1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of etridiazole and two major metabolites (3-carboxylic acid-5-ethoxy-1,2,4-thiadiazole (3-Carb-T) and 3-dichloromethyl-5-ethoxy-1,2,4-thiadiazole (3-DCMT)) in two different water types: ground water and surface water. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), and method detection limit (MDL).

The method was validated by fortification of ground water and surface water with etridiazole, 3-Carb-T, and 3-DCMT at concentrations of 0.100 and 1.00 µg/L. Etridiazole and 3-DCMT recovery samples were extracted with iso-octane followed by dilution into the calibration standard range with matrix matched blank. All samples were analyzed by automated injection using gas chromatography equipped with mass spectrometry detection (GC-MSD). 3-Carb-T recovery samples were extracted by anion exchange solid phase extraction (SPE), and eluted with 2% trifluoroacetic acid in methanol. Samples were diluted into the calibration standard range with 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v). All 3-Carb-T recovery samples were analyzed by automated injection using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 1 November 2017, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted from 1 November to 10 November 2017 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers Viscient's archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this validation study followed those described in the Smithers Viscient protocol entitled "Validation of the Analytical Method for the Determination of Etridiazole and its Metabolites in Surface and Ground Water by LC-MS/MS and GCMS" (Appendix 1). The study was conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR Part 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the guidance documents SANCO/825/00 REV 8.1 (EC, 2010) and OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substances

The test substance, etridiazole, was received on 13 June 2016 from Arysta LifeScience, Canada, Inc., Ontario, Canada. The following information was provided:

Name:	etridiazole
Synonyms:	5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole; etridiazole technical
Lot No.:	2758-31-RRG
CAS No.:	2593-15-9
Purity:	99.5 (± 0.10)% (Certificate of Analysis, Appendix 2)
Expiration Date:	30 November 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 8327) was stored in a refrigerator in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, etridiazole acid, was received on 13 June 2016 from Arysta LifeScience, Canada, Inc., Ontario, Canada. The following information was provided:

Name:	etridiazole acid
Synonyms:	1,2,4-thiadiazole-3-carboxylic acid, 5-ethoxy-; 3-Carb-T
Lot No.:	2840-89-RRG

CAS No.:	67472-43-9
Purity:	99.9% (w/w) (Certificates of Analysis, Appendix 2)
Expiration Date:	28 February 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 8328) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, DCE, was received on 13 June 2016 from Arysta LifeScience, Canada, Inc., Ontario, Canada. The following information was provided:

Name:	DCE
Synonyms:	1,2,4-thiadiazole, 3-(dichloromethyl)-5-ethoxy-; T-03; 3-DCMT
Lot No.:	2840-77-RRG
CAS No.:	Not Listed
Purity:	99.3% (Certificate of Analysis, Appendix 2)
Expiration Date:	27 February 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 8329) was stored in a refrigerator in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substances, and archival of a sample of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

1. Acetonitrile:	EMD, reagent grade
2. Iso-octane:	EMD, reagent grade
3. Acetone:	EMD, reagent grade
4. Methanol:	EMD, reagent grade
5. Trifluoracetic Acid:	Sigma, reagent grade
6. Ammonium Hydroxide:	J.T Baker, reagent grade
7. Purified reagent water:	Prepared from a Millipore Milli-Q Direct 8 water purification system (meets ASTM Type II requirements)

Reagents of similar grade and comparable purity may be substituted for the specific reagents above in future testing with this method as long as acceptable performance is demonstrated.

2.4 Equipment

1. Instrument (LC-MS/MS):	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACHT autoinjector Shimadzu DGU-20A3V vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.4.2 software for data acquisition
2. Instrument (GC-MSD):	Agilent 6890 series gas chromatograph Agilent 7683 series autosampler Agilent 7683 series injector Agilent 5973 series mass selective detector (MSD)
3. Balance:	Mettler Toledo XSE205DU
4. Shaker table:	VWR 3500STD
5. Centrifuge:	Thermo Scientific Sorvall Legend XFR Centrifuge
6. Laboratory equipment:	volumetric flasks, disposable glass pipets, positive displacement pipets, graduated cylinders, stir bars, stir plates, vortexers, autosampler vials, Waters MAX SPE columns, amber Wheaton bottles, low-binding centrifuge tubes, and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used but may require optimization to achieve the desired separation and sensitivity.

2.5 Test Matrix

The matrices used during this method validation were ground water and surface water.

Ground water information:

Ground water used in the study consists of unadulterated water from a 100-meter bedrock well prepared by filtering to remove any potential organic contaminants.

Surface water information:
The surface water used for this method validation analysis was collected from the Weweantic River (SMV Lot No. 12Jul17water-A) in Wareham, Massachusetts. The water was collected from an area of the river with approximately 60 cm of overlying water. Prior to use, the surface water was characterized by Smithers Viscient and was determined to have a pH of 6.18 and a dissolved oxygen content of 5.92 mg/L. All documentation relating to the preparation, storage and handling is maintained by Smithers Viscient.

2.6 Preparation of Liquid Reagent and Mobile Phase Solutions

All volumes can be scaled up or down as necessary; however, the proportions must remain the same.

A 50:50 methanol:purified reagent water (v:v) liquid reagent solution was typically prepared by combining 100 mL of methanol with 100 mL of purified reagent water.

A 0.1% trifluoroacetic acid in purified reagent water mobile phase solution was typically prepared by adding 1.00 mL of trifluoroacetic acid to 1000 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for ten minutes.

A 0.1% trifluoroacetic acid in acetonitrile mobile phase solution was typically prepared by adding 1.00 mL of trifluoroacetic acid to 1000 mL of acetonitrile. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for ten minutes.

A 30:30:40 acetonitrile:methanol:purified reagent water (v:v:v) autosampler needle wash solution was typically prepared by combining 1200 mL of acetonitrile, 1200 mL of methanol, and 1600 mL of purified reagent water.

A 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v) liquid reagent solution was typically prepared by combining 100 mL of acetonitrile, 400 mL of purified reagent water, and 0.500 mL of trifluoroacetic acid. The solution was mixed using a stir bar and stir plate for five minutes.

A 2% trifluoroacetic acid in methanol liquid reagent solution was typically prepared by combining 50.0 mL of methanol, and 1.00 mL of trifluoroacetic acid. The solution was vortex mixed for thirty seconds.

2.7 Preparation of Stock Solutions

All volumes and masses can be scaled up or down as necessary; however, the proportions must remain the same.

Primary stock solutions were typically prepared as described in the table below.

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8327P	0.025165	0.02503	Acetone	25.0	1000	Sub-stock solution
8328M	0.02508	0.02505	Acetonitrile	25.0	1000	Secondary stock solutions
8328N	0.02510	0.02507	Acetonitrile	25.0	1000	Secondary stock solutions
8329J	0.02519	0.02501	Acetone	25.0	1000	Sub-stock solution

Secondary stock solutions were typically prepared as described in the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8328M	1000	0.500	50.0	Acetonitrile	8327M-1	10.0	Sub-stock solution
8328N	1000	0.500	50.0		8328N-1	10.0	Sub-stock solution

Sub-stock solutions were typically prepared as described in the table below.

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8327M-1	10.0	0.100	10.0	Acetonitrile	Tech Stk 1	0.100	LOQ and 10X LOQ recovery samples
8328N-1	10.0	0.100	10.0	Acetonitrile	Ana Stk 1	0.100	Calibration Standards
8327P	1000	0.100	10.0	Acetone	Mixed Stock 1	10.0	Sub-stock solution
8329J	1000	0.100	10.0				
Mix-Stk 1	10.0	0.100	10.0	Acetone	Mixed Stock 2	0.100	LOQ and 10X LOQ samples, calibration standards

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

2.8.1 Extracted Calibration Standards – Etridiazole and 3-DCMT

Calibration standards were prepared in aqueous matrix by fortifying with the 0.100 mg/L test substance mixed sub-stock solution to yield test substance concentrations of 0.0650, 0.135, 0.200, 0.350, 0.500, and 0.650 µg/L. These standard solutions were extracted and reconstituted at the same dilution factor as the controls, and LOQ recovery samples to yield reconstituted test substance concentrations of 2.00, 4.00, 6.00, 10.0, 15.0, and 20.0 µg/L. Extracted standards were prepared to guard against matrix effects. This procedure is detailed in the table below.

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Reconstituted Concentration (µg/L)	Sample ID
NA	NA	NA	60.0	0.00	0.00	MM Std Blk
Mixed Stock 2	0.100	0.0390	60.0	0.0650	2.00	MM-Std 1
		0.0810	60.0	0.135	4.00	MM-Std 2
		0.120	60.0	0.200	6.00	MM-Std 3
		0.210	60.0	0.350	10.0	MM-Std 4
		0.300	60.0	0.500	15.0	MM-Std 5
		0.390	60.0	0.650	20.0	MM-Std 6

2.8.2 Calibration Standards – 3-Carb-T

Calibration standards were prepared in 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v) by fortifying with the 0.100 mg/L test substance sub-stock solution to yield test substance concentrations of 0.250, 0.500, 1.00, 1.50, 2.00, and 2.50 µg/L. This procedure is detailed in the table below.

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.100	0.0250	10.0	0.250	Std 1
		0.0500	10.0	0.500	Std 2
		0.100	10.0	1.00	Std 3
		0.150	10.0	1.50	Std 4
		0.200	10.0	2.00	Std 5
		0.250	10.0	2.50	Std 6

2.8.3 Calibration Standards – Matrix Effects Etridiazole and 3-DCMT

Calibration standards used to assess possible matrix effects were prepared as follows by fortifying iso-octane with the 0.100 mg/L test substance mixed sub-stock solution to yield test substance concentrations of 3.00 µg/L. These standards were quantified using matrix matched standards.

2.8.3.1 Non Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Mixed stock 2	0.100	0.0600	2.00	3.00	Std A
		0.0600	2.00	3.00	Std B
		0.0600	2.00	3.00	Std C

[a] Samples were prepared in iso-octane.

2.8.4 Calibration Standards – Matrix Effects 3-Carb-T

2.8.4.1 Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.100	0.0250	5.00	0.500	MM-Std 1
		0.0250	5.00	0.500	MM-Std 2
		0.0250	5.00	0.500	MM-Std 3

[a] Samples were diluted with the prepared matrix blanks for the ground water or surface water (see Section 2.10 for extract preparation and dilution procedures).

2.8.4.2 Non Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.100	0.0250	5.00	0.500	Std A
		0.0250	5.00	0.500	Std B
		0.0250	5.00	0.500	Std C

[a] Samples were diluted with 20:80:0.1 acetonitrile:purified:trifluoroacetic acid reagent water (v:v:v).

2.9 Sample Fortification and Preparation

2.9.1 Etridiazole and 3-DCMT

The recovery samples were prepared in each matrix (ground water and surface water) with Etridiazole and 3-DCMT at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Recovery samples for each matrix were prepared separately (“de novo”) at these concentrations. Five replicates were produced for the LOQ samples and five replicates were produced for the 10X LOQ. Two samples were left unfortified to serve as controls and were processed in the same fashion as the LOQ concentration recovery samples. One sample was left unfortified to serve as a matrix matched blank and was processed at the same dilution factor as the control samples but on a larger scale in order to be used as a reagent. In addition, one reagent blank was prepared of iso-octane and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water recovery samples:

Sample ID 14088-6157-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
15	Reagent Blank	NA[a]	NA	NA	0.00
16	MM Blank	NA	NA	360	0.00
17, & 18	Control	NA	NA	60.0	0.00
19, 20, 21, 22, & 23	LOQ	0.100	0.0600	60.0	0.100
24, 25, 26, 27, & 28	10X LOQ	0.100	0.600	60.0	1.00

[a] NA = Not Applicable

Surface water recovery samples:

Sample ID 14088-6157-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
01	Reagent Blank	NA[a]	NA	NA	0.00
02	MM Blank	NA	NA	360	0.00
03, & 04	Control	NA	NA	60.0	0.00
05, 06, 07, 08, & 09	LOQ	0.100	0.0600	60.0	0.100
10, 11, 12, 13, & 14	10X LOQ	0.100	0.600	60.0	1.00

[a] NA = Not Applicable

2.9.2 3-Carb-T

The recovery samples were prepared in each matrix (ground water and surface water) with 3-Carb-T at concentrations of 0.100 (LOQ) and 1.00 (10X LOQ) µg/L. Recovery samples for each matrix were prepared separately ("de novo") at these concentrations. Five replicates were produced for the LOQ samples and five replicates were produced for the 10X LOQ.

Two samples were left unfortified to serve as controls and were processed in the same fashion as the LOQ concentration recovery samples. Three samples were left unfortified to serve as matrix blanks and were processed in the same manner as the control samples in order to assess matrix effects. In addition, one reagent blank was prepared using the elution solvent and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water recovery samples:

Sample ID 14088-6157-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
59	Reagent Blank	NA[a]	NA	NA	0.00
60, 61, & 62	Matrix Blank	NA	NA	25.0	0.00
63, & 64	Control	NA	NA	25.0	0.00
65, 66, 67, 68, & 69	LOQ	0.100	0.0250	25.0	0.100
70, 71, 72, 73, & 74	10X LOQ	0.100	0.0800	8.00	1.00

[a] NA = Not Applicable

Surface water recovery samples:

Sample ID 14088-6157-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
43	Reagent Blank	NA[a]	NA	NA	0.00
44, 45, & 46	Matrix Blank	NA	NA	25.0	0.00
47, & 48	Control	NA	NA	25.0	0.00
49, 50, 51, 52, & 53	LOQ	0.100	0.0250	25.0	0.100
54, 55, 56, 57, & 58	10X LOQ	0.100	0.0800	8.00	1.00

[a] NA = Not Applicable

2.10 Sample Extraction

2.10.1 Etridiazole and 3-DCMT

The samples and extracted standards were extracted with 2.00 mL aliquots of iso-octane using 60 mL glass vials with PTFE lined caps, and they were placed on a shaker table for 30 minutes at 250 rpm. At the end of 30 minutes, the samples were allowed to settle for 10 minutes. The matrix matched blank was also extracted with 12.0 mL of iso-octane in a separatory funnel by shaking three times for two minutes each. The entire extraction layer including residual water of each sample was then transferred using a transfer pipet into disposable conical glass vials. If an emulsion was present, then the emulsion was also transferred. The samples were centrifuged at 1200 rpm for 20 minutes to ensure sufficient phase separation. The 10X LOQ-level extracts were further diluted into the calibration standard range with the prepared matrix matched blank. The following tables summarize the extraction procedure for each sample.

Ground water:

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Extraction Volume[a] (mL)	Sample Volume for Dilution (mL)	Diluted Final Volume[a] (mL)	Dilution Factor
15	Reagent Blank	0.00	NA[b]	2.00	NA	NA	0.0333
16	MM Blank	0.00	360	12.0	NA	NA	0.0333
17, & 18	Control	0.00	60.0	2.00	NA	NA	0.0333
19, 20, 21, 22, & 23	LOQ	0.100	60.0	2.00	NA	NA	0.0333
24, 25, 26, 27, & 28	10X LOQ	1.00	60.0	2.00	0.300	1.00	0.111
MM-Std 1-1	Extracted Standard	0.0650	60.0	2.00	NA	NA	0.0333
MM-Std 2-1	Extracted Standard	0.135	60.0	2.00	NA	NA	0.0333
MM-Std 3-1	Extracted Standard	0.200	60.0	2.00	NA	NA	0.0333
MM-Std 4-1	Extracted Standard	0.350	60.0	2.00	NA	NA	0.0333
MM-Std 5-1	Extracted Standard	0.500	60.0	2.00	NA	NA	0.0333
MM-Std 6-1	Extracted Standard	0.650	60.0	2.00	NA	NA	0.0333

[a] Extraction solvent: iso-octane
[b] NA = Not Applicable

Surface water:

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Extraction Volume[a] (mL)	Sample Volume for Dilution (mL)	Diluted Final Volume[a] (mL)	Dilution Factor
01	Reagent Blank	0.00	NA[b]	2.00	NA	NA	0.0333
02	MM Blank	0.00	360	12.0	NA	NA	0.0333
03, & 04	Control	0.00	60.0	2.00	NA	NA	0.0333
05, 06, 07, 08, & 09	LOQ	0.100	60.0	2.00	NA	NA	0.0333
10, 11, 12, 13, & 14	10X LOQ	1.00	60.0	2.00	0.300	1.00	0.111
MM-Std 1-1	Extracted Standard	0.0650	60.0	2.00	NA	NA	0.0333
MM-Std 2-1	Extracted Standard	0.135	60.0	2.00	NA	NA	0.0333
MM-Std 3-1	Extracted Standard	0.200	60.0	2.00	NA	NA	0.0333
MM-Std 4-1	Extracted Standard	0.350	60.0	2.00	NA	NA	0.0333
MM-Std 5-1	Extracted Standard	0.500	60.0	2.00	NA	NA	0.0333
MM-Std 6-1	Extracted Standard	0.650	60.0	2.00	NA	NA	0.0333

[a] Extraction solvent: iso-octane
[b] NA = Not Applicable

2.10.2 3-Carb-T

Oasis Mixed-Mode Strong Anion Exchange (MAX) SPE columns (60 mg, 3 mL) were conditioned by rinsing with two column volumes of methanol followed by two column volumes of purified reagent water under vacuum at approximately 1 drop/sec. The columns were not allowed to go dry until before elution. A 1.0 µL aliquot of ammonium hydroxide was added to each 5 mL of aqueous sample (i.e. 5 µL to a 25.0 mL sample and 1 µL to an 8.00 mL sample). The samples were loaded onto the columns, and allowed to flow through under vacuum at approximately 1 drop/sec. Each sample vessel and column was rinsed with 5.00 mL of purified reagent water and was loaded onto the column and allowed to flow through under vacuum at approximately 1 drop/sec. Each sample vessel and column was rinsed with 5.00 mL of methanol

and was loaded onto the column and allowed to flow through under vacuum at approximately 1 drop/sec. The water and methanol rinses were first used to rinse the recovery sample vessels and were then added to the SPE columns. The water eluates and rinsates were discarded. The columns were quickly dried under full vacuum. The test substance was eluted from the SPE columns with 3.00 mL of 2% trifluoroacetic acid in methanol under vacuum at approximately 1 drop/sec and collected into glass conical vials. When eluting, the sorbent was saturated with elution solvent and allowed to sit for thirty seconds before applying vacuum. The samples were concentrated to incipient dryness under a gentle stream of nitrogen at 50.0˚C. The concentrated extracts were reconstituted in 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v) which was added to each sample with mixing and sonication (5 minutes) to aid in reconstitution. The sample processing is summarized in the table below.

Ground water:

Sample ID	Sample Type	Nominal Concentration (µg/L)	Sample Volume (mL)	Reconstitution Volume[a] (mL)	Dilution Factor
59	Reagent Blank	0.00	NA[b]	5.00	0.200
60, 61, & 62	Matrix Blank	0.00	25.0	5.00	0.200
63, & 64	Control	0.00	25.0	5.00	0.200
65, 66, 67, 68, & 69	LOQ	0.100	25.0	5.00	0.200
70, 71, 72, 73, & 74	10X LOQ	1.00	8.00	5.00	0.625

[a] Reconstitution solvent: 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v)
[b] NA = Not Applicable

Surface water:

Sample ID	Sample Type	Nominal Concentration (µg/L)	Sample Volume (mL)	Reconstitution Volume[a] (mL)	Dilution Factor
59	Reagent Blank	0.00	NA[b]	5.00	0.200
60, 61, & 62	Matrix Blank	0.00	25.0	5.00	0.200
63, & 64	Control	0.00	25.0	5.00	0.200
65, 66, 67, 68, & 69	LOQ	0.100	25.0	5.00	0.200
70, 71, 72, 73, & 74	10X LOQ	1.00	8.00	5.00	0.625

[a] Reconstitution solvent: 20:80:0.1 acetonitrile:purified reagent water:trifluoroacetic acid (v:v:v)
[b] NA = Not Applicable

2.11 Analysis

2.11.1 Instrument Conditions Etridiazole and 3-DCMT

The GC-MS/EI analysis was conducted utilizing the following instrumental conditions

GC Parameters:

Column: Agilent DB-5MS, 15 m × 0.25 mm × 0.25 µm
Temperature: 65 ºC (initial) and held for 2.00 minutes
Ramps:

Rate (ºC/min)	Final Temperature (ºC)	Hold Time (min)
5.00	100	0.00
10.0	125	1.00
20.0	150	0.00

Run Time: 13.75 minutes
Injection Volume: 2.00 µL
Carrier Gas: Helium
Gas Flows: Constant flow of 2.0 mL/minute
Inlet Mode: Splitless, purge flow to 50.0 mL/minute at 1.00 minute
Inlet Temperature: 200 ºC
Retention Time: Etridiazole, approximately 12.9 minutes
3-DCMT, approximately 11.0 minutes

MSD Parameters:

Solvent Delay: 10.5 minutes

Selected Ion Monitoring:

Etridiazole:

Ion (m/z)	Dwell (msec)	Comments
211.00	50	Primary ion
185.00	50	Confirmation ion
183.00	50	Confirmation ion

3-DCMT:

Ion (m/z)	Dwell (msec)	Comments
149.00	50	Primary ion
184.00	50	Confirmation ion
186.00	50	Confirmation ion

Temperatures: MSD Transfer Line: 300 ºC
MS Quad: 150 ºC
MS Source: 230 ºC

2.11.2 Instrumental Conditions 3-Carb-T

The LC-MS/MS analysis was conducted using the following instrumental conditions:

LC Parameters:

Column: Agilent Poroshell 120 EC-C8 2.7 µm 3.0 × 50mm
Mobile Phase A: 0.1% trifluoroacetic acid in water
Mobile Phase B: 0.1% trifluoroacetic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.600	98.0	2.0
0.50	0.600	98.0	2.0
3.00	0.600	0.0	100
4.00	0.600	0.0	100
4.10	0.600	98.0	2.0
5.00	0.600	98.0	2.0

Run Time: 5.0 minutes
Autosampler Wash: 30:30:40 acetonitrile:methanol:purified reagent water (v:v:v)
Column Temperature: 35 °C
Sample Temperature: 5 °C
Injection Volume: 50 µL

Retention Time: approximately 2.2 minutes for 3-Carb-T

MS Parameters:

Instrument:	MDS Sciex API 5000 mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Dwell Time:	200 milliseconds
Resolution Q1/Q3:	Unit/Unit
Source Temperature:	550 °C
Curtain Gas:	15.00
Ion Source – Gas 1/Gas 2:	70.00/70.00
Collision Gas:	4.00
Declustering Potential:	45.00

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	175.16/147.10	175.16/129.00
Dwell Time (milliseconds):	200	200
Entrance Potential:	4.00	10.00
Collision Energy:	15.00	23.00
Collision Cell Exit Potential:	24.00	18.00

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.11.3 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set: one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the LOQ and 10X LOQ recovery samples. Recoveries of 70.0 to 120% were considered acceptable. The precision was reported in terms of the standard deviation and relative standard deviation for the retention time and the percent recovery values of the LOQ- and 10X LOQ recovery samples. RSD values less than or equal to 20% were considered acceptable for the recovery samples, while RSD values less than or equal to 2% were considered acceptable for the retention times. Specificity of the method was determined by examination of the control samples for peaks at the same retention time as etridiazole, 3-Carb-T, and 3-DCMT which might interfere with the quantitation of the analytes. A linear calibration curve was used for this testing. This calibration curve was evaluated based on the correlation coefficient (r^2) and the recoveries of the calibration standards.

2.13 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.14 Limit of Detection (LOD) and Method Detection Limit (MDL)

The Limit of Detection (LOD) was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Calculations.

The Method Detection Limit (MDL) was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Calculations.

3.0 Calculations

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = mx + b$$

$$(2) \qquad DC\,(x) = \frac{(y - b)}{m}$$

$$(3) \qquad A = DC \times DF$$

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The LOD was calculated using the following equation:

$$(4) \qquad LOD = ((3 \times SN_{ctl}) Resp_{LS}) \times Conc_{LS}$$

where:

SN_{ctl}	=	mean signal to noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
LOD	=	limit of detection for the analysis

The MDL was calculated using the following equation.

$$(5)\ MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	the lowest concentration calibration standard
DF_{CNTL}	=	dilution factor of the control samples
MDL	=	minimum detection limit reported for the analysis of etridiazole, 3-Carb-T or 3-DCMT recovery samples