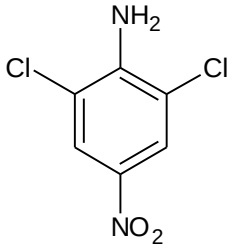
MATERIALS AND METHODS

Protocol Adherence

The study was conducted in accordance with the protocol with two deviations. These deviations did not affect the integrity of the study and are detailed in Appendix 7.

Test Substance

The following test substance was used to fortify the samples, as per the analytical method validated by Smithers ERS (Wareham):

Test Substance Name:	**Dicloran Technical**
IUPAC Name:	2,6-Dichloro-4-nitroaniline
CAS Number:	99-30-9
Structure:	
Molecular Formula:	$C_6H_4Cl_2N_2O_2$
Molecular Weight:	207.0142 g/mol
Sponsor Lot Number:	20130605
Purity:	98.9%
Storage Conditions:	Room Temperature (15-25°C)
Recertification Date:	19 April 2021

The Certificate of Analysis for the test substance is presented in Appendix 1.

Test Matrices

Control surface and ground water were sourced by Smithers ERS. The waters used were CS 14/18 and CS 01/20 Fountains Abbey surface water and CS 13/18 Borehole ground water.

Water characterisation data are listed in the following table:

Water Name	Unique ID	Water Type	Suspended Solids (mg/L)	Conductivity (µS/cm)	Hardness (mg/L $CaCO_3$)	pH	Dissolved Organic Carbon (mg/L)
Fountains Abbey[1]	CS 14/18	Surface	34	154	86	7.44	11.2
Fountains Abbey[2]	CS 01/20	Surface	5	140	132	7.51	8.53
Borehole	CS 13/18	Ground	2	436	349	8.0	0.00

[1] CS 14/18 was used for the initial and repeat matrix assessment.
[2] CS 01/20 was used for the final matrix assessment and surface water validation.

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

Reagents

- Acetonitrile — HPLC grade, Fisher
- Acetonitrile — HPLC grade, Honeywell
- Water — Milli-Q (with LCPAK polisher)
- Dichloromethane (DCM) — HPLC grade, Honeywell
- Acetone — HPLC grade, Honeywell
- Phosphoric acid — Reagent grade, Fisher
- 0.1% Formic acid in water — LC-MS grade, Honeywell
- 0.1% Formic acid in acetonitrile — LC-MS grade, Honeywell

Equipment

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.
- HPLC column: Phenomenex Kinetex 2.6 µm phenyl-hexyl 3 × 50 mm
- Analytical balance
- Separating funnels
- Round bottom flasks
- Glass tubes with screw caps
- Rotary evaporator
- Sample concentrator
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Analytical Method

Analytical method 12791.6319 was supplied by Smithers ERS, Wareham on behalf of the sponsor. The method was re-written in Smithers ERS, Harrogate format as draft method SMI 3202455-01D, including the instrumentation available at Smithers ERS, Harrogate. This was used for method validation, and re-issued as SMI 3202455-02V when the validation was complete. The complete analytical procedure is presented in Appendix 3.

Preparation of Reagents

Acetonitrile: water (20:80 v/v)

200 mL acetonitrile was mixed with 800 mL water.

Reagents were stored at room temperature and given a nominal expiry date of one month.

Preparation of Stock Solutions

Primary Stock Solutions

Primary stock solutions of Dicloran were prepared as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (µg/mL)[1]
Stock 1	10.18	98.9	Acetonitrile	10.068	1000
Stock 2	11.52			11.394	1000
Stock 3	10.23			10.118	1000
Stock 4	10.32			10.207	1000

[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.

Secondary Stock Solutions

Secondary stock solutions of Dicloran were prepared as described in the following table:

Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
1000	0.1	Acetonitrile	10	10

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

Sub-Stock Solutions

Sub-stock solutions of Dicloran were prepared as described in the following table:

Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)
10	0.1	Acetonitrile	1	1
10	0.1		10	0.1[1]

[1] Equivalent to 100 µg/L.

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

Preparation of Non-Matrix Matched Standards for Matrix Assessment

Non-matrix matched standards of Dicloran were prepared in acetonitrile: water (20:80 v/v) for comparison with matrix matched standards.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.1	Acetonitrile: water (20:80 v/v)	10	1
100	0.1		10	1
100	0.1		10	1

Preparation of Matrix Matched Standards for Matrix Assessment
Matrix matched standards of Dicloran were prepared in control water final extract.

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.1	Surface water final extract	10	1
100	0.1		10	1
100	0.1		10	1
100	0.1	Ground water final extract	10	1
100	0.1		10	1
100	0.1		10	1

The three matrix matched standards for each water were analysed with the three non-matrix matched standards and their peak areas compared.

Preparation of Calibration Standards
Matrix matched calibration standards of Dicloran were prepared for the initial validation of surface water as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.25	Surface water final extract	5	5
5	0.8		1	4
5	0.6		1	3
5	0.4		1	2
5	0.2		1	1
5	0.1		1	0.5

Non matrix-matched calibration standards of Dicloran were prepared for the ground water validations and repeat validation of surface water as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.5	Acetonitrile: water (20:80 v/v)	10	5
5	0.8		1	4
5	0.6		1	3
5	0.4		1	2
5	0.2		1	1
5	0.1		1	0.5

A single set of calibration standards was prepared for each validation batch, which was analysed twice during the batch, interspersed with the samples.

Sample Preparation and Fortification
100 mL of water was measured into a separating funnel. Quintuplicate water samples were fortified at the LOQ (0.1 µg/L) and at 10 × LOQ (1 µg/L) with stock solutions of Dicloran. Duplicate unfortified control water samples and a reagent blank were prepared for each validation batch (with the exception of the first ground water validation attempt, where the reagent blank was not prepared in error). Additional control samples were prepared for matrix matched standards and dilutions, as described in the following tables:

Fountains Abbey surface water (CS 14/18)

Sample ID	Sample Type	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Control A, B & C	Control matrix	100	N/A	N/A	N/A
Control G, H & I	Control matrix[1]	100	N/A	N/A	N/A

N/A = Not applicable.
[1] The matrix assessment was repeated because the first matrix assessment was inconclusive due to a poor instrument response.

Fountains Abbey surface water (CS 01/20)

Sample ID	Sample Type	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Reagent Blank A	Reagent blank[1]	100	N/A	N/A	N/A
Control O-Q	Control[2]	100	N/A	N/A	N/A
F0.1 F-J	LOQ	100	0.1	0.1	0.1
F1 F-J	10 × LOQ	100	1	0.1	1
Reagent Blank C	Reagent blank[1]	100	N/A	N/A	N/A
Control R-S	Control	100	N/A	N/A	N/A
F1 K-O	10 × LOQ[3]	100	1	0.1	1

N/A = Not applicable.
[1] Milli-Q water was used for the reagent blanks.
[2] Controls O-Q were used for the matrix assessment for the new batch of surface water and for the initial validation attempt.
[3] The validation was repeated at 10 × LOQ with a new set of controls (R-S) and a reagent blank (C) due to incorrect fortification on the first attempt.

Borehole ground water (CS 13/18)

Sample ID	Sample Type	Sample Volume (mL)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (µg/L)
Control D, E & F	Control matrix	100	N/A	N/A	N/A
Control J, K & L	Control matrix[1]	100	N/A	N/A	N/A
Control M-N	Control	100	N/A	N/A	N/A
F0.1 A-E	LOQ	100	0.1	0.1	0.1
F1 A-E	10 × LOQ	100	1	0.1	1
Reagent Blank D	Reagent blank[2]	100	N/A	N/A	N/A
Control T-U	Control	100	N/A	N/A	N/A
F0.1 K-O	LOQ[3]	100	0.1	0.1	0.1
F1 P-T	10 × LOQ[3]	100	1	0.1	1
Reagent Blank E	Reagent blank[2]	100	N/A	N/A	N/A
Control V-W	Control	100	N/A	N/A	N/A
F0.1 P-T	LOQ[4]	100	0.1	0.1	0.1
F1 U-Y	10 × LOQ[4]	100	1	0.1	1

N/A = Not applicable.
[1] The matrix assessment was repeated because the first matrix assessment was inconclusive due to a poor instrument response.
[2] Milli-Q water was used for the reagent blanks.
[3] The validation was repeated because no reagent blank was prepared in error with the first attempt.
[4] The validation was repeated again because an incorrect concentration of stock solution was used for the second attempt, resulting in no quantifiable data.

Sample Extraction

100 mL of water was measured into a glass separating funnel. 0.10 mL or 0.16 mL of phosphoric acid was added to the surface water (for CS 14/18 and CS 01/20 respectively) and 0.15 mL of phosphoric acid was added to the ground water and mixed, in order to achieve a pH of approximately 2 (the pH was checked using a control sample of each water with a pH paper).

The water was extracted twice with 100 mL DCM by shaking and the two DCM extracts were combined in a round bottom flask. The separating funnel was rinsed with 50 mL DCM and transferred to the round bottom flask containing the combined extracts. The extracts were evaporated to low volume (approximately 2 mL) using a rotary evaporator under vacuum set to < 35°C.

100 mL acetone was added to flask and evaporated to low volume (approximately 5 mL) to remove residual water. The 5 mL extract was transferred into a glass tube and the flask was rinsed using DCM followed by acetone which was also transferred to the glass tube.

The extract was evaporated to incipient dryness (approximately 100 µL) under a gentle stream of nitrogen at room temperature. The extract was reconstituted with 10 mL acetonitrile: water (20:80 v/v), then vortex-mixed for approximately 30 seconds and ultrasonicated for 5 minutes to dissolve the extract. The extract was further diluted into the calibration range using acetonitrile: water (20:80 v/v) or control extract for matrix matching.

The extraction and dilution procedure is outlined in the following tables:

Fountains Abbey surface water (CS 14/18)

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Extract Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Control A, B & C	Control matrix	N/A	100	10	N/A	0.1
Control G, H & I	Control matrix	N/A	100	10	N/A	0.1

N/A = Not applicable.

Fountains Abbey surface water (CS 01/20)

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Extract Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Reagent Blank A	Reagent blank	N/A	100	10	N/A	0.1
Control O-Q	Control	N/A	100	10	N/A	0.1
F0.1 F-J	LOQ	0.1	100	10	N/A	0.1
F1 F-J	10 × LOQ	1	100	10	0.3-1[1]	0.333
Reagent Blank C	Reagent blank	N/A	100	10	N/A	0.1
Control R-S	Control	N/A	100	10	N/A	0.1
F1 K-O	10 × LOQ	1	100	10	0.3-1	0.333

N/A = Not applicable.
[1] Sample dilutions were performed with control extract (matrix matched).

Borehole ground water (CS 13/18)

Sample ID	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Extract Volume (mL)	Sample Dilution (mL to mL)	Overall Dilution Factor
Control D, E & F	Control matrix	N/A	100	10	N/A	0.1
Control J, K & L	Control matrix	N/A	100	10	N/A	0.1
Control M-N	Control	N/A	100	10	N/A	0.1
F0.1 A-E	LOQ	0.1	100	10	N/A	0.1
F1 A-E	10 × LOQ	1	100	10	0.3-1	0.333
Reagent Blank D	Reagent blank	N/A	100	10	N/A	0.1
Control T-U	Control	N/A	100	10	N/A	0.1
F0.1 K-O	LOQ	0.1	100	10	N/A	0.1
F1 P-T	10 × LOQ	1	100	10	0.3-1	0.333
Reagent Blank E	Reagent blank	N/A	100	10	N/A	0.1
Control V-W	Control	N/A	100	10	N/A	0.1
F0.1 P-T	LOQ	0.1	100	10	N/A	0.1
F1 U-Y	10 × LOQ	1	100	10	0.3-1	0.333

N/A = Not applicable.

Instrument Conditions

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

LC Parameters:

Instrument:	Shimadzu Nexera series HPLC system		
Column#:	Phenomenex Kinetex 2.6 µm phenyl-hexyl 3 × 50 mm		
Mobile Phase A#:	0.1% Formic acid in water		
Mobile Phase B#:	0.1% Formic acid in acetonitrile		
Flow Rate:	0.5 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	80	20
	0.5	80	20
	3.0	0	100
	4.0	0	100
	4.1	80	20
	5.5	80	20
Run Time:	5.5 minutes		
Column Temperature:	40°C		
Autosampler Temperature:	4°C		
Injection Volume:	10 µL		
Retention Time:	Approx. 2.5 minutes		
Valco Valve Diverter:	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.5	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:	4500 V		
Collision Gas (CAD):	8		
Curtain Gas (CUR):	20		
Gas Flow 1 (GS1):	40		
Gas Flow 2 (GS2):	40		
Vaporiser Temperature (TEM):	550°C		
Interface Heater (ihe):	On		
Entrance Potential (EP):	10		
Declustering Potential (DP):	50		
Collision Exit Potential (CXP)	25		
Resolution Q1/Q3:	Unit/Unit		
Transition Name:	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Dicloran (Primary):	207/190	23	200
Dicloran (Confirmatory):	207/160	35	200

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

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

$$x = \frac{(y - c)}{m} \times DF$$

Where:

x = concentration of test substance in sample extract (µg/L)
y = peak area due to test substance
c = *y* intercept on calibration graph
m = gradient of the calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$Recovery\ (\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/L)
S = concentration added to fortified sample (µg/L)

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

The Method Detection Limit (MDL) based upon the sample concentration equivalent to the lowest calibration standard was calculated as follows:

MDL (µg/L) = lowest calibration standard (µg/L) × control sample dilution factor

Validation Pass Criteria
The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored for Dicloran:

Mean Recovery and Precision
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 110% and a %RSD (relative standard deviation) ≤ 20%.

Specificity/Selectivity
Specificity was acceptable if no significant interferences at the retention time of Dicloran were found in the control samples at > 30% of the LOQ or at > 50% of the MDL peak area response.

Linearity
The Linear range was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract concentration (after dilution). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

LOD (Limit of Detection) Assessment
An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions for Dicloran.

MDL (Method Detection Limit)
The MDL was calculated as the initial sample concentration equivalent to the lowest calibration standard (based upon a lowest standard concentration of 0.5 µg/L and a dilution factor of 0.1).

Matrix Assessment
An assessment of matrix effects was made by comparison of peak areas for triplicate standards prepared in acetonitrile: water (20:80 v/v) and in each control matrix final extract. This was assessed for Dicloran for both the primary and confirmatory transitions.

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

A difference of ≥ 20% was considered significant.

If matrix effects were determined to be significant, matrix matched calibration standards would be used for method validation.

REVISION HISTORY

SMI 3202455-01D	New method for independent laboratory validation based upon Smithers ERS, Wareham study 12791.6319.
SMI 3202455-01 V	Re-issued following method validation.
SMI 3202455-02V	Minor corrections to text.

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining concentrations of Dicloran in ground water and surface water by LC-MS/MS. Samples are acidified with phosphoric acid and then extracted twice with DCM. Extracts are concentrated then reconstituted with acetonitrile: water (20:80 v/v). The final extracts are quantified by LC-MS/MS.

Matrix effects for Dicloran in ground water and surface water will be determined by comparing peak areas of calibration standards prepared in control water final extract and in acetonitrile: water (20:80 v/v). Matrix effects are considered significant if the matrix matched standard area is ≥ 20% different from the non-matrix standard area. If matrix effects are significant then matrix matched calibration standards should be used.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector.
- HPLC column: Phenomenex Kintex 2.6 µm phenyl-hexyl 3 × 50 mm
- Analytical balance
- Separating funnels
- Round bottom flasks
- Glass tubes with screw caps
- Rotary evaporator
- Sample concentrator
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Equivalent equipment may be used if required

Materials

• Acetonitrile	HPLC grade, Honeywell
• Water	Milli-Q (with LCPAK polisher)
• Dichloromethane (DCM)	HPLC grade, Honeywell
• Acetone	HPLC grade, Honeywell
• Phosphoric acid	Reagent grade, Fisher
• 0.1% Formic acid in water	LC-MS grade, Honeywell
• 0.1% Formic acid in acetonitrile	LC-MS grade, Honeywell

Equivalent materials may be used if required

Reagents

Acetonitrile: water (20:80 v/v)

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

Reagent volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stock

Prepare duplicate stock solutions of Dicloran at 1000 µg/mL in acetonitrile. Accurately weigh ≥ 10 mg test substance, corrected for purity and transfer into a 10 mL volumetric flask. Adjust the volume to give exactly 1000 µg/mL. Transfer into an amber glass bottle. The primary stocks should be stored refrigerated and given a nominal expiry date of 3 months.

Standard Correlation

Dilute the duplicate primary stocks to the mid-point of the calibration line. Correlate the standard solutions by injecting each of the two calibration standards 5 times into the LC-MS/MS. Ensure that the two solutions are injected alternately in the run

sequence. The results for the correlation should be ± 5% of the overall mean calculated by peak areas.

Review of Results
Review the data and document the correlation calculations. If the correlation is out of specification, either repeat the injections, re-dilute, or prepare two new stock standards and repeat the procedures in sections *<<Primary Standard Stock>>* to *<<Review of Results>>*.

If the acceptance criteria from section *<<Standard Correlation>>* have been met, then the calibration solutions are acceptable for use. If required, fortification solutions for method validation will be made from the same stock standard, or its dilutions, from which the calibration line has been prepared.

Secondary Standard Stocks
Prepare secondary stock solutions of Dicloran in acetonitrile. The following dilution scheme is suggested:

Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
1000	0.1	Acetonitrile	10	10

Transfer into amber glass bottles. The secondary stocks should be stored refrigerated and given a nominal expiry date of 1 month.

Sub-Stocks
Prepare mixed sub-stock solutions of Dicloran in acetonitrile. The following dilution scheme is suggested:

Secondary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Sub-Stock Concentration (µg/mL)
10	1	Acetonitrile	10	1
1	1		10	0.1
0.1	1		10	0.01[1]

[1] Equivalent to 10 µg/L.

Transfer into disposable glass vials. The sub-stock solutions should be prepared on the day of use.

Matrix Matched Standards for Matrix Assessment
Prepare surface water or ground water matrix matched standards of Dicloran in control ground water or surface water final extract. The following dilution scheme is suggested:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.1	Control water final extract	10	1
100	0.1		10	1
100	0.1		10	1

Non-Matrix Matched Standards for Matrix Assessment

Prepare mixed non-matrix matched standards of Dicloran in acetonitrile: water (20:80 v/v). The following dilution scheme is suggested:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.1	Acetonitrile: water (20:80 v/v)	10	1
100	0.1		10	1
100	0.1		10	1

Calibration Standards

Prepare calibration standards of Dicloran acetonitrile: water (20:80 v/v). The following dilution scheme is suggested:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
100	0.5	Acetonitrile: water (20:80 v/v)[1]	10	5
5	0.8		1	4
5	0.6		1	3
5	0.4		1	2
5	0.2		1	1
5	0.1		1	0.5

[1] If matrix matched calibration standards are required, use ground water or surface water final extract as the solvent.

A single set of calibration standards should be prepared for each validation batch and injected twice, interspersed with and bracketing the samples.

PROCEDURES

All procedures will be carried out in compliance with departmental SOPs, following departmental safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]

Measure 100 mL of either ground water or surface water into a separating funnel. Fortify samples with Dicloran standard in acetonitrile. The following fortification scheme is suggested:

Number of Replicates	Sample Type	Stock Concentration (mg/L)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
1	Reagent blank[1]	N/A	N/A	100	N/A
2	Control[2]	N/A	N/A	100	N/A
5	LOQ	0.1	0.1	100	0.1
5	10 × LOQ	1	0.1	100	1

N/A = Not Applicable.

[1] Use Milli-Q water for the reagent blank.

[2] Prepare additional controls for matrix matched standards and final dilution as required.

Sample Extraction [1b, 4a]

1. Measure 100 mL of water into a separating funnel.
2. Bring to approximately pH 2 with phosphoric acid, checking with pH paper.
3. Add 100 mL DCM and shake well. Allow to settle.
4. Transfer the bottom layer into a round bottom flask.
5. Repeat steps 3 and 4, combining the two extracts. Discard the water.
6. Rinse the separating funnel with 50 mL DCM and transfer to the round bottom flask.
7. Evaporate to low volume (approximately 2 mL) using a rotary evaporator under vacuum with gentle heating (< 35°C).
8. Add 100 mL acetone to the flask and evaporate to low volume (approximately 5 mL) to remove residual water.
9. Transfer to a glass tube, rinsing the flask into the tube with DCM followed by acetone.
10. Evaporate to incipient dryness (approximately 100 µL) under a gentle stream of nitrogen at room temperature. Do not allow to dry completely.
11. Reconstitute with 10 mL acetonitrile: water (20:80 v/v).
12. Vortex mix for approximately 30 seconds and ultrasonicate for approximately 5 minutes to dissolve the extract.
13. Further dilute into calibration range, if required, with acetonitrile: water (20:80 v/v), or control extract if matrix matching. The suggested dilution scheme is given in the following table.
14. Transfer into an HPLC vial for analysis.

Sample type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank	N/A	100	10	N/A	0.1
Control[1]	N/A	100	10	N/A	0.1
LOQ	0.1	100	10	N/A	0.1
10 × LOQ	1	100	10	0.3-1	0.333

N/A = Not Applicable.

LC-MS/MS CONDITIONS

HPLC Parameters:

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

LC Parameters:

Instrument:	Shimadzu Nexera series HPLC system		
Column#:	Phenomenex Kinetex 2.6 µm phenyl-hexyl 3 × 50 mm		
Mobile Phase A#:	0.1% Formic acid in water		
Mobile Phase B#:	0.1% Formic acid in acetonitrile		
Flow Rate:	0.5 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	80	20
	0.5	80	20
	3.0	0	100
	4.0	0	100
	4.1	80	20
	5.5	80	20
Run Time:	5.5 minutes		
Column Temperature:	40°C		
Autosampler Temperature:	4°C		
Injection Volume:	10 µL		
Retention Time:	Approx. 2.5 minutes		
Valco Valve Diverter:	Time (min)	Position	
	0	A (to waste)	
	0.5	B (to MS)	
	5.5	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:	4500 V		
Collision Gas (CAD):	8		
Curtain Gas (CUR):	20		
Gas Flow 1 (GS1):	40		
Gas Flow 2 (GS2):	40		
Vaporiser Temperature (TEM):	550°C		
Interface Heater (ihe):	On		
Entrance Potential (EP):	10		
Declustering Potential (DP):	50		
Collision Exit Potential (CXP)	25		
Resolution Q1/Q3:	Unit/Unit		
Transition Name:	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Dicloran (Primary):	207/190	23	200
Dicloran (Confirmatory):	207/160	35	200

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

CALCULATION OF RESULTS

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where the calibration fit is linear as in this study, Analyst uses the following formula to calculate the concentration of test substance present in the sample extract.

$$x = \frac{(y - c)}{m} \times DF$$

Where:-

x = concentration of test substance in sample (µg/L)
y = area of peak due to test substance
m = gradient
c = Y intercept on calibration graph
DF = sample dilution factor

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/L)
S = concentration added to fortified sample (µg/L)

METHOD CRITERIA

For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.995 with a 1/x weighting.
- All sample extracts should be within the appropriate range of calibration standards.
- Mean recovery from fortified samples should be within the range of 70 to 110% at each concentration.
- Precision of fortified sample recoveries should be ≤ 20% RSD at each concentration.
- The control sample should not contain interference > 30% of the LOQ at the retention time of the test substance.

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main	Division	CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shield to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON – ensure antidote is available and is within its expiry date (must specify details)