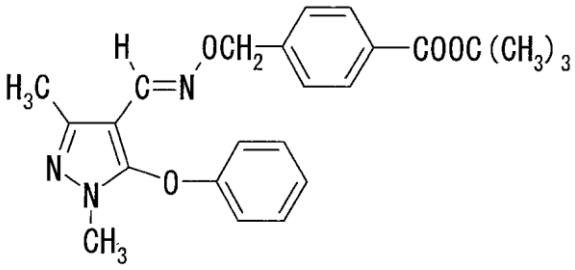
2 Reference Item Details

The following reference item was used in the study. The Certificate of Analysis is shown in Appendix A.

Common name	Fenpyroximate
IUPAC Name	*tert*-butyl(E)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate
CAS-Registry-No.	134098-61-6
Empirical formula	$C_{24}H_{27}N_3O_4$
Molar mass	421.5 g/mol
Structure	
ResChem Lot. No.	RAL 039/2015
Batch Identification	5AA0023P
Purity	99.4%
Expiry date	06 October 2021

3 Test System Details

3.1 Specimen Origin

The validation was undertaken on surface water taken from the River Derwent in Derby, UK. Characterisation of the water was conducted by CEMAS. Results are presented in Appendix B.

4 Analytical Methodology

The analytical method involves extraction of a sub-sample of surface water using a liquid-liquid partition into hexane. After organic solvent removal, the residue is redissolved in an acetonitrile/water solvent mix prior to final determination by LC-MS/MS.

4.1 Reagents Used

Reagent	Description	Supplier
Acetonitrile (for standard preparation)	AR Grade	Rathburns
Acetonitrile (for LC-MS mobile phase)	LC-MS Grade	VWR
Deionised Water (for sample and standard preparation)	AR Grade	Rathburns
Deionised Water (for LC-MS mobile phase)	LC-MS Grade	VWR
Formic Acid (for LC-MS mobile phase)	Optima LC-MS Grade	Fisher Scientific

4.2 Equipment and Apparatus used

Item	Description
Laboratory Balance's	A&D GR-202
Ultrasonic Bath	GT Sonic, 10 L Capacity
Adjustable Pipettes	Gilson P100, P200, P1000, P10mL
General Laboratory Supplies	Volumetric Flasks, Pipette's, Beakers, GC Vials, Centrifuge tubes, Measuring Cylinders etc.
DriBlock heater	Techne model DB-3A
LC-MS/MS	AB Sciex API 4000 Mass Spectrometer with an Agilent 1100 Binary HPLC Pump, Phenomenex DG-4400 Degasser, CTC Analytics HTS PAL Autosampler, Phenomenex Thermasphere TS-130 Column Oven and a Peak Scientific NM300DR Gas Generator
HPLC Column	Ascentis Express C18, 50 x 2.1 mm, 2.7 µm Particle Size, Sigma

4.3 Standard Preparation

Individual fenpyroximate stock solutions were prepared in acetonitrile with the aid of an ultrasonic bath, by dissolving 10 mg in 10 mL of solvent. The standards were allocated unique reference numbers i.e. FEN1-19.5.16 and FEN1-31.5.16

The fenpyroximate stock solutions were further diluted for use as fortification standards in the procedural recovery process and for the preparation of intermediate standards for instrument set-up and matrix-matched standard preparation.

4.3.1 Preparation of Fortification Solutions

Fortification standard solutions were prepared by serial dilution of the fenpyroximate stock solution using acetonitrile as listed below.

Standard Ref.	Standard Conc. (µg/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (µg/mL)	Standard Ref.
FEN1-19.5.16, FEN1-31.5.16	1000	1	10	100	FEN2-19.5.16, FEN2-31.5.16
FEN2-19.5.16, FEN2-31.5.16	100	1	10	10	FEN3-19.5.16, FEN3-31.5.16
FEN3-19.5.16, FEN3-31.5.16	10	1	10	1.0	FEN4-19.5.16, FEN4-31.5.16
FEN4-19.5.16, FEN4-31.5.16	1.0	1	10	0.1	FEN5-19.5.16, FEN5-31.5.16
FEN5-19.5.16, FEN5-31.5.16	0.1	1	10	0.01	FEN6-19.5.16, FEN6-31.5.16

4.3.2 Preparation of Intermediate Standard Solutions

Intermediate standard solutions were prepared by serial dilution using acetonitrile/water, 1/1, v/v as listed below

Standard Ref.	Standard Conc. (µg/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (µg/mL)	Standard Ref.
FEN4-19.5.16, FEN4-31.5.16	1.0	1	10	0.1	FEN7-23.5.16, FEN7-31.5.16
FEN4-19.5.16, FEN4-31.5.16	1.0	0.4	10	0.04	FEN8-23.5.16, FEN8-31.5.16
FEN4-19.5.16, FEN4-31.5.16	1.0	0.2	10	0.02	FEN9-23.5.16, FEN9-31.5.16
FEN7-23.5.16, FEN7-31.5.16	0.1	1	10	0.01	FEN10-23.5.16, FEN10-31.5.16
FEN7-23.5.16, FEN7-31.5.16	0.1	0.4	10	0.004	FEN11-23.5.16, FEN11-31.5.16
FEN10-23.5.16, FEN10-31.5.16	0.01	1	10	0.001	FEN12-23.5.16, FEN12-31.5.16

4.3.3 Preparation of Matrix-Matched Calibration Standard Solutions

Dilutions were performed in untreated final water extract (acetonitrile/water, 1/1, v/v) as follows;

Standard Ref.	Standard Conc. (µg/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (µg/mL)	Standard Ref.
FEN7-23.5.16	0.1	0.05	1.0	0.005	SW1-23.5.16
FEN8-23.5.16	0.04	0.05	1.0	0.002	SW2-23.5.16
FEN9-23.5.16	0.02	0.05	1.0	0.001	SW3-23.5.16
FEN10-23.5.16	0.01	0.05	1.0	0.0005	SW4-23.5.16
FEN11-23.5.16	0.004	0.05	1.0	0.0002	SW5-23.5.16
FEN12-23.5.16	0.001	0.05	1.0	0.00005	SW6-23.5.16

4.3.4 Preparation of Solvent Standard Solutions equivalent to the LOQ

Solutions were prepared for matrix effect and standard stability assessments (in acetonitrile/water, 1/1, v/v) as follows;

Standard Ref.	Standard Conc. (µg/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (µg/mL)	Standard Ref.
FEN11-23.5.16	0.004	0.05	1.0	0.0002	SOL1-23.5.16, SOL2-19.5.16
FEN11-31.5.16	0.004	0.05	1.0	0.0002	SOL1-31.5.16

4.4 Extraction

1. An aliquot of water (20 mL) was dispensed into a polypropylene tube (50 mL).
2. Procedural recoveries were prepared by fortifying aliquots of untreated water with the appropriate fenpyroximate fortification solution as detailed in the table below.

Matrix	Sample Weight (g)	Standard Reference	Standard Conc. (µg/mL)	Volume added (µL)	Fortification Level (µg/L)
Surface Water	20.0	FEN6-19.5.16	0.01	20	0.01
	20.0	FEN5-19.5.16	0.1	20	0.1
	20.0	FEN4-19.5.16	1.0	20	1.0

3. The tube was manually shaken and an aliquot of hexane (2.5 mL) added to the tube.
4. The tube was vortex mixed for 30 seconds.
5. The tube was then placed in a centrifuge (3000 rpm, 5 minutes) to separate the phases.
6. The hexane phase was removed by pasteur pipette and placed in a clean 15 mL polypropylene centrifuge tube.
7. Further hexane (2.5 mL) was added to the aqueous phase and partitioned as before. The organic phases were combined.
8. The hexane phase was evaporated to dryness using a Dri-block heater at 50 ˚C using a gentle stream of air.
9. The residue was redissolved in acetonitrile/water (1/1, v/v, 1.0 mL) using an ultrasonic bath and vortex mixer.
10. The final extract was transferred to an autosampler vial prior to quantitation of fenpyroximate by LC-MS/MS.

4.5 LC-MS/MS Conditions

An AB Sciex API 4000 Mass Spectrometer with an Agilent 1100 Binary HPLC Pump, Phenomenex DG-4400 Degasser, CTC Analytics HTS PAL Autosampler, Phenomenex Thermasphere TS-130 Column Oven and a Peak Scientific NM300DR Gas Generator was used for quantitation.

4.5.1 Chromatography Parameters for Fenpyroximate

Parameter	Description		
HPLC Column	Ascentis Express C18, 50 x 2.1 mm, 2.7 µm Particle Size, Sigma		
Column Temperature	45 °C		
Injection Volume	5 µL		
Retention Time	2.4 minutes (approx.)		
Mobile Phase	A: 0.1% Formic Acid in Water B: 0.1% Formic Acid in Acetonitrile		
Flow Rate	0.5 mL/min		
Gradient	Time (minutes)	A (%)	B (%)
	0.0	40	60
	3.5	40	60

4.5.2 Mass Spectrometry Parameters for Fenpyroximate

Parameter	Description			
Ionisation Mode	Turbospray (Electrospray)			
Probe Position	5 Horizontal, 5 Vertical			
Polarity	Positive			
Curtain Gas	40			
CAD Gas	8			
Gas 1	45			
Gas 2	40			
Source Temperature	550 °C			
Spray Voltage	5500			
Declustering Potential	70			
Entrance Potential	12			
Mass Transitions	Ions monitored (*m/z*)	Collision Energy	Cell Exit Potential	Primary / Confirmatory
	422 → 366	37	10	Primary
	422 → 135	45	6	Confirmatory

4.6 Quantitation

The quantitative determination of all analytical samples was carried out by external standardisation using calibration standards in matrix.

A linear calibration curve was constructed using the method of least squares (1/x weighting) using 6 different concentrations of fenpyroximate ranging from 0.00005 to 0.005 µg/mL.

The calibration curves were calculated from the area of the calibration solutions with their corresponding concentrations using the following equation:

$$Y = A \times C + B$$

where:

Y = Peak area (integration units)

A = slope of the linear least squares fit of the calibration curve

C = concentration determined from the standard curve (µg/mL)

B = Y-intercept of the linear least squares fit of the calibration curve

The amount of fenpyroximate in each specimen was calculated from the generated calibration curve using Analyst® software.

The concentration (C) of fenpyroximate determined from the standard curve is

$$C = (Y - B) / A$$

The residue of fenpyroximate in each test specimen is calculated as follows;

$$\text{Residue, µg/L} = \frac{V_f}{V_1} \times n \times C$$

Where: V_1 = Initial extraction volume (20 mL)

V_f = Final volume (1 mL)

n = dilution factor (if applicable)

Percent recovery from fortified samples was calculated as described below:

$$\text{Recovery (\%)} = \frac{(R_{fortified})}{F} \times 100$$

where:

$R_{fortified}$ = Residue determined in fortified sample (µg/mL)

F = Fortification rate (µg/mL)

4.7 Example Calculations

For a 0.01 µg/L fortified water sample (sample no. 1259 (422>366 *m/z*)) the percent recovery found was calculated as follows:

Recovery (%) = (0.010394 µg/L / 0.01 µg/L) x 100

= 104 %

4.8 Method Flow Chart