INTRODUCTION

The objective of this study was to independently validate the analytical method 14088.6157, for measuring residues of Etridiazole and its metabolites Etridiazole acid and DCE in accordance with EPA 850.6100 (2012) and SANCO/825/00 rev.8.1 (2010) guidelines.

Control samples of surface and ground water were simultaneously fortified with Etridiazole and DCE at 0.0001 and 0.001 µg/mL in quintuplicate and analysed. Samples were extracted once with iso-octane. Samples were further diluted into the calibration range if necessary with iso-octane.

Control samples of surface and ground water were fortified with Etridiazole acid at 0.0001 and 0.001 µg/mL in quintuplicate and analysed. Samples were extracted by anion exchange solid phase extraction (SPE) and eluted with 2% trifluoroacetic acid in methanol, evaporated and reconstituted with acetonitrile: water: trifluoroacetic acid (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). 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 water 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 17 January 2018 (stock dilution) and completed on 01 February 2018 (GC-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.

Test System
Control samples of surface and ground water were sourced by Smithers Viscient (ESG). The control waters used were Fountains Abbey surface water (supplied by Smithers Viscient (ESG)) and Borehole (ground) water (supplied by Agrochemex).

Water characterisation data are listed in the following table:

Water Name	Water Type	Suspended Solids (mg/L)	Conductivity (µS/cm)	Hardness (mg/L $CaCO_3$)	pH	Dissolved Organic Carbon (mg/L)
Fountains Abbey (CS 04/17)	Surface	15	49	200	7.9	4.94
Borehole Water (CS 12/17)	Ground	4	467	176	7.6	0.00

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

Reagents

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

Equivalent or better reagents may have been used.

Equipment

Shimadzu Nexera series UHPLC 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 14088.6157 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

Acetonitrile: water: TFA (20:80:0.1 v/v/v) was prepared by mixing 400 mL LC-MS grade 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 LC-MS grade 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.

Some batches of reagents were prepared under GLP study 3202057 (Etridiazole – Independent Laboratory Validation of Analytical Method 14088.6158 for the Determination of Etridiazole and its metabolites Etridiazole acid and DCE in Soil.) when they were used by both studies 3202057 and 3202058.

Preparation of Stock Solutions

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

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

[1] Corrected for Purity.
Duplicate stocks were prepared for correlation purposes.
Primary stocks were prepared under GLP study 3202057.

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months.

Secondary stock solutions of Etridiazole, DCE and Etridiazole acid 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

[1] Mixed stock of Etridiazole and DCE.
Secondary stocks were prepared under GLP study 3202057.

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 following table:

Test Substance	Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
Etridiazole and DCE	10	0.1	Acetone	10	0.1
Etridiazole acid	10	0.1	Acetonitrile	10	0.1

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	10	0.02	Iso-octane	10	0.02
	0.02	0.75		1	0.015
	0.02	0.5		1	0.01
	0.02	0.3		1	0.006
	0.02	0.2		1	0.004
	0.02	0.1		1	0.002
Etridiazole acid	0.1	0.25	Acetonitrile: water: TFA (20:80:0.1 v/v/v)	10	0.0025
	0.0025	0.8		1	0.002
	0.0025	0.6		1	0.0015
	0.0025	0.4		1	0.001
	0.0025	0.2		1	0.0005
	0.0025	0.1		1	0.00025

A single set of calibration standards was prepared for each validation batch, which was analysed once before the samples and once after the samples. An exception to this was the first validation attempt for Etridiazole and DCE in Borehole ground water, where the GC-MS stopped after the first calibration standard run after the samples. In this case, only the first set of calibration standards were used or quantification, and the single calibration standard run after the samples was used to verify that instrument response had not significantly drifted after analysis of the samples. In the validation attempt for Etridiazole acid in Borehole ground water, the LC-MS/MS stopped before the last three calibration standards run after the samples. The first three calibration standards which ran after the samples were accepted. When samples required re-injection due to failure, the same full set of 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 60 mL control water and extracted in exactly the same manner as the samples. Matrix matched standards of Etridiazole acid were prepared in control final extract 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	0.1	0.2	Ground water, extracted in iso-octane	2	0.01
	0.1	0.2		2	0.01
	0.1	0.2		2	0.01
	0.1	0.2	Surface water, extracted in iso-octane	2	0.01
	0.1	0.2		2	0.01
	0.1	0.2		2	0.01
Etridiazole acid	0.1	0.05	Ground water final extract	5	0.001
	0.1	0.05		5	0.001
	0.1	0.05		5	0.001
	0.1	0.05	Surface water final extract	5	0.001
	0.1	0.05		5	0.001
	0.1	0.05		5	0.001

Preparation of Non-Matrix Matched Standards for Matrix Assessment

Non-matrix 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	0.02	0.5	Iso-octane	1	0.01
	0.02	0.5		1	0.01
	0.02	0.5		1	0.01
Etridiazole acid	0.1	0.05	Acetonitrile: water: TFA (20:80:0.1 v/v/v)	5	0.001
	0.1	0.05		5	0.001
	0.1	0.05		5	0.001

Sample Fortification

Etridiazole and DCE

60 mL of water was measured into glass jars. Quintuplicate water samples were fortified at the LOQ (0.0001 µg/mL) and at 10 × LOQ (0.001 µg/mL) with a mixed stock solution of Etridiazole and DCE. Duplicate control water samples and a reagent blank (without water) were also prepared, as described in the following tables:

Ground water (CS 12/17 Borehole water)

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/mL)
Reagent Blank A	N/A	N/A	N/A	N/A
Reagent Blank E	N/A	N/A	N/A	N/A
Control A-C	60	N/A	N/A	N/A
Control G-H	60	N/A	N/A	N/A
Control U-V	60	N/A	N/A	N/A
F0.0001 A-E	60	0.1	0.06	0.0001
F0.0001 U-Y	60	0.1	0.06	0.0001
F0.001 A-E	60	0.1	0.6	0.001

N/A = Not applicable.
Control A-C were used to prepare matrix matched standards for matrix assessment.
Reagent Blank E, Control U-V and F0.0001 U-Y were prepared for the second validation attempt of Etridiazole acid at the LOQ.

Surface water (CS 04/17 Fountains Abbey water)

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/mL)
Reagent Blank B	N/A	N/A	N/A	N/A
Control D-F	60	N/A	N/A	N/A
Control I-J	60	N/A	N/A	N/A
F0.0001 F-J	60	0.1	0.06	0.0001
F0.001 F-J	60	0.1	0.6	0.001

N/A = Not applicable.
Control D-F were used to prepare matrix matched standards for matrix assessment.

Etridiazole acid

25 mL of water was measured into glass tubes for the controls and LOQ samples.
8 mL of water was measured into glass tubes for the 10 × LOQ samples.
Quintuplicate water samples were fortified at the LOQ (0.0001 µg/mL) and at 10 × LOQ (0.001 µg/mL) with Etridiazole acid stock solution. Duplicate control water samples and a reagent blank (without water) were also prepared, as described in the following tables:

Ground water (CS 12/17 Borehole water)

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/mL)
Reagent Blank C	N/A	N/A	N/A	N/A
Control K-M	25	N/A	N/A	N/A
Control Q-R	25	N/A	N/A	N/A
F0.0001 K-O	25	0.1	0.025	0.0001
F0.001 K-O	8	0.1	0.08	0.001

N/A = Not applicable.
Control K-M were used to prepare matrix matched standards for matrix assessment.

Surface water (CS 04/17 Fountains Abbey water)

Sample ID	Sample Volume (mL)	Fortification Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/mL)
Reagent Blank D	N/A	N/A	N/A	N/A
Control N-P	25	N/A	N/A	N/A
Control S-T	25	N/A	N/A	N/A
F0.0001 P-T	25	0.1	0.025	0.0001
F0.001 P-T	8	0.1	0.08	0.001

N/A = Not applicable.
Control N-P were used to prepare matrix matched standards for matrix assessment.

Sample Extraction

Etridiazole and DCE

2 mL iso-octane was added to the water and placed on a shaker for 30 minutes. After allowing to settle for approximately 10 minutes, the top layer was transferred to a glass tube and centrifuged at 1200 rpm for 20 minutes. The iso-octane was transferred to a vial for analysis. The 0.001 µg/mL samples were further diluted in iso-octane. Sample extracts were stored refrigerated in case further analysis was required. The extraction procedure is summarised in the following tables:

Ground water (CS 12/17 Borehole water)

Sample ID	Fortified Concentration (µg/mL)	Sample Volume (mL)	Extraction Volume (mL)	Sample Dilution (mL to mL)
Reagent Blank A	N/A	N/A	2	N/A
Reagent Blank E	N/A	N/A	2	N/A
Control A-C	N/A	60	2	N/A
Control G-H	N/A	60	2	N/A
Control U-V	N/A	60	2	N/A
F0.0001 A-E	0.0001	60	2	N/A
F0.0001 U-Y	0.0001	60	2	N/A
F0.001 A-E	0.001	60	2	0.3 to 1

N/A = Not applicable.
Control A-C were used to prepare matrix matched standards for matrix assessment.
Reagent Blank E, Control U-V and F0.0001 U-Y were prepared for the second validation attempt of Etridiazole acid at the LOQ.

Surface water (CS 04/17 Fountains Abbey water)

Sample ID	Fortified Concentration (µg/mL)	Sample Volume (mL)	Extraction Volume (mL)	Sample Dilution (mL to mL)
Reagent Blank B	N/A	N/A	2	N/A
Control D-F	N/A	60	2	N/A
Control I-J	N/A	60	2	N/A
F0.0001 F-J	0.0001	60	2	N/A
F0.001 F-J	0.001	60	2	0.3 to 1

N/A = Not applicable.
Control D-F were used to prepare matrix matched standards for matrix assessment.

Etridiazole acid

5 µL ammonium hydroxide was added to the control and 0.0001 µg/mL samples, 1.6 µL ammonium hydroxide was added to the 0.001 µg/mL samples. Oasis MAX cartridges (3cc, 60 mg) were conditioned with two column volumes of methanol followed by two column volumes of water. The sample was loaded onto the column. The sample tube and SPE column were rinsed with 5 mL water then 5 mL methanol and full vacuum applied. The samples were eluted into glass tubes using 3 mL 2% TFA in methanol, allowing the sorbent to saturate under gravity before applying vacuum. The samples were concentrated under a gentle stream of nitrogen in a heating block set to 50°C, re-constituted with 5 mL acetonitrile: water: TFA (20:80:0.1 v/v/v) and ultrasonicated to dissolve. Sample extracts were stored refrigerated in case further analysis was required. The extraction and dilution procedure is summarised in the following tables:

Ground water (CS 12/17 Borehole water)

Sample ID	Fortified Concentration (µg/mL)	Sample Volume (mL)	Reconstitution Volume (mL)
Reagent Blank C	N/A	N/A	5
Control K-M	N/A	25	5
Control Q-R	N/A	25	5
F0.0001 K-O	0.0001	25	5
F0.001 K-O	0.001	8	5

N/A = Not applicable.
Control K-M were used to prepare matrix matched standards for matrix assessment.

Surface water (CS 04/17 Fountains Abbey water)

Sample ID	Fortified Concentration (µg/mL)	Sample Volume (mL)	Reconstitution Volume (mL)
Reagent Blank D	N/A	N/A	5
Control N-P	N/A	25	5
Control S-T	N/A	25	5
F0.0001 P-T	0.0001	25	5
F0.001 P-T	0.001	8	5

N/A = Not applicable.
Control N-P were used to prepare matrix matched standards for matrix assessment.

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	2.0 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 65°C for 2 minutes. Ramp at 5°C/minute to 100°C Ramp at 10°C/minute to 125°C, hold for 1 minute. Ramp at 20°C/minute to 150°C.
Run Time	13.75 minutes
Retention Time	DCE: Approx. 9.9 minutes Etridiazole: Approx. 11.8 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)	149	20
DCE (Confirmatory)	184	20
DCE (Confirmatory)	186	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	Agilent Poroshell 120 EC-C8, 3 × 50 mm, 2.7 µm		
Mobile Phase A	0.1% TFA in water		
Mobile Phase B	0.1% TFA in acetonitrile		
Flow Rate	0.6 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	98	2
	0.5	98	2
	3.0	0	100
	4.0	0	100
	4.1	98	2
	5.0	98	2

Run Time	5 minutes	
Column Temperature	35°C	
Autosampler Temperature	5°C	
Injection Volume	50 µL[1]	
Retention Time	Approx. 1.8 minutes	
Valco Valve Diverter	Time (min)	Position
	0	A (to waste)
	0.5	B (to MS)
	4.5	A (to waste)

[1] A 10 µL injection volume was used for the first attempt of the matrix assessment, but sensitivity and precision was insufficient at this volume, therefore a 50 µL was used for the re-injection of the matrix assessment and validation attempts.

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
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 (µg/mL) = Extract concentration (µg/mL) × Dilution factor

Dilution factor = Final extract volume (mL) / volume of water in final extract (mL)

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

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 14088.6157 was ≥ 0.995 for r and ≥ 0.99 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 14088.6157 is defined as the MDL (see below). Therefore the LOD was calculated from baseline noise as in study 14088.6157.

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.