.

2.2 Test and Reference Items

The following analytical reference standards are used:

Common Name of Active	Etoxazole
Company Experimental Name	Etoxazole
IUPAC Name	(*R, S*)-5-*tert*-butyl-2-[2-(2,6-difluorophenyl)-4,5-dihydro-1,3-oxazol-4-yl]phenetole
CAS Name	2-(2,6-difluorophenyl)-4-[4-(1,1-dimethylethyl)-2-ethoxyphenyl]-4,5-dihydrooxazole
CAS Number	153233-91-1
Lot Number	AS 1800e
Manufacturer's Sample Identity	Valent Reference VTC-1372-49
Date of Analysis	10-Apr-17
Expiration Date	10-Apr-19
Storage Conditions	Freezer
Molecular Formula	$C_{21}H_{23}F_2NO_2$
Molecular Weight	359.4
Structural Formula	
Purity (%)	99.3

Common Name of Active	R-8
Company Experimental Name	R-8 (Etoxazole metabolite)
IUPAC Name	N.A.
CAS Name	2-amino-2-(4-*tert*-butyl-2-ethoxyphenyl)ethanol
CAS Number	153281-81-3
Lot Number	AS 1858e
Manufacturer's Sample Identity	Valent Reference VTC-701-6
Date of Analysis	21-Aug-17
Expiration Date	21-Aug-19
Storage Conditions	Freezer
Molecular Formula	$C_{14}H_{23}NO_2$
Molecular Weight	237.3
Structural Formula	
Purity (%)	98.9

Common Name of Active	R-13
Company Experimental Name	R-13 (S-1) (Etoxazole metabolite)
IUPAC Name	N.A.
CAS Name	4-(4-*tert*-butyl-2-ethoxyphenyl)-2-(2,6-difluorophenyl)oxazol
CAS Number	N.A.
Lot Number	AS 1860e
Manufacturer's Sample Identity	Valent Reference VDL-701-7
Date of Analysis	10-Aug-17
Expiration Date	10-Aug-19
Storage Conditions	Freezer
Molecular Formula	$C_{21}H_{21}F_2NO_2$
Molecular Weight	357.4
Structural Formula	
Purity (%)	99.7

Standard substances are stored in a freezer until use.

VALENT has retained a reserve sample of this chemical, and has documentation specifying the location of the synthesis and characterization information for this compound and is available to the VALENT.

2.3 Reagents and Chemicals

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included.

1) Ammonium bicarbonate, reagent grade or equivalent
2) Ammonium formate, reagent grade or equivalent
3) Formic acid, reagent grade or equivalent
4) Methanol, HPLC grade or equivalent
5) Water, HPLC grade with purified in-house HYDRO™ purification system or equivalent.
6) Mobile phase A: 0.1% formic acid + 5 mM ammonium formate in water
   Add 1 mL concentrated formic acid to ultra-pure water in a 1 L volumetric flask. Adjust to the 1L mark with ultra-pure water. Stopper the flask securely and shake to mix thoroughly.
7) Mobile phase B: 0.1% formic acid + 5 mM ammonium formate in methanol
   Add 1 mL concentrated formic acid and 5 mM ammonium formate in a 1 L volumetric flask. Adjust to the 1L mark with methanol. Stopper the flask securely and shake to mix thoroughly.
8) 10 mM ammonium bicarbonate buffer in methanol/water, 9/1 (v/v)
   <u>500mM NH_4CO3 solution at pH 9</u>
   500 mM solution make by 1.97 g of NH_4HCO_3 into 50 mL volumetric flask brought to volume with ultra-pure water. The pH of the 500 mM solution of NH_4HCO_3 was adjusted to pH at 9 by addition of NH_4OH using a pH probe.
   <u>MeOH/water, 9/1 (v/v) containing 10 mM NH_4HCO_3</u>
   An 20 mL aliquot of the pH adjusted 500 mM NH_4HCO_3 solution was added to 1L volumetric flask and followed by addition of 80 mL of ultra-pure water and then brought to volume with methanol.
9) Methanol/water, 9/1 (v/v)
   Add 900 mL methanol and 100 mL ultra-pure water into 1L volumetric flask and mix well to ensure complete homogeneous solution
10) Methanol/water, 1/1 (v/v)
    Add 500 mL methanol and 500 mL ultra-pure water into 1L volumetric flask and mix well to ensure complete homogeneous solution

2.4 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.4.1 Stock Solution Preparation

Prepare individual 1000 µg/mL stock solutions for etoxazole and its metabolites R-8 and R-13.

Weigh out accurately, using a five-figure balance, sufficient etoxazole and its metabolites R-8 and R-13 analytical standard into separate amber "Class A" volumetric flasks. Dilute to the mark with methanol to give individual 1000 µg/mL stock solutions of etoxazole, R-8 and R-13.

Note that the amount weighed out must be corrected for its chemical purity.

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example using one of the following approaches:

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

2.4.2 Intermediate and Fortification Solution

Fortification solutions for etoxazole, R-8 and R-13 should be prepared by mixing equal amounts of aliquots from the stock solutions and then followed by volumetric serial dilutions in methanol. It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL. All intermediate solutions are stored under freezer condition.

2.4.3 Working Standard and Calibration Standard Solution

Working standard solution (0.1 µg/mL) for etoxazole, R-8 and R-13 is prepared from 1.0 µg/mL of standard solution mixed with equal amounts of standards in MeOH/H_2O, 90/10

(v/v) containing 10 mM ammonium bicarbonate. The working standard solution is stored under refrigerator conditions (nominally 0-10 °C).

Calibration standards for HPLC-MS/MS analysis are prepared by volumetric dilutions from the 0.1 µg/mL mixed working standard solution using MeOH/H_2O, 50/50 (v/v).

Final calibration standard solutions are prepared as follows for both sediment and water samples:

Final Concentration (ng/mL)	µL of Standard (100 ng/mL)	µL of Standard (10 ng/mL)	µL of Standard (1 ng/mL)	Solvent Volume (MeOH/H2O, 50/50, v/v) (µL)
10*	100	--	--	900
2	--	200	--	800
1	--	100	--	900
0.6	--	60	--	940
0.3	--	30	--	970
0.125	--	--	125	875
0.06	--	--	60	940
0.02	--	--	20	980

* used for calibration standards (2 ng/mL, 1 ng/mL, 0.5 ng/mL, and 0.3 ng/mL) and not used for calibration curve.

There are no known interferences originating from both water and sediment samples. However, interferences can be originated from impure chemicals, solvents, contaminated glassware, and particularly the HPLC water supply. Solvent matched calibration standards should normally be used for quantitation of all analytes if significant matrix effects >20% of suppression or enhancement of the instrument response is observed.

2.4.4 Standard Solution Storage and Expiration

During method development, no analyte stability problem has been observed in the standard solutions. The mixed standards are used for fortifications and calibration standards.

All stock and fortification solutions in methanol should be stored in a freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use. It is recommended 6 months of expiration date for all stock and fortification solutions. A working standard solution for calibration solutions should be stored in a refrigerator (2-10 °C) when not in use to prevent decomposition and/or concentration of standard. It is recommended 2 weeks of expiration date.

2.5 Safety

The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Store work clothing separately. Keep away from food, drink and animal feed stuffs. No eating, drinking, smoking or tobacco use at the place of work. Hands and/or face should be washed before breaks and at the end of the shift.

Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples have to be sufficiently homogenized beforehand, in order to assure that the aliquot taken for residue analysis is representative for the whole sample. If water and sediment samples are received deep frozen they should be allowed to defrost completely at room temperature. Defrosted water sample should be shaken thoroughly to ensure sample homogeneity prior to analysis. Defrosted entire soil sample should be homogenized with dry ice in a commercial blender.

3.2 Procedure

A summary of the method is included in flow-chart form in Appendix.

At least one untreated control and one fortified control samples should be analysed with each sample set. 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.

Note: All glassware should be thoroughly cleaned and followed with a rinse of acetonitrile or methanol prior to use. The analysis system is very sensitive and may detect contamination from previous samples if all glassware is not properly cleaned prior to each use.

Water

1. Aliquot a 15 mL sample and place into a 50 mL graduated polypropylene test tube.
2. If required, fortify control samples for method recovery with etoxazole, R-8 and R-13 at LOQ and 5 or 10 times the LOQ of the method.
3. Add 15 mL of extract solvent, i e. MeOH/H_2O (90/10, v/v) containing 10 mM ammonium bicarbonate. Vortex and shake for one minute to extract sample.
4. Centrifuge the extract samples at approximately 3500 RPM for 10 minutes.

5. Aliquot 0.5 mL of sample from the extract sample and transfer to 50 mL graduated polypropylene test tube and make up to 20 mL volume using MeOH/H_2O(50/50, v/v). Sonicate and vortex for 1 minute. If dilution is required, prepare any necessary dilutions with MeOH/H_2O (50/50, v/v).
6. Transfer the final sample into 1.5 mL HPLC vial and analyze by LC-MS/MS.
7. Use LC-MS/MS method as detailed in section 4.0 to determine Etoxazole and its metabolites R-8 and R-13.

Sediment

1. Weigh 5 g (±0.01 g) of sample into 50 mL graduated polypropylene test tube.
2. If required, fortify control samples for method recovery with etoxazole, R-8 and R-13 at LOQ and 5 or 10 times the LOQ of the method.
3. Add 20 mL of extract solvent, i e. MeOH/H_2O (90/10, v/v) containing 10 mM ammonium bicarbonate.
4. Samples were swirled and sonicated for 5 minutes, and then extracted by shaking twice for 30 seconds on the Omni Bead Ruptor Homogenizer at speed setting of 4 m/s at room temperature (addition of beads is not required for extraction).
5. The extract samples were placed on the centrifuge at 3500 RPM for 15 minutes and carefully decanted supernatant to a clean 50 mL graduated polypropylene test tube.
6. Take original test tube with sediment and repeat step 3 – 5 once more.
7. Combined final extract was vortexed and sonicated for a minute and centrifuged at 350 RPM for 5 min.
8. Take 1 mL aliquot of the final extract into 15 mL graduated centrifuge tube, make up to 10 mL volume using MeOH/H_2O (50/50, v/v). Sonicate and vortex the samples for 0.5 minute.
9. Transfer the sample into 1.5 mL HPLC vial and analyze by LC-MS/MS. If dilution is required, prepare any necessary dilutions with MeOH/H_2O (50/50, v/v)
10. Use LC-MS/MS method as detailed in section 4.0 to determine Etoxazole and its metabolites R-8, and R-13.

3.3 Experimental Precautions

a) Bottled HPLC grade ultra-pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

b) To prevent contamination of the instrument and to minimize possible carry-over issues, it is recommended that high level recoveries and samples with expected residues greater than 1.6 ng/mL (80% of highest calibration standard) should be diluted so that the final analyte concentration does not exceed 1.6 ng/mL. It may also

be useful to include blank injections of methanol/ultra-pure water, 50/50 (v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ level.

c) Additional needle and valve washes with an organic solvent such as acetonitrile and methanol may help to reduce any significant carry-over of etoxazole, R-8 and R-13.

3.4 Time Required for Analysis

The methodology is normally performed with a batch of 20 samples. One person can complete the bench analysis of 20 samples in 0.5 day (4 hour working period). Additional time is required for LC-MS/MS analysis, integration and reporting.

3.5 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an AB Sciex API 5500. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description and Chromatography Conditions

Chromatographic System:	Agilent 1290 UPLC			
Analytical Column:	Acquity UPLC BEH C18, 50 mm X 2.1 mm, 1.7 µm; part number: 186002350			
Column Temperature:	60 °C			
Injection Volume:	5.0 µL			
Mobile Phase A:	0.1% Formic Acid + 5 mM Ammonium formate in HPLC Water			
Mobile Phase B:	0.1% Formic Acid + 5 mM Ammonium formate in MeOH			
Gradient:	Time (min.)	Flow Rate (µL/min)	A (%)	B (%)
	0.0	500	90	10
	0.2	500	90	10
	0.7	500	5	95
	2.0	500	5	95
	2.1	500	90	10
	3.0	500	90	10

4.2 Mass Spectrometer Conditions for Etoxazole, R-8 metabolite, and R-13 metabolite

MS/MS Conditions						
Interface:	AB SCIEX 5500 QTrap					
Ionization mode	Electrospray ionization (ESI) interface					
Polarity:	Positive					
Curtain gas (CUR):	Nitrogen set at 25.0 (arbitrary units)					
Temperature (TEM):	450 °C					
Collision gas setting (CAD):	Nitrogen set at 8.0 (arbitrary units)					
GS1:	40.0					
GS2:	60.0					
Entrance potential (EP):	10.0					
Scan type:	MRM					
MRM Conditions	**Etoxazole**		**R-8 metabolite**		**R-13 metabolite**	
	Primary	Secondary	Primary	Secondary	Primary	Secondary
Q1 m/z:	360.203		238.087		358.145	
Q3 m/z:	141.000*	177.100	165.100*	147.000	140.800*	274.000
Expected Retention (min.):	Approx. 1.3		Approx. 1.1		Approx. 1.4	
Declustering potential (DP):	110.00		46.00		99.00	
Collision energy (CE):	41.00	27.00	16.00	24.00	45.00	33.00
Collision cell exit potential (CXP):	14.00	10.00	15.00	13.00	14.00	14.00

* proposed as quantitation transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.

A divert valve may be used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.

Other parameters like gas flows and voltages are dependent of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

4.3 Confirmatory Procedures for Etoxazole, R-8 metabolite and R-13 metabolite

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

Etoxazole, R-8 etoxazole metabolite, and R-13 etoxazole metabolite residues may be calculated for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (e.g. 20% LOQ to at least 20% above the highest fortified level as a minimum). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to etoxazole, R-8 and R-13. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit ("X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of m and c should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for x gives

$$x = \frac{y - c}{m}$$

e) Calculate the etoxazole, R-8 and R-13 residues in the sample, expressed as µg/L, as follows

$$\text{Residue } (\mu g/L) = \frac{\text{Analyte found } (\mu g/mL)}{\text{Sample conc. } (L/mL)}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in L/mL.

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu g/L)$$

5.1 Detector Linearity

For accurate quantitation of residue concentrations, analyses should be carried out within the linear range of the detector. For multi-point calibration, detector range and linearity will be demonstrated within each sample set.

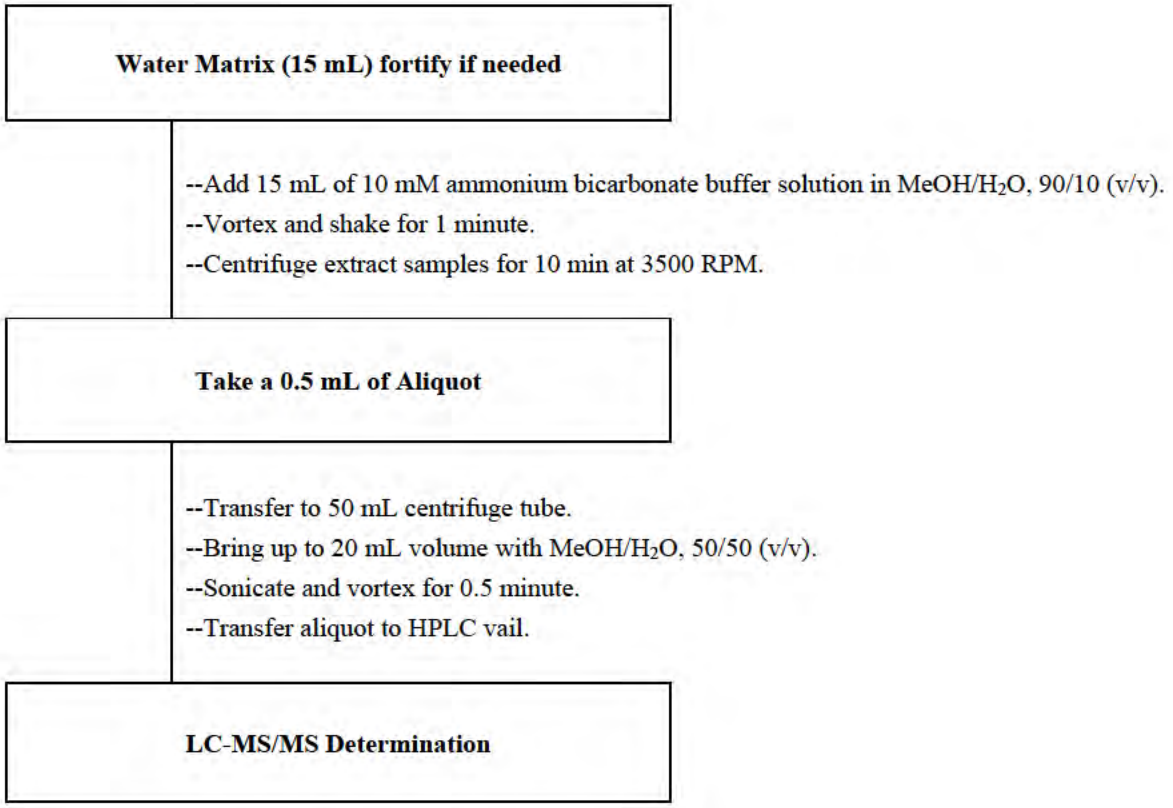
In the method development, standards over the concentration range 0.02 ng/mL – 2 ng/mL to cover at least 20% of LOQ (LOD) and 10 x LOQ for water sample and 50% of LOQ (LOD) and 10x of LOQ for sediment sample are analysed by LC-MS/MS and peak area plotted against the standard concentration.

The linearity of the LC-MS/MS detector response for etoxazole, R-8 and R-13 was examined in the range from 0.02 ng/mL to 2 ng/mL injected on column (equivalent to 0.0001 ng to 0.01 ng standards when using at 5 µL injection volume) and was found to be linear.

If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantitation.

FLOWCHART

Water Sample



Sediment Sample