INTRODUCTION

The objective of this study was to independently validate the analytical method 14088.6158, for measuring residues of Etridiazole and its metabolites Etridiazole acid and DCE in two soils of differing USDA Textural Classification in accordance with EPA 850.6100 (2012) and SANCO/825/00 rev.8.1 (2010) guidelines.

Control samples of Brierlow and Speyer 5M soil were fortified with Etridiazole and DCE at 0.05 and 0.5 mg/kg in quintuplicate and analysed. Samples were extracted with dichloromethane: acetone (75:25 v:v). An aliquot was evaporated and reconstituted with acetone containing Benzophenone internal standard.

Control samples of Brierlow and Speyer 5M soil were fortified with Etridiazole acid at 0.05 and 0.5 mg/kg in quintuplicate and analysed. Samples were extracted with acetonitrile: water (20:80 v/v). An aliquot was extracted using anion exchange solid phase extraction and eluted with 2% TFA in methanol, followed by evaporation and reconstitution with acetonitrile: water: TFA (20:80:0.1 v/v/v).

To assess matrix effects, calibration standards were prepared in control extract.

Samples were analysed for Etridiazole and DCE using gas chromatography with mass spectrometry detection (GC-MS) using Benzophenone as an internal standard. Samples were analysed for Etridiazole acid using high performance liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy was calculated at each validation level in each soil for Etridiazole, Etridiazole acid, and DCE. One primary and two confirmatory GC-MS fragment ions were analysed for Etridiazole and DCE. One primary and one confirmatory LC-MS/MS transition was analysed for Etridiazole acid.

The study was initiated on 04 December 2017 (date the protocol was signed by the Study Director) and completed on the date the final report was signed by the Study Director. The practical phase of the study was conducted by Smithers Viscient (ESG) and was started on 10 January 2018 (stock preparation) and completed on 21 February 2018 (LC-MS/MS analysis).

MATERIALS AND METHODS

Test Substances

Test substance name:	**Etridiazole Tech.**
IUPAC name:	Ethyl 3-trichloromethyl-1,2,4-thiadiazol-5-yl ether
Molecular formula:	$C_5H_5Cl_3N_2OS$
Sponsor Lot Number:	GN20160403
Purity:	99.5%
Molecular mass:	247.53
Storage conditions:	Room Temperature (15-30°C)
Expiry date:	7 June 2018

Test substance name:	**Etridiazole acid (also known as 3-Carb-T)**
IUPAC name:	5-ethoxy-1,2,4-thiadiazole-3-carboxylic acid
Molecular formula:	$C_5H_6N_2O_3S$
Sponsor Lot Number:	2840-89-RRG
Purity:	99.9%
Molecular weight:	174.18
Storage conditions:	Frozen (< -10°C, nominally -20°C)
Expiry date:	28 February 2020

Test substance name:	**DCE (also known as 3-DCMT or DCE (T-03))**
IUPAC name:	3-dichloromethyl-5-ethoxy-1,2,4-thiadiazole
Molecular formula:	$C_5H_6Cl_2N_2OS$
Sponsor Batch Number:	2840-77-RRG
Purity:	99.3%
Molecular weight:	213.08
Storage conditions:	Refrigerated (2°C to 8°C)
Expiration date:	27 February 2020

Certificates of Analysis for the test substances are presented in Appendix 1.

Internal Standard

Internal standard name:	**Benzophenone**
Molecular formula:	$C_{13}H_{100}$
Sponsor Lot Number:	LC18173V
Purity:	99.9%
Molecular mass:	182.2
Storage conditions:	Room Temperature (15-30°C)
Expiry date:	28 February 2019

The Certificate of Analysis for the internal standard is presented in Appendix 1.

Test System

Control samples of soil with differing USDA Textural Classification were sourced by Smithers Viscient (ESG). The soils used were CS 30/16 Brierlow (Silt loam) and CS 27/16 Speyer 5M (Sandy loam).

Soil characterisation data are listed in the table below:

Soil Name	Textural class[1]	% Sand, Silt, Clay[2]	CEC (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
Brierlow	Silt loam	26, 58, 16	20.0	2.5	6.4	5.6
Speyer 5M	Sandy loam	59, 30, 11	17.7	1.0	8.5	7.3

[1, 2] USDA classification.

The certificates of analysis for each soil are presented in Appendix 2.

Reagents

Acetonitrile	LC-MS grade, Honeywell
Acetonitrile	HPLC grade, Honeywell
Dichloromethane (DCM)	HPLC grade, Honeywell
Acetone	HPLC grade, Honeywell
Methanol	HPLC grade, Honeywell
Water	Milli-Q with LCPAK polisher, In House
Trifluoroacetic acid (TFA)	LC-MS grade, Fisher
Trifluoroacetic acid (TFA)	HPLC grade, Sigma
Ammonium hydroxide	Reagent grade, Fisher
Oasis MAX cartridge (3cc, 60 mg)	Waters (186000367)

Equivalent or better reagents may have been used.

Equipment

Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector

Thermo Trace 1300 Gas Chromatograph with ISQ LT single quadrupole mass spectrometer detector

Analytical Method

Analytical method 14088.6158 was supplied by the sponsor. The method used GC-MS analysis for Etridiazole and DCE, and LC-MS/MS analysis for Etridiazole acid.

Preparation of Reagents

DCM: acetone (75:25 v/v) was prepared by mixing 250 mL HPLC grade acetone with 750 mL HPLC grade DCM.

Acetonitrile: water (20:80 v/v) was prepared by mixing 200 mL HPLC grade acetonitrile with 800 mL water.

Acetonitrile: water: TFA (20:80:0.1 v/v/v) was prepared by mixing 400 mL water with 100 mL HPLC grade acetonitrile and 0.5 mL HPLC grade TFA.

2% TFA in methanol was prepared by mixing 50 mL HPLC grade methanol with 1 mL HPLC grade TFA.

0.1% TFA in water was prepared by mixing 1000 mL water with 1 mL LC-MS grade TFA.

0.1% TFA in acetonitrile was prepared by mixing 1000 mL LC-MS grade acetonitrile with 1 mL LC-MS grade TFA.

0.002 µg/mL Benzophenone in acetone (internal standard diluent) was prepared by adding 0.1 mL of 10 µg/mL Benzophenone in acetone to 500 mL acetone.

Preparation of Stock Solutions

Primary stock solutions of Etridiazole, DCE, Etridiazole acid and Benzophenone were prepared as described in the table below:

Stock ID	Test Substance	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Stock 1	Etridiazole	29.26	99.5	Acetone	10	2911
Stock 2		13.80	99.5		10	1373
Stock 3	DCE	15.33	99.3		10	1522
Stock 4		11.81	99.3		10	1173
Stock 5	Etridiazole acid	10.11	99.9	Acetonitrile	10.100	1000
Stock 6		10.27	99.9		10.260	1000
Stock 7	Benzophenone	14.20	99.9	Acetone	10	1419

[1] Corrected for Purity.
Duplicate stocks were prepared for correlation purposes.

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months for Etridiazole, DCE and Etridiazole acid, and one year for Benzophenone internal standard.

Secondary stock solutions of Etridiazole, DCE, Etridiazole acid and Benzophenone were prepared as described in the table below:

Primary Stock ID	Test Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)[1]	Solvent	Final Volume (mL)	Concentration (µg/mL)
Stock 1	Etridiazole	2911	0.0344	Acetone	10	10[1]
Stock 3	DCE	1522	0.0657			
Stock 2	Etridiazole	1373	0.0728	Acetone	10	10[1]
Stock 4	DCE	1173	0.0853			
Stock 5	Etridiazole acid	1000	0.1	Acetonitrile	10	10
Stock 6		1000	0.1	Acetonitrile	10	10
Stock 7	Benzophenone	1419	0.0705	Acetone	10	10

[1] Mixed stock of Etridiazole and DCE.

Secondary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of one month.

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

Test Substance	Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Etridiazole and DCE	10	1	Acetone	10	1
Etridiazole acid	10	1	Acetonitrile	10	1

Sub-stock solutions were prepared on the day of use and stored refrigerated until the corresponding analysis was complete.

Preparation of Calibration Standards

Calibration standards of Etridiazole, DCE and Etridiazole acid were prepared as described in the following table:

Test Substance	Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Etridiazole and DCE	1	0.1	0.002 µg/mL Benzophenone in acetone	10	0.01[1]
	0.01	0.75		1	0.0075
	0.01	0.5		1	0.005
	0.01	0.2		1	0.002
	0.01	0.15		1	0.0015
	0.01	0.1		1	0.001
	0.01	0.075		1	0.00075
Etridiazole acid	1	0.1	MeCN: H_2O: TFA (20:80:0.1 v/v/v)	10	0.01
	0.01	0.75		1	0.0075
	0.01	0.5		1	0.005
	0.01	0.3		1	0.003
	0.01	0.2		1	0.002
	0.01	0.1		1	0.001
Etridiazole acid	1	0.1	Brierlow soil final extract	10	0.01
	0.01	0.75		1	0.0075
	0.01	0.5		1	0.005
	0.01	0.3		1	0.003
	0.01	0.2		1	0.002
	0.01	0.1		1	0.001

[1] Used as an intermediate standard (not analysed).
Etridiazole acid in Brierlow soil used matrix matched calibration standards.

A single set of calibration standards was prepared for each validation batch, which was analysed once before the samples and once after the samples. When samples required re-injection due to failure, the same calibration standards were used as the initial injection, so that the calibration standards and sample extracts were equally aged. Suitability of aged calibration standards was verified by an acceptable correlation coefficient or coefficient of determination. When samples required re-dilution from the stored extracts, fresh calibration standards were prepared.

Preparation of Matrix Matched Standards for Matrix Assessment
Matrix matched standards of Etridiazole and DCE were prepared in control soil final extract. Matrix matched standards of Etridiazole acid were prepared in control soil final extract.

Test Substance	Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Etridiazole and DCE	1	0.02	Speyer 5M soil final extract	10	0.002
	1	0.02		10	0.002
	1	0.02		10	0.002
	1	0.02	Brierlow soil final extract	10	0.002
	1	0.02		10	0.002
	1	0.02		10	0.002
Etridiazole acid	1	0.025	Speyer 5M soil final extract	5	0.005
	1	0.025		5	0.005
	1	0.025		5	0.005
	1	0.025	Brierlow soil final extract	5	0.005
	1	0.025		5	0.005
	1	0.025		5	0.005

Preparation of Non-Matrix Matched Standards for Matrix Assessment
Non-matrix matched standards of Etridiazole, DCE and Etridiazole acid were prepared in blank solvent for comparison with matrix matched standards.

Test Substance	Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Etridiazole and DCE	1	0.02	0.002 µg/mL Benzophenone in acetone	10	0.002
	1	0.02		10	0.002
	1	0.02		10	0.002
Etridiazole acid	1	0.025	Acetonitrile: water: TFA (20:80:0.1 v/v/v)	5	0.005
	1	0.025		5	0.005
	1	0.025		5	0.005

Sample Fortification
Etridiazole and DCE
A sample amount equivalent to 5 g dry weight (±0.05 g) was weighed into a glass tube. Quintuplicate soil samples were fortified at the LOQ (0.05 mg/kg) and at 10 × LOQ (0.5 mg/kg) with a mixed stock solution of Etridiazole and DCE. Duplicate control soil samples and a reagent blank (without soil) were also prepared, as described in the following tables:

CS 27/16 Speyer 5M soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank A	N/A	N/A	N/A	N/A
Reagent Blank E	N/A	N/A	N/A	N/A
Control A	5	N/A	N/A	N/A
Control C-D	5	N/A	N/A	N/A
Control M-N	5	N/A	N/A	N/A
F0.05 A-E	5	10	0.025	0.05
F0.05 U-Y	5	10	0.025	0.05
F0.5 A-E	5	10	0.25	0.5
F0.5 U-Y	5	10	0.25	0.5

N/A = Not applicable.
Control A was used to prepare matrix matched standards for matrix assessment.
In deviation to the method, Reagent Blank A, Control A, Control C-D, F0.05 A-E and F0.5 A-E were weighed into plastic rather than glass tubes, resulting in validation failure (it was suspected that the test substances adsorbed to the vessel.
Reagent Blank E, Control M-N and F0.05 U-Y and F0.5 U-Y were prepared for the second validation attempt of Etridiazole and DCE (and were weighed into glass tubes).

CS 30/16 Brierlow soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank B	N/A	N/A	N/A	N/A
Reagent Blank F	N/A	N/A	N/A	N/A
Control B	5	N/A	N/A	N/A
Control E-F	5	N/A	N/A	N/A
Control O-P	5	N/A	N/A	N/A
F0.05 F-J	5	10	0.025	0.05
F0.05 Z-AD	5	10	0.025	0.05
F0.5 F-J	5	10	0.25	0.5

N/A = Not applicable.
Control B was used to prepare matrix matched standards for matrix assessment.
Reagent Blank F, Control O-P and F0.05 Z-AD were prepared for the second validation attempt of DCE at the LOQ.

Etridiazole acid

The moisture content of the soil was determined. The sample amount equivalent to 5 g dry weight (±0.05 g) was weighed into a Nalgene centrifuge tube. Quintuplicate soil samples were fortified at the LOQ (0.05 mg/kg) and at 10 × LOQ (0.5 mg/kg) with a stock solution of Etridiazole acid. Duplicate control soil samples and a reagent blank (without soil) were also prepared, as described in the following tables:

CS 27/16 Speyer 5M soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank C	N/A	N/A	N/A	N/A
Reagent Blank G	N/A	N/A	N/A	N/A
Control G	5	N/A	N/A	N/A
Control I-J	5	N/A	N/A	N/A
Control Q-R	5	N/A	N/A	N/A
F0.05 K-O	5	1	0.25	0.05
F0.05 AE-AI	5	1	0.25	0.05
F0.5 K-O	5	10	0.25	0.5

N/A = Not applicable.
Control G was used to prepare matrix matched standards for matrix assessment.
Reagent Blank G, Control Q-R and F0.05 AE-AI were prepared for the second validation attempt of Etridiazole acid at the LOQ.

CS 30/16 Brierlow soil

Sample ID	Sample Weight (g)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank D	N/A	N/A	N/A	N/A
Reagent Blank H	N/A	N/A	N/A	N/A
Control H	5	N/A	N/A	N/A
Control K-L	5	N/A	N/A	N/A
Control S-T	5	N/A	N/A	N/A
F0.05 P-T	5	1	0.25	0.05
F0.05 AJ-AN	5	1	0.25	0.05
F0.5 P-T	5	10	0.25	0.5

N/A = Not applicable.
Control H was used to prepare matrix matched standards for matrix assessment.
Reagent Blank H, Control S-T and F0.05 AJ-AN were prepared for the second validation attempt of Etridiazole acid at the LOQ.

Sample Extraction

Etridiazole and DCE

30 mL DCM: acetone (75:25 v/v) was added to the soil, placed on a shaker for 30 minutes at 150 rpm and centrifuged for 15 minutes at 1200 rpm. A portion of extract was removed and evaporated to approximately 100 µL volume under a gentle stream of nitrogen and reconstituted with 0.002 µg/mL Benzophenone in acetone (internal standard diluent). Sample extracts were stored refrigerated in case further analysis was required. The extraction procedure is summarised in the following tables:

CS 27/16 Speyer 5M soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extraction Volume (mL)	Sample Dilution (mL to mL)
Reagent Blank A	N/A	N/A	30	3-10
Reagent Blank E	N/A	N/A	30	3-10
Control A	N/A	5	30	3-10
Control C-D	N/A	5	30	3-10
Control M-N	N/A	5	30	3-10
F0.05 A-E	0.05	5	30	3-10
F0.05 U-Y	0.05	5	30	3-10
F0.5 A-E	0.5	5	30	0.3-10
F0.5 U-Y	0.5	5	30	0.3-10

N/A = Not applicable.
Three portions of Control A extract were used to prepare matrix matched standards for matrix assessment.
Reagent blank A, Con C-D, F0.05 A-E and F0.5 A-E were re-injected against the original calibration standards due to an instrument/software failure during the initial injection sequence.
Reagent Blank E, Control M-N and F0.05 U-Y and F0.5 U-Y were prepared for the second validation attempt of Etridiazole and DCE due to initial validation failure, suspected to be due to plastic tubes being used instead of glass.

CS 30/16 Brierlow soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extraction Volume (mL)	Sample Dilution (mL to mL)
Reagent Blank B	N/A	N/A	30	3-10
Reagent Blank F	N/A	N/A	30	3-10
Control B	N/A	5	30	3-10
Control E-F	N/A	5	30	3-10
Control O-P	N/A	5	30	3-10
F0.05 F-J	0.05	5	30	3-10
F0.05 Z-AD	0.05	5	30	3-10
F0.5 F-J	0.5	5	30	0.3-10

N/A = Not applicable.
Three portions of Control B extract were used to prepare matrix matched standards for matrix assessment.
Control E-F, F0.05 F-J and F0.5 F-J were re-diluted using glass pipettes and re-analysed with fresh calibration standards due to initial validation failure, suspected to be due to plastic pipettes being used.
Control E-F, F0.05 F-J and F0.5 F-J re-dilutions were re-injected using the same calibration standards due to validation failure for Etridiazole LOQ and DCE LOQ & 10×LOQ.
F0.05 F-J were re-diluted a second time due to validation failure at the LOQ with the first re-dilution.
Reagent Blank F, Control O-P and F0.05 Z-AD were prepared for the second validation attempt of DCE at the LOQ.

Etridiazole acid

20 mL acetonitrile: water (20:80 v/v) was added to each soil, placed on a shaker for 30 minutes at 150 rpm and centrifuged at 3000 rpm for 10 minutes and the supernatant removed. An additional 20 mL acetonitrile: water (20:80 v/v) was added to each soil, placed on a shaker for 30 minutes at 150 rpm and centrifuged at 3000 rpm for 10 minutes and the supernatant removed. The two extracts were combined and made to 50 mL volume. 5 mL extract was removed for solid phase extraction.

Oasis MAX cartridges (3cc, 60 mg) were conditioned by filling twice with methanol then twice with water. 1 µL ammonium hydroxide was added to each sample before loading onto the cartridge. The sample vessel and cartridge was rinsed with 5 mL water then 5 mL methanol. The columns were dried under full vacuum and the rinses discarded. The columns were eluted with 3 mL 2% TFA in methanol and full vacuum applied. The eluate was evaporated to approximately 100 µL volume under a gentle stream of nitrogen at 50°C. The extracts were reconstituted in acetonitrile: water: TFA (20:80:0.1 v/v/v) and ultrasonicated for 5 minutes. Sample extracts were stored refrigerated in case further analysis was required. The extraction and dilution procedure is summarised in the following tables:

CS 27/16 Speyer 5M soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extraction Volume (mL)	Sample Dilution (mL to mL)	Sample Dilution (mL to mL)
Reagent Blank C	N/A	N/A	50	5-5	N/A
Reagent Blank G	N/A	N/A	50	5-5	N/A
Control G	N/A	5	50	5-5	N/A
Control I-J	N/A	5	50	5-5	N/A
Control Q-R	N/A	5	50	5-5	N/A
F0.05 K-O	0.05	5	50	5-5	N/A
F0.05 AE-AI	0.05	5	50	5-5	N/A
F0.5 K-O	0.5	5	50	5-5	0.1-1

N/A = Not applicable.
Three portions of Control G extract were used to prepare matrix matched standards for matrix assessment.
Reagent Blank C, Con I-J and F0.5 K-O were re-diluted and re-analysed using fresh calibration standards due to initial validation failure, suspected to be due to calibration standard preparation issues (loss of test substance in volumetric flask).
Reagent Blank G, Control Q-R and F0.05 AE-AI were prepared for the second validation attempt of Etridiazole acid at the LOQ.

CS 30/16 Brierlow soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extraction Volume (mL)	Sample Dilution (mL to mL)	Sample Dilution (mL to mL)
Reagent Blank D	N/A	N/A	50	5-5	N/A
Reagent Blank H	N/A	N/A	50	5-5	N/A
Control H	N/A	5	50	5-5	N/A
Control K-L	N/A	5	50	5-5	N/A
Control S-T	N/A	5	50	5-5	N/A
F0.05 P-T	0.05	5	50	5-5	N/A
F0.05 AJ-AN	0.05	5	50	5-5	N/A
F0.5 P-T	0.5	5	50	5-5	0.1-1

N/A = Not applicable.
Three portions of Control H extract were used to prepare matrix matched standards for matrix assessment.
Three portions of Con K-L were used to prepare matrix matched calibration standards.
Three portions of Control S-T were used to prepare matrix matched calibration standards.
F0.5 P-T were re-injected using the same calibration standards to confirm initial validation failure.
Reagent Blank D, Con K-L and F0.5 P-T were re-extracted by SPE and re-analysed using fresh calibration standards due to initial validation failure, suspected to be due to calibration standard preparation issues (loss of test substance in volumetric flask).
Reagent Blank H, Control S-T and F0.05 AJ-AN were prepared for the second validation attempt of Etridiazole acid at the LOQ.

Instrument Conditions

Etridiazole and DCE

GC-MS analysis was performed using the following instrument conditions:

GC Parameters:

Column	Agilent DB-5ms 15m × 0.25 mm 0.25 µm film
Carrier Gas	Helium
Flow Rate	1.0 mL/min
Inlet Temperature	200°C
Injection mode	Splitless
Split flow	50 mL/min
Splitless time	1 minute
Injection Volume	2 µL
Oven Temperature	Hold at 50°C for 2 minutes. Ramp at 45°C/minute to 250°C Ramp at 125°C/minute to 300°C, hold for 8 minutes.
Run Time	15.556 minutes
Retention Time	DCE: Approx. 4.8 minutes Etridiazole: Approx. 5.1 minutes Benzophenone (internal standard): Approx.5.6 minutes

MS Parameters:

Instrument	Thermo ISQ Single Quad Mass Spectrometer	
Ionisation Mode	Electron Ionisation (EI)	
Polarity	Positive	
Scan Type	Selected Ion Monitoring (SIM)	
MS Transfer Line Temperature	300°C	
Ion Source Temperature	230°C	
Compound Name	SIM Ions Monitored	Dwell Time (ms)
Etridiazole (Primary)	211	20
Etridiazole (Confirmatory)	185	20
Etridiazole (Confirmatory)	183	20
DCE (Primary)	143	20
DCE (Confirmatory)	184	20
DCE (Confirmatory)	186	20
Benzophenone (internal standard)	105	20

GC-MS data was collected using Chromeleon 7.

Etridiazole acid

LC-MS/MS analysis was performed using the following instrument conditions:

LC Parameters:

Column	Phenomenex Kinetex 5 µm EVO C18 50 × 2.1 mm		
Mobile Phase A	0.1% TFA in water		
Mobile Phase B	0.1% TFA in acetonitrile		
Flow Rate	0.5 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	98	2
	0.5	98	2
	2.0	0	100
	3.0	0	100
	3.1	98	2
	4.0	98	2

Run Time	4 minutes	
Column Temperature	35°C	
Autosampler Temperature	15°C	
Injection Volume	10 µL	
Retention Time	Approx. 1.35 minutes	
Valco Valve Diverter	Time (min)	Position
	0	A (to waste)
	0.5	B (to MS)
	3.5	A (to waste)

MS/MS Parameters:

Instrument	AB Sciex API 5000 Triple Quadrupole Mass Spectrometer			
Ionisation Type	Electrospray (ESI)			
Polarity	Positive			
Scan Type	Multiple reaction monitoring (MRM)			
Ion Spray Voltage	5500 V			
Collision Gas (CAD)	5			
Curtain Gas (CUR)	25			
Gas Flow 1 (GS1)	40			
Gas Flow 2 (GS2)	40			
Vaporiser Temperature (TEM)	550			
Interface Heater (ihe)	On			
Entrance Potential (EP)	10			
Collision Exit Potential (CXP)	13			
Compound Name	MRM Transition Ions Monitored	Declustering Potential (DP) (V)	Collision Energy (CE) (V)	Dwell Time (ms)
Etridiazole acid (Primary)	174.9/146.9	80	17	200
Etridiazole acid (Confirmatory)	174.9/129.0	80	21	200

LC-MS/MS data was collected using Analyst 1.6.2.

Calculation of Results

GC-MS data were calculated using Chromeleon 7.
LC-MS/MS data were calculated using Analyst 1.6.2.

When the calibration fit is linear as in this study, Chromeleon/Analyst uses the following formula to calculate the concentration of test substance present in the sample extract:

$x = (y - c) / m$

Where:

x = concentration of test substance in sample extract (µg/mL)
y = peak area due to test substance (or internal standard peak area ratio for Etridiazole and DCE)
c = y intercept on calibration graph
m = gradient of the calibration graph

The concentration of test substance in the initial sample is calculated as follows:

Sample concentration (mg/kg) = Extract concentration (µg/mL) × Dilution factor

Dilution factor = Final extract volume (mL) / weight of soil in final extract (g)

Procedural recovery from fortified samples is calculated as follows:

Recovery (%) = Sample concentration / Fortified concentration × 100

95% confidence intervals were calculated for each validation level as follows:

95% confidence interval (±) =

1.96 × standard deviation of results / square root of the number of replicate results

The limit of detection (LOD) based upon the sample concentration equivalent to three times the baseline noise of a control sample was calculated as follows:

LOD = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (µg/mL) / height of calibration standard peak

In deviation to the study protocol, percentages were presented to the nearest whole number and RSDs were recorded to one decimal place, which were not three significant figures in some cases. This was because a validated spreadsheet had been used to calculate precision and accuracy of recoveries, which did not allow manual correction of significant figures.

Validation Pass Criteria
The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions or fragment ions monitored for each compound:

Mean Recovery and Precision
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 120% and a %RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if the amounts found in blank samples were ≤ 50% of cited method limit of detection (LOD) and ≤ 30% of the LOQ.

Linearity
Linearity was acceptable if the lowest calibration standard concentration was at least 30% of the equivalent LOQ final extract concentration. The highest calibration standard concentration was at least 120% of the 10 × LOQ extract concentration (after dilution if applicable). If matrix effects were determined to be significant, matrix matched standards would be used. The correlation coefficient (r) was acceptable if it was ≥ 0.99 and the coefficient of determination (r^2) was ≥ 0.98 (in agreement with local SOPs). The criteria given in the method in study 14088.6158 was ≥ 0.995 for r and ≥ 0.990 for r^2.

LOD (Limit of Detection) Assessment
An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions or fragment ions for all compounds.

The protocol stated that the LOD would be calculated as the sample concentration equivalent to the lowest calibration standard, which in study 14088.6158 is defined as the MDL (see below). Therefore, in deviation from the study protocol, the LOD was calculated from baseline noise as in study 14088.6158.

MDL (Method Detection Limit)
The MDL was calculated as the sample concentration equivalent to the lowest calibration standard.

Matrix Assessment
An assessment of matrix effects was made by comparison of triplicate standards prepared in blank solvent and in each control matrix final extract. This was assessed for all compounds and for the primary and confirmatory transitions or fragment ions.

Results were presented as a % difference from the mean non-matrix standard value.

A difference of ≥ 20% was considered significant.