1.0 INTRODUCTION

1.1 Background and Purpose of Study

Etoxazole is a narrow spectrum systemic acaricide and also an insecticide. A residue analytical method (RM-37S-3), for the analysis of etoxazole and metabolites, R-8 and R-13 in sediment and surface water was validated at ADPEN Laboratories, Inc., Jacksonville, Florida.

The purpose of this study is to validate Valent Analytical Method Number RM-37S-3, "The Determination of Residues of Etoxazole and its Metabolites, R-8 and R-13 in Sediment and Surface Water Matrices Using LC-MS/MS".

1.2 Principle of the Method

Residues of etoxazole, R-8 etoxazole metabolite, and R-13 etoxazole metabolite are extracted from surface water and sediment using an aqueous buffer solution, methanol/water, 90/10 (v/v) with 10 mM ammonium bicarbonate. Water samples are extracted by shaking (vortexing) and sediment sample are extracted by an Omni Beadruptor, which is high speed extraction technique. 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 final volume is analysed by LC/MS/MS.

Transitions for etoxazole, R-8 etoxazole metabolite and R-13 etoxazole metabolite were monitored in positive ion mode for primary and confirmation quantification.

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
R-8	Positive	238→165	238→147
R-13	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. No matrix suppression or enhancement was found to affect the analyte.

2.0 MATERIALS AND METHODS

2.1 Test Systems

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

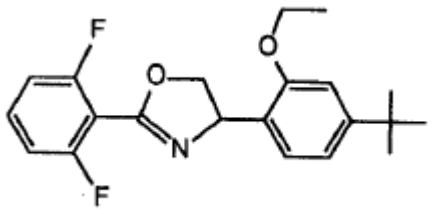
Each analytical set was identified with an analytical set number, which consisted of a unique number (e.g., WO-17111702). The test system samples were assigned unique lab code numbers according to ADPEN SOP 3.2, and these were recorded in the raw data (e.g., control sediment, PA.CA.T.Bulk.Sediment, was identified as 171004001-001). 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/reference standards shown below were provided by Valent USA LLC and used during the analytical portion of this study. The test/reference items were maintained frozen until use in this study. Valent USA LLC determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to Valent and are located in their Dublin, California facility.

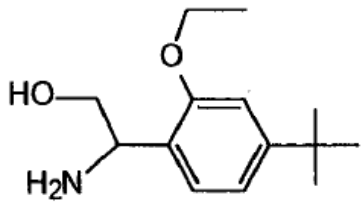
The certificates of analysis are presented in **Appendix B**. A detailed summary of the reference substances are presented below:

Common Name:	**Etoxazole**
CAS Name:	2-(2,6-difluorophenyl)-4-[4-(1,1-dimethylethyl)-2-ethoxyphenyl]-4,5-dihydrooxazole
CAS Number	153233-91-1
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:	

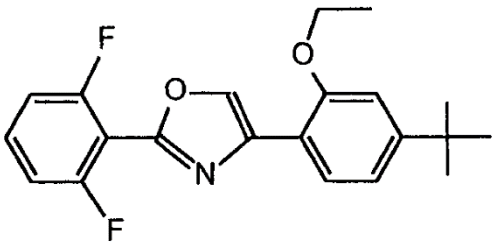


Test and Reference Substance (continued)

Common Name:	**R-8 (Etoxazole Metabolite)**
CAS Name:	2-amino-2-(4-tert-butyl-2-ethoxyphenyl)ethanol
CAS Number	153281-81-3
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:	**R-13 (Etoxazole Metabolite)**
CAS Name:	4-(4-tert-butyl-2-ethoxyphenyl)-2-(2,6-difluorophenyl)oxazol
CAS Number	N.A.
Lot Identification:	AS 1860e
Molecular Formula:	$C_{21}H_{21}F_2NO_2$
Molecular Weight:	357.4 g/mol
Purity:	99.7%
Expiration Date:	August 10, 2019
Storage:	Freezer
Source:	Valent USA, LLC
Structural Formula:	



The test substances in solution were used in the study to generate data for both instrument and method performance. Quantitation of residues in all samples was achieved using calibration curves calculated by linear regression (weighted by 1/x) of instrument responses for the reference items. The performance of the instrument was evaluated during each injection set.

2.3 Route of Administration

In this method validation study, the test items were applied to the test system as analytical standard solutions (etoxazole, R-8 and R-13 in methanol) by micropipette to ensure precise delivery of a small amount of the test items.

2.4 Reagents

All solvents and other reagents used high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care was taken to avoid contamination of the reagents used. A list of reagents used in this method along with details of preparation of solutions is provided below.

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^{TM} purification system or equivalent.
6) Mobile phase A: 0.1% formic acid + 5 mM ammonium formate in water:
   Added 1 mL concentrated formic acid to ultra-pure water in a 1 L volumetric flask. Adjusted to the 1 L 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:
   Added 1 mL concentrated formic acid and 5 mM ammonium formate in a 1 L volumetric flask. Adjusted 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):
   500 mM NH_4CO_3 solution at pH 9: Prepared by mixing 1.97 g of NH_4HCO_3 into 50 mL volumetric flask brought to volume with ultra-pure water. Adjust the pH to 9.0 using NH_4OH.
   MeOH/water (9/1, v/v) containing 10 mM NH_4HCO_3: Added a 20 mL aliquot of the pH adjusted 500 mM NH_4HCO_3 solution to 1L volumetric flask and add 80 mL of ultra-pure water. Brought to volume with methanol.
9) Methanol/water, 9/1 (v/v):
   Added 900 mL methanol and 100 mL ultra-pure water into 1L volumetric flask and mixed well to ensure complete homogeneous solution.
10) Methanol/water, 1/1 (v/v):
    Added 500 mL methanol and 500 mL ultra-pure water into 1L volumetric flask and mixed well to ensure complete homogeneous solution.

2.5 Preparation of Analytical Standard Solutions

Stock and Fortification Solutions

Individual 1000 µg/mL stock solutions for etoxazole, R-8 etoxazole metabolite and R-13 etoxazole metabolite were prepared as follows:

Weighed out accurately, using a five figure balance, sufficient analytical standards into separate "Class A" volumetric flasks. Diluted to the mark with methanol to give individual 1000 µg/mL stock solutions for etoxazole, R-8 etoxazole metabolite and R-13 etoxazole metabolite. Note that the amount weighed out was corrected for its chemical purity.

Intermediate standard solution containing etoxazole, R-8 and R-13 was prepared by taking 1 mL aliquot of each of the stock standard solutions made above (1000 µg/mL) in a 10 mL volumetric flask and bringing to volume using methanol yielding a 100 ng/µL solution.

Fortification standards were prepared by taking 2 mL aliquot of the intermediate standard solution made above (100 ng/µL) in a 20 mL volumetric flask and bringing to volume using methanol yielding a 10 ng/µL solution, which was also used to prepare 1 ng/µL solution.

Preparation Calibration Standards

A mixed standard solution (0.1 µg/mL) of etoxazole, R-8 etoxazole metabolite and R-13 etoxazole metabolite was prepared from a 1.0 µg/mL standard solution in MeOH/H_2O, 90/10 (v/v) containing 10 mM ammonium bicarbonate. The calibration standard solution was stored under refrigerator conditions (between 2-10 °C).

Standard calibration solutions for HPLC-MS/MS analysis were prepared by pipet dilutions from the 0.1 µg/mL mixed working standard solution using MeOH/H_2O, 50/50 (v/v) as exemplified in the table below.

Final Concentration (ng/mL)	**µL of Standard (100 ng/mL)**	**µL of Standard (10 ng/mL)**	**µL of Standard (1 ng/mL)**	**µL of Standard (0.1 ng/mL)**	**Solvent Volume (MeOH/H_2O, 50/50, v/v) (µL)**
10*	100	--	--	--	900
1.0	--	100	--	--	900
0.6	--	60	--	--	940
0.3	--	30	--	--	970
0.1	--	--	100	--	900
0.06	--	--	60	--	940
0.02	--	--	--	200	800
0.01	--	--	--	100	900

* Standard solution was used for preparing calibration standards and not used for calibration curve.

The dilution scheme for calibration standard solution preparation was modified prior to the method validation due to observed saturation of parent analyte, etoxazole, at the highest calibration standard concentration injected on the LC-MS/MS; therefore, the final sample volume solutions were modified accordingly.

Standard Solution Storage and Expiration

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

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

and/or concentration of standard. All stock and fortification solutions have a recommended expiration of 6 months.

2.6 Safety and Health

All standard substances, reagents, and samples were handled in accordance with ADPEN's laboratory safety policies and standard operating procedures SOPs for practicing good industrial hygiene and safety. Standard substances, etoxazole, R-8 etoxazole metabolite, and R-13 etoxazole metabolite were stored according to the certification of analysis provided by Valent prior to use. All reagents were stored and handled in accordance with material safety data sheet (MSDS) provided by Valent.

3.0 ANALYTICAL METHOD

3.1 Principle of the Method

Using Valent Method RM-37S-3, residues of etoxazole and its metabolites, R-8 and R-13 in sediment and surface water matrices were determined using LC-MS/MS at positive mode. The working method validated in this study is provided in **Appendix C**. A brief description of the methodology is referenced below:

Etoxazole and its metabolites were extracted from 15 mL of surface water and from 5 grams of sediment using (twice 20 mL for sediment and 15 mL for surface water) an aqueous buffer solution containing 10 mM ammonium bicarbonate in methanol/water, 90/10 (v/v). For water samples extraction was performed by shaking (vortexing) method, for sediment a high speed extraction method using beadruptor homogenizer was used at speed setting of 4m/s (addition of beads is not required). The extracts of water and sediment were centrifuged for 10 minutes at 3500 rpm. The supernatant was diluted with methanol/water, 50/50 (v/v) and the analytes residues was analysed by LC-MS/MS with quantitation based on a comparison of peak areas with those of known standards.

The MRM transitions were monitored in positive mode for primary and confirmation quantification, and are shown below.

Analyte	Mode	Quantitation (*m/z*)	Confirmation (*m/z*)
Etoxazole	Positive	360→141	360→177
R-8	Positive	238→165	238→147
R-13	Positive	358→141	358→274

The results were calculated by direct comparison of the sample peak responses to those of external standards.

3.2 Validation of Method

For validation, untreated sediment and surface water samples were fortified with residues of etoxazole and metabolites, R-8 and R-13 then analyzed according to the established method validation guidelines. To test the repeatability of the method, the analytical sets for each matrix consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.01 ppm, and five replicates fortified at a higher level, corresponding to 10x the limit of quantitation, 0.1 ppm. The example of recovery calculation is provided in **Appendix D**.

The validation data including the detail analytical data for each matrix types are provided in **Appendix E**.

3.3 Sample Preparation

Samples were sufficiently homogenized beforehand, in order to assure that the aliquot taken for residue analysis is representative for the whole sample. The water and sediment samples were received deep frozen, they were defrosted completely to room temperature. Water sample was thawed and shaken thoroughly to ensure sample homogeneity prior to analysis. Sediment sample was completely thawed prior to homogenization with dry ice in a commercial blender.

3.4 Sample Weighing and Fortifications

For procedural recovery samples weighed 5 ± 0.01 g of sediment and 15 ± 0.1 mL of surface water, respectively, into an appropriate sample container. Untreated control sediment and surface water samples were fortified using microliter amounts of the following fortification standards to yield 0.01 ppm for LOQ and 0.1 ppm for 10x LOQ concentrations as per method.

Fortifications used in this method validation are as follows:

Matrix	Sample Type	Sample Size	Concentration of Fortification Solution (µg/mL)	Volume of Spiking Fortification Solution (µL)	Level of Fortification (ppm)
Sediment	Control	5 g	--	--	--
	Fortification (LOQ*)	5 g	1	50	0.01
	Fortification (10x LOQ*)	5 g	10	50	0.1
Surface Water	Control	15 mL	--	--	--
	Fortification (LOQ*)	15 mL	1	150	0.01
	Fortification (10x LOQ*)	15 mL	10	150	0.1

*limit of quantitation

3.5 Extraction Procedure

At least one untreated control and one fortified control samples were 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 glasswares were 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 Procedure:

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 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 40 mL volume using MeOH/H_2O(50/50, v/v). Sonicate and vortex for 30 seconds. 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 3.6 to determine etoxazole and its metabolites R-8 and R-13.

Sediment Procedure:

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 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 to 5 once more.
7. Combined final extract was vortexed and sonicated for a minute and centrifuged at 3500 RPM for 5 min.
8. Take 1 mL aliquot of the final extract and dilution with 1 mL of MeOH/H_2O, 10/90 (v/v) without buffer concentration.
9. Take 1 mL aliquot of the final extract into 25 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.
10. 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). No buffer concentration required.
11. Use LC-MS/MS method as detailed in section 3.6 to determine etoxazole and its metabolites R-8, and R-13.

3.6 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 optimization 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.

Instrument Description and Chromatography Conditions

HPLC 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

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:	40.0					
Entrance potential (EP):	9.0					
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*:	141.0*	177.1	165.1*	147.1	140.8*	274.0
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

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

Note: A divert valve was 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. Following table used for valco valve diverter setting.

Valco valve diverter table

Event	Time (min)	Position
1	0.0	waste
2	0.8	Mass Spectrometer
3	1.6	waste

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.

Confirmatory Procedures for Etoxazole and its metabolites, R-8 and R-13

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

The residues of etoxazole were determined by LC-MS/MS, monitoring (in the positive ion mode) ion transitions at *m/z* 360 → *m/z* 141 (proposed as the primary transition for quantitation) and *m/z* 360 → *m/z* 177 (typically for confirmatory purposes).

The residues of R-8 etoxazole metabolite were determined by LC-MS/MS, monitoring (in the positive mode) ion transitions at *m/z* 238 → *m/z* 165 (proposed as the primary transition for quantitation) and *m/z* 238 → *m/z* 147 (typically for confirmatory purposes).

The residues of R-13 etoxazole metabolite were determined by LC-MS/MS, monitoring (in the positive mode) ion transitions at *m/z* 358 → *m/z* 141 (proposed as the primary transition for quantitation) and *m/z* 358 → *m/z* 274 (typically for confirmatory purposes).

The results were calculated by direct comparison of the sample peak responses to those of external standards. The MRM transitions used to identify etoxazole and its metabolites were determined by product ion spectra (**Appendix K**). As LC-MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary.

3.7 Influence of Matrix Effects on Analysis

In conjunction with the subject study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC/MS/MS analysis. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with only solvent at three concentration levels; half LOQ, LOQ and two times LOQ. The matrix-matched standards corresponding to three different concentration levels of solvent-based standards were prepared by fortifying an aliquot of mixed standard solution into final condition of control samples for each sediment and surface water Each set of matrix-matched standards (for each matrix) was injected 3 times. The data generated were evaluated by comparing the average area response of the standards for three injections without matrix and three injections with matrix, for each of the three standard

concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) required a difference in area of <20%, calculated as the "Mean Area Change (%)". For each analyte/matrix/ion transition, an overall average "Mean Matrix Interference (%)" across the three tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects.

The results of the extensive testing on all matrices (for both transitions) demonstrated that the matrix load in the samples from sediment and surface water had no significant influence on the analysis. Matrix effects, calculated as the overall mean percent area count difference between matrix-matched standards and solvent-based standards, at three standard concentration levels, were less than 20%. No matrix effects were noticed during these experiments for any analyte/matrix/ion transition; therefore, validation samples were quantitated against calibration standard solutions prepared in solvent. A summary of the matrix effects data is provided in **Tables 3** and **4** for surface water and sediment matrices, respectively. The detail data from matrix effect evaluation is provided in **Appendix F**.

3.8 Experimental Precautions

a) Bottled HPLC grade ultra-pure water was 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 0.8 ng/mL (80% of highest calibration standard) should be diluted so that the final analyte concentration does not exceed 0.8 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 metabolite and R-13 metabolite.

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

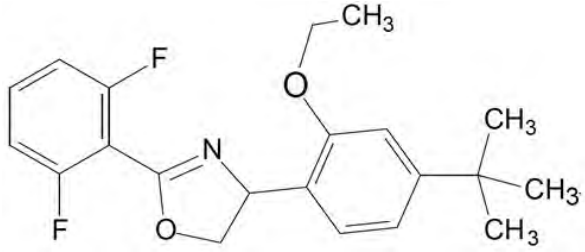
The recommended equipment and apparatus are listed in. Equipment with equivalent performance specifications may be substituted.

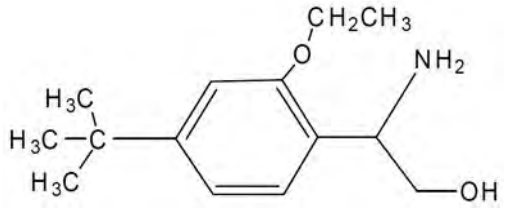
Bead Ruptor Homogenizer, OMNI
Balance, analytical or equivalent.
Cylinder, graduated, 10-mL, 25-mL, 50-mL, 100-mL, 500 mL, and 1000-mL or equivalent.

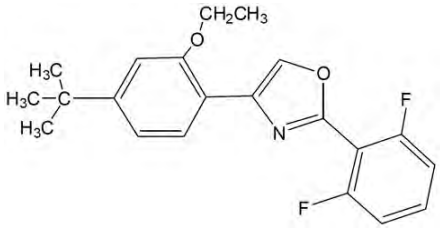
Pipets, glass, Class A certified, assorted volumes. These pipets are used when an exact addition of liquid is required (i.e., sample fortification, standard solution preparation and dilutions).
Pipetter, Eppendorf Repeater, 100 – 1000 µL variable volume range and 500-5000 µL variable volume range, or 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^{TM} 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

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

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.

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

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.

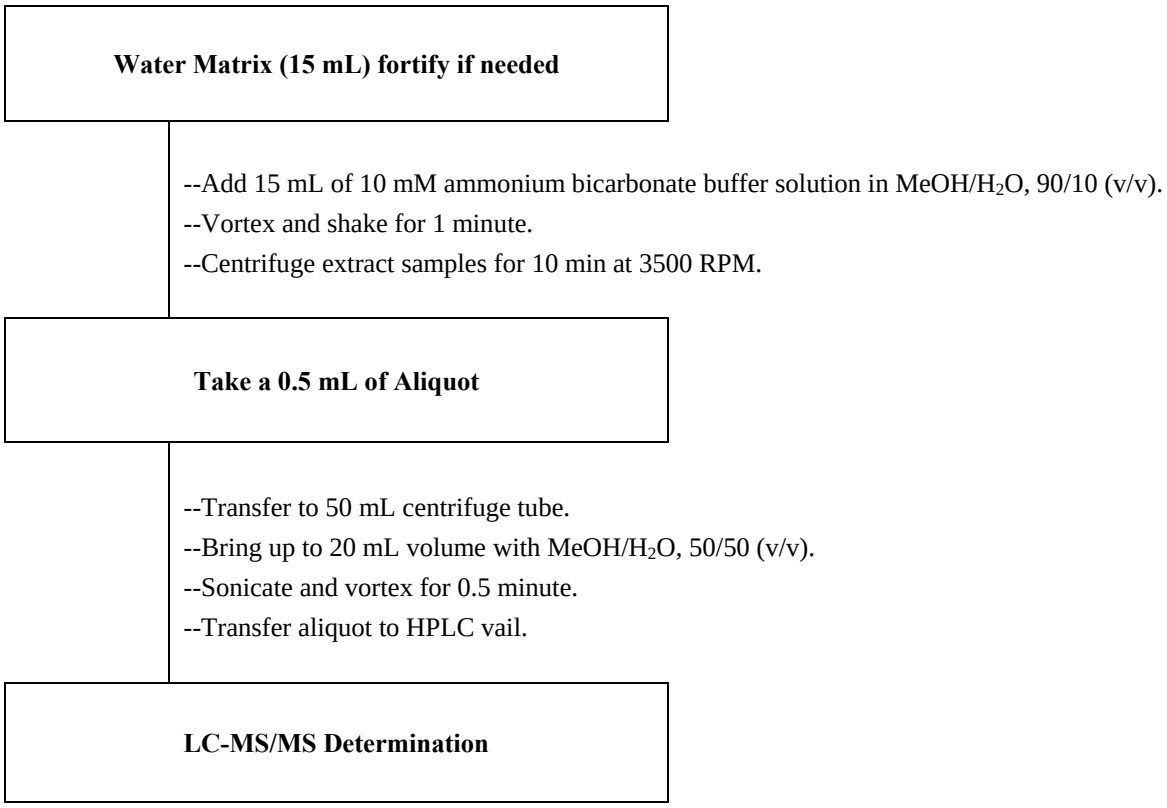
In the method development, standards over the concentration range 0.02 ng/mL – 2 ng/mL to cover at least 20% of LOQ (LOD) and 10 x LOQ for water sample and 50% of LOQ (LOD) and 10x of LOQ for sediment sample are analysed by LC-MS/MS and peak area plotted against the standard concentration.

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.

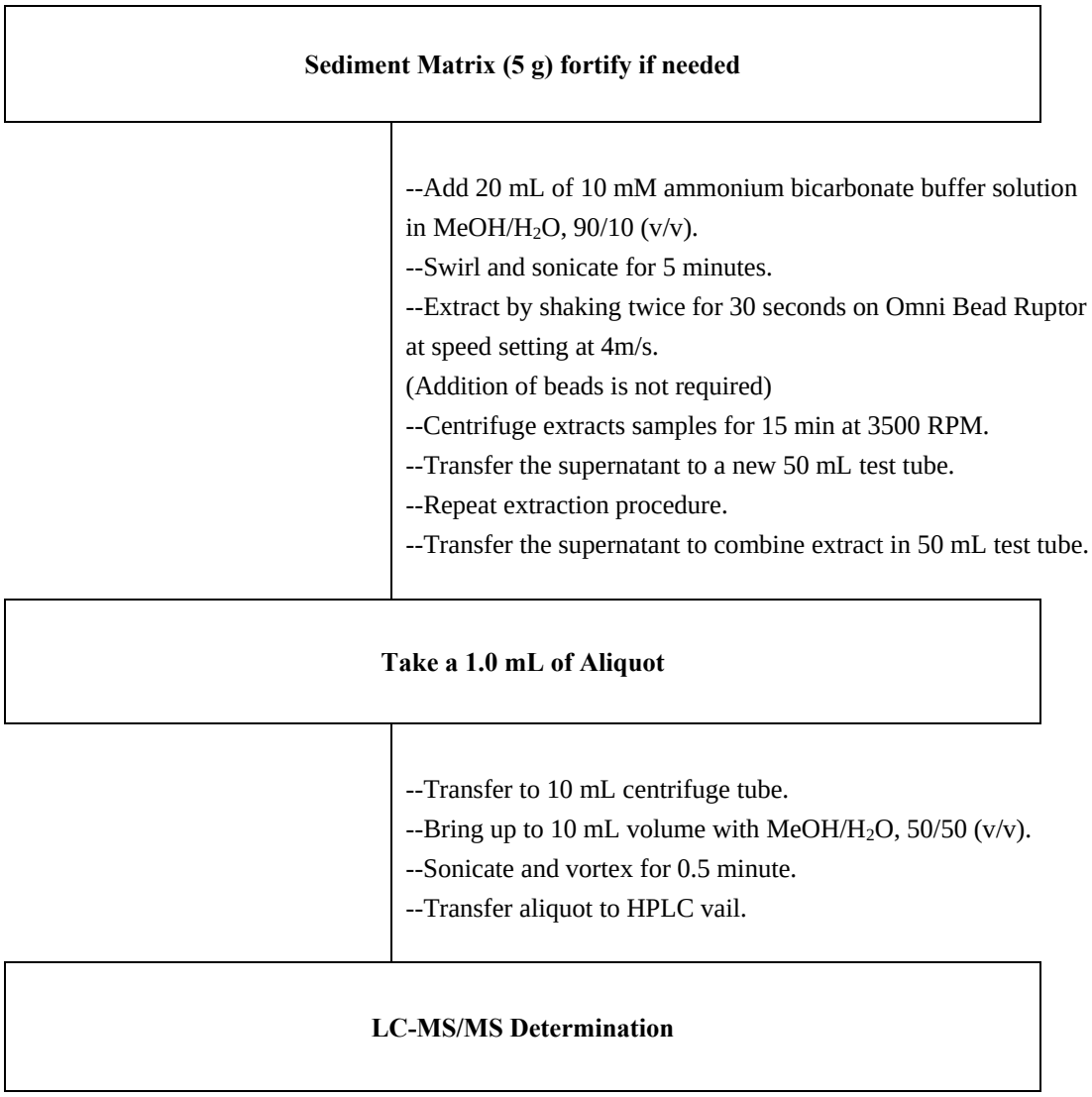
If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantitation.

FLOWCHART

Water Sample



Sediment Sample



Appendix D. Example Calculations

Typical Recovery Calculation for LC-MS/MS Quantitation

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 and transcribed into the detailed analytical data report sheets (Appendix E). The recoveries and residues of etoxazale, R-8 and R-13 in ppm are 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

Where,

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

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

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

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

Set ID: WO-17111702
Lab Code: 17111702-Recovery1-1
Sample Name: Control + 0.01 ppm

a) Calibration curve: y = (1.1e+009)x – 1.22e+004

Solving for x: 121384 = (1.1e+009)x – 1.22e+004
x =(121384 + 1.22e+004)/(1.1e+009) = 0.000121 ng

b) $\text{Amount of sample injected (mg)} = \frac{0.005\ \text{mL}}{2000\ \text{mL}} \times 5\ \text{g} \times 1000\ \text{mg} = 0.0125\ \text{mg}$

c) $\text{Amount found (ppm)} = \frac{0.000121\ \text{ng}}{0.0125\ \text{mg}} = 0.00971\ \text{ppm}$

No residue above the LOD was found in the corresponding control sample (171004001-001B)

d) $\text{Recovery (\%)} = \frac{0.00971\ \text{ppm}}{0.01\ \text{ppm}} \times 100 =$ **97.1%**