2. INTRODUCTION

The purpose of this study was to independently validate the Smithers (Wareham, USA) analytical method 12791.6405 (Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Spirodiclofen and Spirodiclofen Enol in Aqueous Matrices by LC-MS/MS) in ground and surface water in accordance with the EPA OCSPP 850.6100 (2012), EPA OPPTS 860.1340 (1996) and SANTE/2020/12830 rev.1 (2021) guidelines.

The analytical method 12791.6405 was provided by the Sponsor. The method was re-written in Smithers ERS Limited, Harrogate format to include details of the equivalent equipment and reagents used. The independently validated method was then issued as SMV 3202885-01V.

Five control samples each of ground and surface water were fortified with Spirodiclofen and Spirodiclofen-enol at both 0.1 µg/L (LOQ) and 1 µg/L (10xLOQ) and analysed. An aliquot of 0.11% formic acid in acetonitrile:Milli-Q water (56:44 v/v) was added to each sample to give a final composition of 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v). The 10xLOQ samples were diluted 1:1 v/v with 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v).

Samples were analysed for Spirodiclofen and Spirodiclofen-enol using Liquid Chromatography with tandem Mass Spectrometry detection (LC-MS/MS).

Assay specificity, linearity, assay limit of detection (LOD), assay limit of quantitation (LOQ), accuracy and precision, and matrix effects of the method were determined. Precision and accuracy were calculated at each validation level in each water for Spirodiclofen and Spirodiclofen-enol. One primary and one confirmatory LC-MS/MS transition were analysed for Spirodiclofen and Spirodiclofen-enol.

To assess matrix effects of both water types, standards were prepared using both final matrix extracts and non-matrix standards and the peak areas were compared.

3. STUDY TIMETABLE

Study initiation:	12 April 2021 (date the protocol was signed by the Study Director).
Experimental start:	19 April 2021 (the date of the first recording of study specific experimental raw data).
Experimental completion:	17 May 2021 (the date of the last recording of study specific experimental raw data).
Study completion:	Date the final report was signed by the Study Director.

4. MATERIALS AND METHODS

4.1. Protocol Adherence

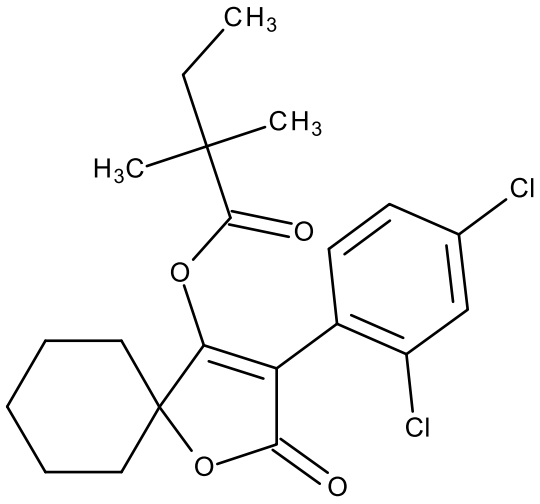
The study was conducted in accordance with the protocol with no amendments and one deviations.

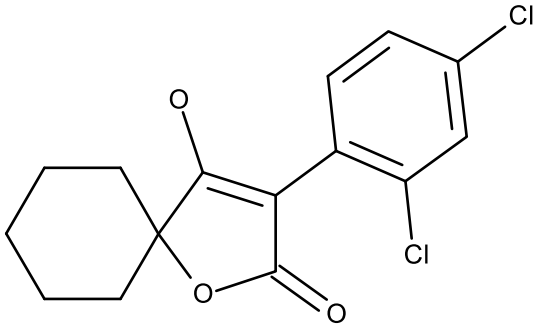
4.1.1. Protocol Deviation Details

The protocol stated that the confirmatory transition for Spirodiclofen would be 411.3/71.1. During the course of the study it was determined that there was an interference peak at this transition, likely caused by laboratory solvents, so a new one was employed (411.3/295.1) to avoid the interference peak. There is no impact to the study because the transition used mitigated the effects of the interference peak, and the method was validated successfully in both waters tested.

4.2. Test Substances

The information below is presented as detailed in the test substance information provided by the supplier.

Test Substance Name:	**Spirodiclofen**
IUPAC Name:	[3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl] 2,2-dimethylbutanoate
CAS Number:	148477-71-8
Structure:	
Molecular Formula:	$C_{21}H_{24}Cl_2O_4$
Molecular Weight:	411.32 g/mol
Lot Number:	BCCB8700
Purity:	99.2%
Storage Conditions:	Room temperature (15-25°C)
Expiry Date:	31 July 2024

Test Substance Name:	**Spirodiclofen-enol**
IUPAC Name:	3-(2,4-Dichlorophenyl)-4-hydroxy-1-oxaspiro[4.5]dec-3-en-2-one
CAS Number:	148476-22-6
Structure:	
Molecular Formula:	$C_{15}H_{14}Cl_2O_3$
Molecular Weight:	313.18 g/mol
Lot Number:	0218200501
Purity:	99.9%
Storage Conditions:	Frozen (nominal -20°C)
Expiry Date:	24 June 2027

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

4.3. Test Matrices

Control samples of ground and surface water were sourced by Smithers ERS Limited. The unique identification numbers and water characterisation data of the ground water and the surface water are listed in the following table:

Water Type	**Ground Water**	**Surface Water**
Test System Name	Borehole water	Fountains Abbey
Unique ID	CS38/20	CS06/21
Dissolved Organic Carbon (DOC, mg/L)	3.68	9.49
Suspended Solids (mg/L)	1	8
Hardness (mg/L $CaCO_3$)	312	69
Electrical Conductivity (µS/cm)	631	153
pH	8.4	7.53

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

4.4. Reagents

- Acetonitrile — HPLC grade, Honeywell
- Methanol — HPLC grade, Honeywell
- Formic acid — ≥ 98%, Honeywell
- Water — Milli-Q (with LCPAK polisher)
- 0.1% formic acid in water — LC-MS grade, Honeywell
- 0.1% formic acid in acetonitrile — LC-MS grade, Honeywell

Equivalent or better grade materials may be used if required.

4.5. Equipment

- Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector
- Phenomenex Kinetex Biphenyl, 2.6 µm, 3×50 mm
- Analytical balance
- Polypropylene centrifuge tubes
- Polypropylene bottles
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- HPLC vials
- Glass scintillation vials
- Various disposable laboratory glassware

4.6. Analytical Method

4.6.1. Preparation of Reagents

4.6.1.1. 0.1% Formic Acid in Acetonitrile:Milli-Q Water (50:50 v/v)
Mix 500 mL HPLC grade acetonitrile with 500 mL Milli-Q water and 1 mL formic acid.

4.6.1.2. 0.11% Formic Acid in Acetonitrile:Milli-Q Water (56:44 v/v)
Mix 560 mL HPLC grade acetonitrile with 440 mL Milli-Q water and 1.1 mL formic acid.

4.6.1.3. 0.1% Formic Acid in Acetonitrile:Ground Water:Milli-Q Water (50:10:40 v/v)
Mix 50 mL HPLC grade acetonitrile with 10 mL of ground water, 40 mL Milli-Q water and 0.1 mL formic acid.

4.6.1.4. 0.1% Formic Acid in Acetonitrile:Surface Water:Milli-Q Water (50:10:40 v/v)
Mix 50 mL HPLC grade acetonitrile with 10 mL of surface water, 40 mL Milli-Q water and 0.1 mL formic acid.

4.6.1.5. Acetonitrile:Milli-Q Water (90:10 v/v)
Mix 90 mL HPLC grade acetonitrile with 10 mL Milli-Q water.

4.6.1.6. Acetonitrile/Methanol/Milli-Q water (30:30:40 v/v/v)
Mix 300 mL LC/MS grade acetonitrile, 300 mL LC/MS grade methanol and 400 mL Milli-Q water.

Reagents were stored at room temperature and volumes were scaled as appropriate.

4.6.2. Preparation of Stock Solutions

4.6.2.1. Primary Stock Solutions

Primary stock solutions of Spirodiclofen were prepared in acetonitrile and primary stock solutions of Spirodiclofen-enol were prepared in acetonitrile:Milli-Q water (90:10 v/v) as described in the following table:

Test Substance	Stock ID	Amount Weighed (mg)	Purity (%)	Final Volume (mL)	Primary Stock Concentration (µg/mL)
Spirodiclofen	Stock A	10.28	99.2	10.198	1000
	Stock B	10.30		10.218	
Spirodiclofen-enol	Stock A	10.05	99.9	10.040	1000
	Stock B	10.34		10.330	

Primary stock solutions were stored refrigerated (2-8°C) in amber glass bottles and given a nominal expiry of one month.

4.6.2.2. Secondary Standard Stocks

Secondary stock solutions of Spirodiclofen and Spirodiclofen-enol were prepared in acetonitrile as described in the following table:

Test Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
Spirodiclofen	1000	0.5	Acetonitrile	50	10
Spirodiclofen-enol	1000	0.5		50	10

Secondary standard stocks were stored refrigerated (2-8°C) in amber glass bottles and given a nominal expiry of one month.

4.6.2.3. Calibration Sub-Stocks and Fortification Standards

Using the secondary standards prepared from the “A” stocks of Spirodiclofen and Spirodiclofen-enol, one set of mixed standard solutions was prepared to be used for sample fortification purposes and using the secondary standards prepared from the “B” stocks of Spirodiclofen and Spirodiclofen-enol, one set of mixed standard solutions was prepared to be used for the preparation of the calibration lines and for matrix assessment.

The calibration sub-stock standards and fortification standards from primary and secondary stocks were prepared in acetonitrile as described in the following table:

Test Substance	Standard Concentration (µg/mL)	Volume Taken (mL)	Final Volume (mL)	Mixed Standard Concentration (µg/mL)
Spirodiclofen	10	0.1	10	0.1[1,3]
Spirodiclofen-enol	10	0.1		
Mixed	0.1	1	10	0.01[2,4,5]
Mixed	0.01	1	10	0.001[4,6]

[1]Prepared from secondary stock (from Stock A) for fortification of high level recovery samples and for further dilutions.
[2](From mixed standard prepared from Stock A secondary stock) used for fortification of LOQ recovery samples.
[3]Prepared from secondary stock (from Stock B) for further dilutions.
[4](From mixed standard prepared from Stock B secondary stock) used for calibration standards and matrix effects assessment.
[5]0.01 µg/mL is equivalent to 10 ng/mL
[6]0.001 µg/mL is equivalent to 1 ng/mL

Fortification and calibration sub-stock standards were prepared fresh for each assay in disposable glass scintillation vials.

4.6.2.4. Preparation of Calibration Standards

Calibration solutions of Spirodiclofen and Spirodiclofen-enol were prepared in 0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v) in HPLC autosampler vials as described in the following table:

Sub-Stock Concentration (ng/mL)	Volume Taken (mL)	Volume of Solvent (mL)	Final Volume (mL)	Calibration Standard Concentration (ng/mL)
10	0.02	0.98	1	0.2
10	0.01	0.99	1	0.1
1	0.05	0.95	1	0.05
1	0.03	0.97	1	0.03
1	0.02	0.98	1	0.02
1	0.01	0.99	1	0.01
0.1	0.05	0.95	1	0.005
0.1	0.03	0.97	1	0.003
0.1	0.02	0.98	1	0.002

Calibration standards were prepared freshly from the calibration sub-stock standards on the day of analysis in 1.5 mL glass HPLC vials.

4.6.2.5. Preparation of Matrix Assessment Standards

Triplicate matrix-matched standards of Spirodiclofen and Spirodiclofen-enol were prepared in HPLC autosampler vials by fortifying 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v) (ground water and surface water control final extract) with the 10 ng/mL mixed solution to produce matrix-matched standards at 0.01 ng/mL. Non-matrix matched standards were prepared in the same way in triplicate using 0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v) for the matrix assessment. The peak areas of the matrix-matched and non-matrix matched standards were compared and used to calculate matrix effects.

The matrix assessment standards were prepared as described in the following table:

Mixed Standard Concentration (ng/mL)	Volume Taken (mL)	Dilution Solvent	Final Volume (mL)	Standard Concentration (ng/mL)
10	0.02	0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v) (Control matrix final extract)	20	0.01
10	0.02		20	0.01
10	0.02		20	0.01
10	0.02	0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)	20	0.01
10	0.02		20	0.01
10	0.02		20	0.01

These standards were prepared in disposable glass scintillation vials.

4.6.3. Sample Preparation and Fortification

5 mL (10 mL for control samples) of each water type was measured into either 50 mL polypropylene centrifuge tubes (PPCT) or 125 mL polypropylene bottles, as appropriate. Five control samples each of ground and surface water were fortified with Spirodiclofen and Spirodiclofen-enol at both 0.1 µg/L (LOQ) and 1 µg/L (10xLOQ) with the appropriate solution of Spirodiclofen and Spirodiclofen-enol. Two control water samples and a reagent blank sample were also prepared per water type, as described in the following table:

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
1	Reagent Blank	N/A	N/A	N/A	N/A
2	Control	N/A	N/A	10[1]	N/A
5	LOQ	0.01	0.05	5	0.1
5	10×LOQ	0.1	0.05	5	1.0

N/A = Not applicable.
[1]Volume increased for use in matrix effects assessment.

4.6.4. Sample Extraction

45 mL (or 90 mL for control samples) of 0.11% formic acid in acetonitrile:Milli-Q water (56:44 v/v) was added to each sample to give a final composition of 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v).

The 10xLOQ samples were further diluted 1:1 v/v with 0.1% formic acid in acetonitrile:control test matrix:Milli-Q water (50:10:40 v/v/v).

Samples were transferred to HPLC vials for LC-MS/MS analysis.

Full details of sample extraction are presented in the analytical method SMV 3202885-01V in Appendix 3.

The method is summarised in the following table:

Sample ID	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Extract Volume (mL)	Further Dilution	Dilution Factor
Reagent Blank	N/A	5	50	N/A	10
Control A+B	N/A	10	100	N/A	10
F0.1 A-E	0.1	5	50	N/A	10
F1 A-E	1.0	5	50	1:1	20

N/A = Not applicable.

4.6.5. Instrument Conditions

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

LC Parameters:

Instrument:	Shimadzu Nexera series HPLC system
Column#:	Phenomenex Kinetex Biphenyl, 2.6 µm, 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 (%)
	0.5	95	5
	0.6	50	50
	4.0	0	100
	5.0	0	100
	5.1	95	5
	6.0	95	5

Run Time:	6 minutes
Autosampler Wash Solvent:	Acetonitrile/methanol/water (30:30:40 v/v/v)
Column Temperature:	40°C
Autosampler Temperature:	10°C
Injection Volume:	50 µL
Retention Time:	Spirodiclofen – approximately 2.8 minutes Spirodiclofen-enol – approximately 1.6 minutes

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

MS/MS Parameters:

Instrument:	AB Sciex API 5000 mass spectrometer
Ionisation Type#:	Electrospray (ESI)
Polarity#:	Positive
Ion Spray Voltage:	5500 V
Scan Type#:	Multiple reaction monitoring (MRM)
Source Temperature:	400°C
Curtain Gas:	20.0
Ion Source – Gas 1/Gas 2:	70.0/70.0
Collision Gas:	6.0
Collision Cell Entrance Potential:	10.0
Collision Cell Exit Potential:	10.0
Declustering Potential:	50.0
Resolution (Q1/Q3):	Unit/Unit

Test Substance	**Spirodiclofen**		**Spirodiclofen-enol**	
Instrument Parameter	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	411.3/313.0	411.3/295.1*	313.2/212.9	313.2/184.8
Collision Energy:	18.0	35.0*	35.0	43.0
Dwell Time:	150	150	200	200

*The confirmatory transition for Spirodiclofen in study 12791.6405 was 411.3/71.1 with a collision energy of 27.0. The transition was updated for this study in order to mitigate the effects produced by an interference peak from the laboratory solvents. The collision energy was optimised for the new transition but no other parameters required modification.

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

4.6.6. Calculation of Results

When the calibration fit is linear as in this study, Analyst 1.6.2 uses the following formula to calculate the concentration of test substance present in the sample:

$$\text{Amount } (\mu g/L) = \left[\frac{y - c}{m}\right] \times \left[\frac{fv \times sd}{sv}\right]$$

Where:
y = peak area
c = Y intercept on calibration graph
m = slope of the linear calibration graph
sv = sample volume equivalent in the final extract (mL)
fv = final volume of the extract (mL)
sd = additional sample dilution of the final extract (if required)

The dilution factor in this case was 10 (prior to any further dilutions) or 20 (10xLOQ samples only with a 1:1 v/v further dilution).

Procedural recovery data from fortified samples were 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)

The 95% confidence intervals were calculated as follows:

95% confidence intervals (±) = $t_{n-1}s/\sqrt{n}$

Where:
t_{n-1} = 2.78 (for n=5)
t_{n-1} = 3.18 (for n=4)
s = standard deviation
n = number of samples (5 or 4)

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 sample dilution factor × lowest calibration standard concentration (ng/mL) / 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 concentration (ng/mL) × control sample dilution factor.

Matrix effects (%) were calculated as follows:

Matrix effects (%) = ((mean peak area with matrix - mean peak area with solvent) / mean peak area with solvent) x 100.

4.6.7. Validation Pass Criteria

The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored for Spirodiclofen and Spirodiclofen-enol:

4.6.7.1. 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%.

4.6.7.2. Specificity/Selectivity

Specificity was acceptable if interferences at the retention time of Spirodiclofen and Spirodiclofen-enol were found in the control samples at ≤ 30% of the LOQ peak area response.

4.6.7.3. Linearity
The linear range was acceptable if the lowest calibration standard concentration was ≤ 30% of the equivalent LOQ final extract concentration and if the highest calibration standard concentration was ≥ 120% of the 10×LOQ final extract concentration.

The suitability of the calibration line was demonstrated by a residual plot of the regression residuals (d_i):

$d_i = y_i - y_{yi}$

Where:
y_i = the measured value i.
y_{yi} = the estimated value which corresponds to yi and is derived from the calibration function.

The residual plot was visually inspected to decide if the regression residuals were randomly distributed, indicating linear calibration. If a trend was observed in the residuals, the calibration was not acceptable.

4.6.7.4. Limit of Detection (LOD) and Method Detection Limit (MDL) Assessments
An estimate of the LOD was made at 3 × baseline noise for primary and confirmatory transitions for Spirodiclofen and Spirodiclofen-enol.

The MDL was calculated as the initial sample concentration equivalent to the lowest calibration standard (based upon the lowest standard concentration of 0.002 ng/mL and a dilution factor of 10).

Note that in SANTE/2020/12830 rev.1 (2021), the LOD, rather than the MDL (as in this report), is expressed as the lowest calibration standard.

4.6.7.5. Matrix Assessment
An assessment of matrix effects was made by comparison of the peak area response of standards prepared using final matrix extracts against non-matrix standards.
This applied to the primary and confirmatory transitions.

Results were presented as a % difference from the mean non-matrix standard value and a difference of ≥ 20% was considered significant.

REVISION HISTORY

SMV 3202885-01V	Update after validation. Minor typographical corrections. Use of disposable glassware for fortification and calibration sub-stocks added. Extra calibration standard added.
SMV 3202885-02D	Update to wording of Reagents section and update to new transition for Spirodiclofen (confirmatory).
SMV 3202885-01D	New method for independent laboratory validation based upon the Smithers (Wareham, USA) analytical method in study 12791.6405 (Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Spirodiclofen and Spirodiclofen Enol in Aqueous Matrices by LC-MS/MS).

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 Spirodiclofen and Spirodiclofen-enol in ground water and surface water by LC-MS/MS.

Samples are fortified with Spirodiclofen and Spirodiclofen-enol before being subjected to the prescribed method and quantified by LC-MS/MS.

Matrix effects for Spirodiclofen and Spirodiclofen-enol in ground water and surface water will be determined by comparing peak areas of calibration standards prepared in control final extract and in the relevant final extract solvent. Matrix effects are considered significant if the matrix-matched standard area is $\geq$ 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
- Phenomenex Kinetex Biphenyl, 2.6 µm, 3×50 mm
- Analytical balance
- Polypropylene centrifuge tubes
- Polypropylene bottles
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- HPLC vials
- Various disposable laboratory glassware

With the exception of the HPLC column, equivalent equipment may be used if required.

Materials

• Acetonitrile	HPLC grade, Honeywell
• Methanol	HPLC grade, Honeywell
• Formic acid	≥ 98%, Honeywell
• Water	Milli-Q (with LCPAK polisher)
• 0.1% formic acid in water	LC-MS grade, Honeywell
• 0.1% formic acid in acetonitrile	LC-MS grade, Honeywell

Equivalent or better grade materials may be used if required.

Reagents

0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)
Mix 500 mL HPLC grade acetonitrile, 500 mL Milli-Q water and 1 mL formic acid.

0.11% formic acid in acetonitrile:Milli-Q water (56:44 v/v)
Mix 560 mL HPLC grade acetonitrile, 440 mL Milli-Q water and 1.1 mL formic acid.

0.1% formic acid in acetonitrile:ground water:Milli-Q water (50:10:40 v/v)
Mix 50 mL HPLC grade acetonitrile, 10 mL of ground water, 40 mL Milli-Q water and 0.1 mL formic acid.

0.1% formic acid in acetonitrile:surface water:Milli-Q water (50:10:40 v/v)
Mix 50 mL HPLC grade acetonitrile, 10 mL of surface water, 40 mL Milli-Q water and 0.1 mL formic acid.

Acetonitrile:Milli-Q water (90:10 v/v)
Mix 90 mL HPLC grade acetonitrile with 10 mL Milli-Q water.

Acetonitrile/methanol/Milli-Q water (30:30:40 v/v/v)
Mix 300 mL LC/MS grade acetonitrile, 300 mL LC/MS grade methanol and 400 mL Milli-Q water.

Reagent volumes may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]

Primary Standard Stocks

Prepare duplicate stock solutions (stocks A and B) of Spirodiclofen and Spirodiclofen-enol, separately. Accurately weigh ≥ the required amount of each test substance, corrected for purity and transfer into an appropriately sized volumetric flask. Adjust the volume to give the exact concentration required. The following dilution scheme is suggested:

Test Substance	Target Weight (mg)	Solvent	Final Volume (mL)	Primary Stock Concentration (µg/mL)
Spirodiclofen	10	Acetonitrile	10	1000
Spirodiclofen-enol	10	Acetonitrile:Milli-Q water (90:10 v/v)	10	1000

Transfer into amber glass bottles with PTFE-lined caps. One stock is for preparing calibration standards and the other is for preparing quality control (QC) standards. Should further primary stocks be required, these should also be prepared in duplicate (stocks C and D) where one is for preparing calibration standards and the other is for preparing quality control (QC) standards.

The primary stocks of Spirodiclofen and Spirodiclofen-enol should be stored refrigerated (2-8°C) and given a nominal expiry date of 1 month.

Secondary Standard Stocks

Prepare secondary stock solutions from the duplicate primary stocks of Spirodiclofen and Spirodiclofen-enol, separately, at 10 µg/mL in acetonitrile. The following dilution scheme is suggested:

Test Substance	Primary Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Primary Stock Concentration (µg/mL)
Spirodiclofen	1000	0.5	Acetonitrile	50	10
Spirodiclofen-enol	1000	0.5		50	10

Transfer into amber glass bottles with PTFE-lined caps. The secondary stocks should be stored refrigerated (2-8°C) and given a nominal expiry date of 1 month.

Calibration Sub-Stocks and Fortification Standards

Using the secondary standards prepared from the "A" stocks of Spirodiclofen and Spirodiclofen-enol, prepare one set of mixed standard solutions to be used for sample fortification purposes and using the secondary standards prepared from the "B" stocks of Spirodiclofen and Spirodiclofen-enol, prepare one set of mixed standard solutions to be used for the preparation of the calibration lines and for matrix assessment.

The following dilution scheme is suggested for the preparation of calibration sub-stock standards and fortification standards from primary and secondary stocks:

Test Substance	Standard Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Mixed Standard Concentration (µg/mL)
Spirodiclofen	10	0.1	Acetonitrile	10	0.1[1,3]
Spirodiclofen-enol	10	0.1			
Mixed	0.1	1		10	0.01[2,4,5]
Mixed	0.01	1		10	0.001[4,6]

[1]Prepare from secondary stock (from Stock A) for fortification of high level recovery samples and for further dilutions.
[2](From mixed standard prepared from Stock A secondary stock) use for fortification of LOQ recovery samples.
[3]Prepare from secondary stock (from Stock B) for further dilutions.
[4](From mixed standard prepared from Stock B secondary stock) use for calibration standards and matrix effects assessment.
[5]0.01 µg/mL is equivalent to 10 ng/mL
[6]0.001 µg/mL is equivalent to 1 ng/mL

Fortification and calibration sub-stock standards should be prepared fresh for each assay in disposable glass scintillation vials.

Calibration Standards

Prepare calibration solutions of Spirodiclofen and Spirodiclofen-enol in 0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v) in HPLC autosampler vials.
The following dilution scheme is suggested:

Sub-Stock Concentration (ng/mL)	Volume Taken (mL)	Volume of Solvent (mL)	Final Volume (mL)	Calibration Standard Concentration (ng/mL)
10	0.02	0.98	1	0.2
10	0.01	0.99	1	0.1
1	0.05	0.95	1	0.05
1	0.03	0.97	1	0.03
1	0.02	0.98	1	0.02
1	0.01	0.99	1	0.01
0.1	0.05	0.95	1	0.005
0.1	0.03	0.97	1	0.003
0.1	0.02	0.98	1	0.002

Matrix-Matched Standards for Matrix Assessment

Prepare triplicate matrix-matched standards of Spirodiclofen and Spirodiclofen-enol in HPLC autosampler vials by fortifying 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v) ground water and surface water control final extract with the 10 ng/mL mixed solution to produce matrix-matched standards at 0.01 ng/mL. Non-matrix matched standards will be prepared in the same way in triplicate using 0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v) for the matrix assessment. The peak areas of the matrix-matched and non-matrix matched standards will be compared and used to calculate matrix effects.

The following dilution scheme is suggested for preparation of the standards for matrix assessment:

Mixed Standard Concentration (ng/mL)	Volume Taken (mL)	Dilution Solvent	Final Volume (mL)	Calibration Standard Concentration (ng/mL)
10	0.02	0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v) Control matrix final extract	20	0.01
10	0.02		20	0.01
10	0.02		20	0.01
10	0.02	0.1% formic acid in acetonitrile:Milli-Q water (50:50 v/v)	20	0.01
10	0.02		20	0.01
10	0.02		20	0.01

PROCEDURES

All procedures will be carried out in compliance with local SOPs, following local 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 5 mL of control ground water or surface water into 50 mL polypropylene centrifuge tubes (PPCT) or 125 mL polypropylene bottles, as appropriate (note that 10 mL is required for the control samples). Fortify samples with the appropriate Spirodiclofen and Spirodiclofen-enol standards. The following fortification scheme is suggested:

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Volume (mL)	Fortified Concentration (µg/L)
1	Reagent Blank	N/A	N/A	5	N/A
2	Control	N/A	N/A	10[1]	N/A
5	LOQ	0.01	0.05	5	0.1
5	10xLOQ	0.1	0.05	5	1

N/A = Not Applicable.
[1]Volume increased for use in matrix effects assessment.

Sample Extraction [1b, 4a]

1. Measure 5 mL (or 10 mL for control samples) of control ground water or surface water into 50 mL polypropylene centrifuge tubes (PPCT) or 125 mL polypropylene bottles, as appropriate.
2. Fortify samples as described in the table above.
3. Add 45 mL (or 90 mL for control samples) of 0.11% formic acid in acetonitrile:Milli-Q water (56:44 v/v) to give a final composition of 0.1% formic acid in acetonitrile:test matrix:Milli-Q water (50:10:40 v/v/v).
4. Dilute the 10xLOQ samples 1:1 v/v with 0.1% formic acid in acetonitrile:control test matrix:Milli-Q water (50:10:40 v/v/v).
5. Transfer into HPLC vials for LC-MS/MS analysis.

Dilution Factor Calculation Summary

The following calculations are required to calculate the software dilution factor:

$$Software\ Dilution\ Factor\ = \frac{Final\ Extract\ Volume\ (mL) \times Sample\ Dilution}{Sample\ Amount\ (g\ or\ mL)} \times Unit\ Conversions$$

Where (for samples which have no further dilutions performed):

Sample amount = 5 mL (or 10 mL for control samples)
Final extract volume = 50 mL (or 100 mL for control samples)
Sample dilution = 1 (no further dilution)
Unit conversion = 1 (no unit conversion)

Software dilution factor = 50/5 (or 100/10 for control samples) = 10.

Where (for 10xLOQ samples):

Sample amount = 5 mL
Final extract volume = 50 mL
Sample dilution = 2 (1:1 v/v dilution)
Unit conversion = 1 (no unit conversion)

Software dilution factor = (50x2)/5 = 20.

LC-MS/MS CONDITIONS

HPLC Parameters:

Instrument:	Shimadzu Nexera series HPLC system
Column#:	Phenomenex Kinetex Biphenyl, 2.6 µm, 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 (%)
0.5	95	5
0.6	50	50
4.0	0	100
5.0	0	100
5.1	95	5
6.0	95	5

Run Time:	6 minutes
Autosampler Wash Solvent:	Acetonitrile/methanol/water (30:30:40 v/v/v)
Column Temperature:	40°C
Autosampler Temperature:	10°C
Injection Volume:	50 µL
Retention Time:	Spirodiclofen – approximately 3.6 minutes Spirodiclofen-enol – approximately 2.5 minutes

MS/MS Parameters:

Instrument:	AB Sciex API 5000 mass spectrometer
Ionisation Type#:	Electrospray (ESI)
Polarity#:	Positive
Ion Spray Voltage:	5500 V
Scan Type#:	Multiple reaction monitoring (MRM)
Source Temperature:	400°C
Curtain Gas:	20.0
Ion Source – Gas 1/Gas 2:	70.0/70.0
Collision Gas:	6.0
Collision Cell Entrance Potential:	10.0
Collision Cell Exit Potential:	10.0
Declustering Potential:	50.0
Resolution (Q1/Q3):	Unit/Unit

Test Substance	**Spirodiclofen**		**Spirodiclofen-enol**	
Instrument Parameter	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	411.3/313.0	411.3/295.1	313.2/212.9	313.2/184.8
Collision Energy:	18.0	35.0	35.0	43.0
Dwell Time:	150	150	200	200

Note: Instrument parameters are provisional and may be subject to change after optimisation.

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 1.6.2 uses the following formula to calculate the concentration of test substance present in the sample extract.

$$\text{Amount } (\mu g/L) = \left[\frac{y - c}{m}\right] \times \left[\frac{fv \times sd}{sw}\right]$$

Where:
y = Peak area
c = Y intercept on calibration graph
m = Slope of the linear calibration graph
sw = Sample volume equivalent in the final extract (mL)
fv = final volume of the extract
sd = Additional sample dilution of the final extract (if required)

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

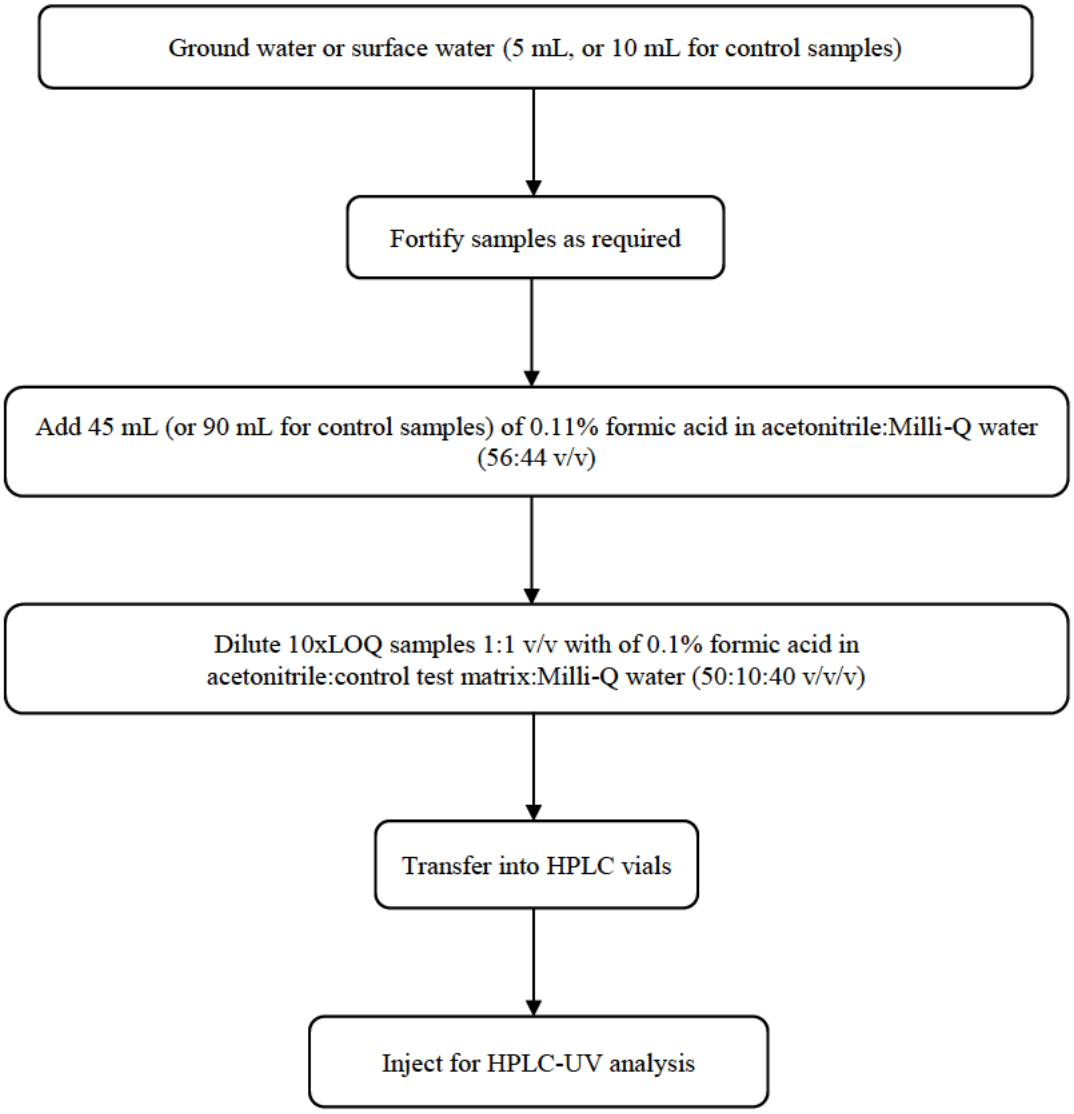
For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The residual plot will have a visually random distribution of regression residuals.
- 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 120% 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)

Flowchart


Ground water or surface water (5 mL, or 10 mL for control samples)
Fortify samples as required
Add 45 mL (or 90 mL for control samples) of 0.11% formic acid in acetonitrile:Milli-Q water (56:44 v/v)
Dilute 10xLOQ samples 1:1 v/v with of 0.1% formic acid in acetonitrile:control test matrix:Milli-Q water (50:10:40 v/v/v)
Transfer into HPLC vials
Inject for HPLC-UV analysis