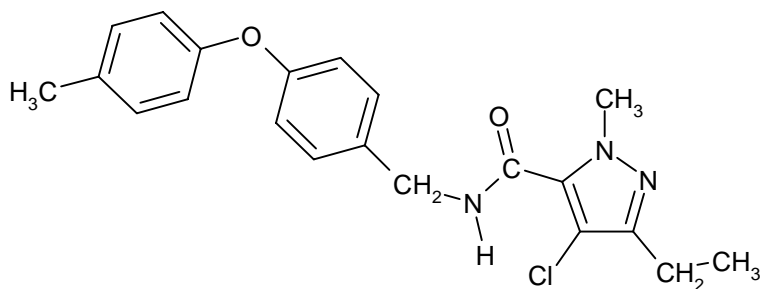


INTRODUCTION

Tolfenpyrad has the following chemical structure:



Common name:	Tolfenpyrad
Code name:	OMI-88
Chemical name (CAS):	4-chloro-3-ethyl-1-methyl-N-[[4-(4-methylphenoxy)phenyl]methyl]-1 <i>H</i> -pyrazole-5-carboxamide
CAS number:	129558-76-5

The study was undertaken to comply with the known data requirements and study guidelines stated on the front cover of the report. These requirements will also satisfy EU post registration enforcement method validation, as provided in the guidance document on residue analytical methods, SANCO/825/00 rev.7 (17/03/04). An analytical method validated to these guidelines is considered suitable for regulatory submission in areas outside of the EU, e.g. USA.

OBJECTIVES

The objective of this study was to develop and validate an analytical method for measuring residues of tolfenpyrad in surface and distilled water.

PROCEDURES

Protocol Adherence

The study was performed in accordance with the protocol and one amendment. Deviations that did not affect the integrity of the study are detailed in Appendix 6.

Analytical Standards

Analytical grade tolfenpyrad was supplied by the Sponsor. A Certificate of Analysis including purity, expiry date and lot number was supplied.

All precautions required in the handling, storage and disposal of the test substance were outlined by the supplier.

The date of receipt and expiry date, plus Lot number and purity details are recorded in the table below along with the allocated Covance identification number. The analytical standard was stored under refrigeration (2 to 8°C). The Certificate of Analysis is presented in [Appendix 1](#).

Analytical standard	Covance ID number	Date received	Supplier Lot no.	Chemical purity	Expiry date
Tolfenpyrad	ESTS 74/11	31 March 2011	7HO0001A	100.0%	28 June 2015

Receipt and Storage of Study Samples

Control samples of surface water (Oak Beck, Harrogate) was sourced locally by Covance and distilled water was commercially sourced from Rathburns, UK.

The surface water was given unique Covance identification number on receipt (CS22/11) in accordance with departmental Standard Operating Procedures (SOPs), and were stored under refrigeration (2 to 8°C). The distilled water was sourced commercially from Rathburns, UK and the sample was not booked in and given a unique number. The distilled water was however identified in the raw data by the Lot number of the bottled water (11C28TA). The distilled water used directly from the laboratory store for which the storage conditions were room temperature (10 to 30°C).

Water Characterisation

Water characteristics are presented in [Appendix 2](#). A summary of these data is shown below.

Water type	Source	pH	DOC (ppm)	Total hardness (mg/L as CaCO ₃)	Conductivity (µS/cm)	Suspended solids (mg/L)
Surface	Oak Beck, Harrogate	8.1	8.0	112	281	<0.05

DOC = dissolved organic carbon

Water type	Source	Non-volatile residue (ppm)	Heavy metals (ppb)
Distilled ⁵	Rathburns, UK	<2	<2

Preparation of Study Samples

Control samples of surface water and distilled water did not require any preparation prior to analysis.

Analytical Procedures

Analytical procedure CLE 8245058-01V was developed by Covance. The analytical method employed UPLC-MS/MS.

Summary of the Analytical Method for Surface Water and Distilled Water. Method CLE 8245058-01V

The method involved dilution of the water sample with methanol : water (1:1, v/v) prior to determination of tolfenpyrad by UPLC-MS/MS.

A copy of the analytical procedure is presented in [Appendix 3](#).

Validation Procedure

The analytical method was validated by fortifying aliquots of untreated control surface water and distilled water with known amounts of tolfenpyrad. The fortified samples were then analysed using the defined method and recovery of tolfenpyrad from each sample was determined.

Validation included consideration of the criteria in the following sections.

Linearity

In order to establish linearity of response of the analytical chromatographic system to tolfenpyrad, at least six standard solutions of increasing concentration were prepared over the range 0.001 to 0.10 µg/L for validation of surface water and distilled water. The lowest concentration was equivalent to less than 50% of a sample at the limit of quantification (LOQ) and the highest concentration was equivalent to greater than 120% of the highest level to be analysed.

⁵ This data is not captured under GLP

Solutions were injected into the chromatograph in random order and concentration/response curves were prepared.

Specificity

The ability of the method to distinguish between tolfenpyrad and other substances present in the control samples was investigated. Components present in a control sample that interfered with the analysis should not have been present at levels greater than 30% of the limit of quantification.

Precision

Repeatability of the method was demonstrated by analysing each validation level in quintuplicate. Relative standard deviation (RSD) was determined at each validation level and was considered acceptable if $\leq 20\%$.

Recovery

Recovery from control surface water and distilled water fortified at 0.01 (LOQ) and 0.10 $\mu\text{g/L}$ ($10 \times \text{LOQ}$) of tolfenpyrad was determined in quintuplicate.

In addition, control samples of surface water and distilled water were extracted and analysed in duplicate.

Mean recoveries of tolfenpyrad at each level and overall, were considered acceptable if they fell within the range 70 to 110%.

Limit of Quantification (LOQ)

The limit of quantification was defined as the lowest fortification level where an acceptable mean recovery (70 to 110%) for tolfenpyrad was obtained and a relative standard deviation of $\leq 20\%$ was achieved. For this analytical method, the limit of quantification for tolfenpyrad was proposed as 0.01 $\mu\text{g/L}$ in both surface water and distilled water.

Limit of Detection (LOD)

Although not specifically required according to the EU guidance documents stated on the front cover of this report, an analytical LOD was estimated for each matrix. The limit of detection was defined as 3 times baseline noise measurable in each control water matrix extract, estimated from the lowest concentration of the calibration standards. The LOD should be 30% of the LOQ or lower, and its signal to noise ratio (S/N) should be greater than three.

Method Confirmation

Residues were confirmed by UPLC-MS/MS using a second ion transition. The same criteria for precision and recovery (see above) were used to assess this confirmatory method.

Matrix	Precision (RSD, %)		
	0.01 µg/L	0.1 µg/L	Overall
Surface water	4.4	1.6	3.6
Distilled water	2.6	3.2	2.8

Limit of Quantification (LOQ)

Acceptable mean recovery (70 to 110%) and a relative standard deviation (RSD) of ≤ 20% was obtained for the analysis of tolfenpyrad at both fortification levels in surface water and distilled water. Therefore, the LOQ was confirmed as 0.01 µg/L tolfenpyrad.

Limit of Detection (LOD)

The LOD was calculated to be 0.0008 µg/L tolfenpyrad in surface water and 0.0011 µg/L tolfenpyrad in distilled water. These values were derived by estimation of the equivalent concentration of tolfenpyrad for a peak response equal to 3 times baseline noise (height) in control matrix extracts when compared with the lowest calibration standard (height of peak equivalent to 0.001 µg/L).

The LOD for both matrices was less than 30% of the LOQ or lower, and its signal to noise ratio (S/N) should be greater than three.

CONCLUSIONS

An analytical method, employing UPLC-MS/MS, for the determination and confirmation of tolfenpyrad in surface water and distilled water was validated at Covance Laboratories Limited.

Control samples of surface water and distilled water were fortified with tolfenpyrad at 0.01 and 0.10 µg/L in quintuplicate and analysed. Recovery of tolfenpyrad was determined and the validity of the analytical procedure was assessed.

The analytical procedure was successfully validated for the determination and confirmation of tolfenpyrad in surface water and distilled water within the concentration range 0.01 to 0.10 µg/L.

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8.2.2. With each batch of samples, a procedural recovery experiment should be carried out to check the method performance. This will be performed by analysing an appropriate blank control sample, and two samples fortified with Tolfenpyrad at LOQ and $10 \times$ LOQ (0.01 and 0.1 $\mu\text{g/L}$). The amount of Tolfenpyrad recovered from the two fortified samples will be compared to the amount added to determine the performance of the analytical procedure. The performance of the analytical procedure will be considered acceptable if the mean procedural recovery of Tolfenpyrad falls within 70 – 110% and the relative standard deviation is $\leq 20\%$. See also Section 15 'Method Criteria'.

9. PROCEDURE

9.1. Extraction

9.1.1 Frozen samples should be thoroughly defrosted (e.g. left in a fume cupboard) prior to analysis.

9.1.2 Measure 10 mL \pm 0.05 mL of surface or distilled water into 15 mL centrifuge tubes. Fortify samples if appropriate with solutions prepared in section 6.3.2.

9.1.3 Transfer the whole sample into a 20 mL volumetric flask.

9.1.4 Add 4 mL of methanol to the centrifuge tube and ultrasonicate briefly before transferring washing to 20 mL volumetric flask.

9.1.5 Repeat step 9.1.4 and make to 20 mL with methanol. Dilute 0.5 mL sample extract with 0.5 mL methanol: water (1:1 v/v) in 1.5 mL HPLC vials. A larger dilution may be done if necessary, to bring the sample solution into calibration range.

Note - See Section 12 for the limitations of the methodology.

9.2 UPLC-MS/MS conditions

	UPLC Conditions
UPLC column#	Phenomenex Luna C18 (2)-HST 2.5 μm particle size 10cm x 2.0mm i.d.
UPLC Guard column	Phenomenex C18 4mm x 2mm i.d
In line filter (Acquity)	Supplier: Waters Part n/o 700002775
Column oven temperature#	Nominal 40°C

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Autosampler temperature	Nominal 4°C	
Mobile phase A#	0.1% formic acid	
Mobile phase B#	Acetonitrile	
Flow rate#	0.2 mL/min	
Gradient settings:	See table below	
Time (minutes)	A (%)	B (%)
0.0	75	25
0.5	75	25
10.25	0	100
16.26	0	100
20.25	75	25
Switching Valve times	0 – 3 mins – To waste 3 – 16 mins – To MS 16 – 20.25 mins – To waste	
Slave pump solvent	0.1% formic acid: acetonitrile (75:25 v/v)	
Wash solvent 1#	0.1% formic acid: acetonitrile (75:25 v/v)	
Weak Wash (Acquity)		
Injection mode (Acquity)	Partial loop with needle over-fill	
Injection loop volume (Acquity)	50 µL	
Needle placement	1.0 mm from bottom	
Injection volume (Recommended)	50 µL (this may vary depending on instrument performance)	

Mass Spectrometer Parameters API 5000

Mode of operation#	Turbo IonSpray (positive ion) (MS/MS)
Collision gas setting (CAD)	6 (± 5)
Curtain gas setting (CUR)	20 psi (± 5)
Ion source gas 1 (GS1)	50 psi
Ion source gas 2 (GS2)	70 psi
IonSpray Voltage (IS)	5500 V
Nebuliser Current (NC)	3.0 µA
Temperature (TEM)	550°C
Q1 Resolution	Unit
Q3 Resolution	Unit
Interface Heater Status	On
Analysis time	20.25 minutes (± 1 minute)

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Compound name	Ions monitored (± 0.5 Da)	Dwell time (ms)	Declustering Potential (DP) Volts	Collision Energy (CE) Volts	Collision Cell Exit Potential (CXP) Volts
Tolfenpyrad (quantitative)	384.2 \rightarrow 197.1	150	150	29	14
Tolfenpyrad (confirmatory)	384.2 \rightarrow 153.1	150	150	83	14

Voltages and dwell times may require optimisation therefore the above values are to be used for reference only.

Entrance Potential (EP)	10 V (± 5 V)
Pause time#	5 ms
Collision gas#	Nitrogen

no alternative to be used

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

10. CONFIRMATORY TECHNIQUES

LC-MS/MS is highly specific, therefore a confirmatory method is not required to confirm presence of Tolfenpyrad in water.

11. TIME REQUIRED FOR ANALYSIS

The methodology is normally performed with a batch of 12 samples. One person can do a batch in 0.5 days, excluding UPLC-MS/MS instrument time (8 hours working period).

12. LIMITATIONS

This method has only been validated on the water types specified. Although it can be reasonable assumed this methodology would work for other water types, further validation would be required.

During method development, Tolfenpyrad in water was found to adhere to plastic. Samples of a larger volume should be analysed whole and vessel rinsed with appropriate volumes of methanol to ensure accurate results.

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13. CALCULATION OF RESULTS

The presence of Tolfenpyrad in a sample is confirmed if the peak arising from the test sample has the same chromatographic retention time as a standard.

Determination of the concentration of Tolfenpyrad in surface and distilled water
Residues of Tolfenpyrad in surface and distilled water are determined by peak area using a calibration line of Tolfenpyrad peak area vs. solution concentration of Tolfenpyrad ($\mu\text{g/L}$). All peak area measurements and calculations are performed using a data system (Analyst 1.5.1). A calibration line will be determined by plotting the response from the calibration solutions (R) against the concentration of test article injected (A) to generate a straight-line graph.

$R = B0 + (B1 \times A)$ where B1 is the gradient and B0 is the intercept.

Concentrations of test article (A) in sample extracts are calculated from their response using the equation:

$$A (\mu\text{g/mL}) = \frac{(R - B0)}{B1}$$

The use of weighted least squares regression ($1/x$) is recommended for calibration lines with a range in excess of 100.

Residues of Tolfenpyrad in test samples are calculated as follows:

$$\text{Residue } (\mu\text{g/L}) = \frac{\text{extract concentration } (\mu\text{g/L}) \times \text{dilution factor} \times \text{final volume (mL)}}{\text{sample volume (mL)}}$$

Accuracy values from fortified samples are calculated using the following equation:

$$\text{Accuracy (\%)} = \left(\frac{(A - C)}{S} \times 100 \right) - 100$$

Where:-

A = concentration found in fortified control sample ($\mu\text{g/L}$)

C = concentration found (or interference) in control sample ($\mu\text{g/L}$)

S = concentration added to fortified control sample ($\mu\text{g/L}$)

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14. NOTE FOR VALIDATION EXPERIMENTS

14.1 Linearity of response

The linearity of response of the UPLC-MS/MS system is determined with at least six different concentration standard solutions over the range 0.001 to 0.1 µg/L.

14.2 Accuracy and precision (repeatability)

Analytical recoveries are determined by fortifying, in quintuplicate, untreated control surface or distilled water with known amounts of Tolfenpyrad at concentrations equivalent to LOQ and 10 × LOQ, as indicated in Section 8.1, and then applying the appropriate analytical procedure. The amount of test substance recovered is calculated as a percentage of the amount added.

14.2.1 Accuracy

Recommended values of accuracy, indicated in the Note for Guidance, are:

True (or fortified) value	Limits (%)
0.01 µg/L	70% to 110%
0.1 µg/L	70% to 110%

14.2.2. Precision (repeatability)

Precision is determined from analysis of the fortified samples prepared in Section 8.1, by determining the coefficient of variation (CV) of the observed concentrations of the fortified matrices.

Recommended values of CV indicated in the Note for Guidance are:

True Value	CV
0.01 µg/L	≤ 20%
0.1 µg/L	≤ 20%

14.3. Specificity

Specificity is determined from analysis of the non-fortified (blank) extract. Any peaks with the same retention time as Tolfenpyrad will be considered to interfere if they are equivalent to or greater than 20% of the limit of quantification (LOQ).

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14.4. Limit of detection (LOD)

The experimental LOD is determined from the analysis of 2 control samples per matrix, and is equal to the concentration equivalent to 3 x baseline noise height.

14.5. Limit of quantification (LOQ)

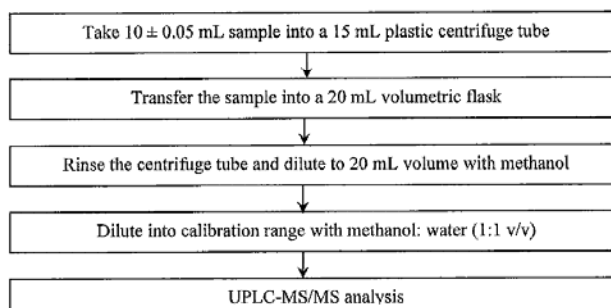
The limit of quantification is the lowest concentration with which surface or distilled water may be fortified to achieve acceptable precision and accuracy data as defined in Section 14.2 (above).

15. METHOD CRITERIA

The analysis will be considered to be successful only if the following criteria are met.

- The two procedural fortification samples meet the 70% to 110% criteria for accuracy.
- At least six calibration standards will be used in the determination of the calibration line.
- A correlation of coefficient (r) for each calibration line will be ≥ 0.995 or, if using a 1/x weighing, a coefficient of determination (r^2) for each calibration line will be ≥ 0.99
- All samples will be within the range of the calibration standards in the UPLC-MS/MS analysis.

16. FLOW DIAGRAM SUMMARISING THE PROCEDURE



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17. LIMITATIONS OF THE METHOD

This method uses polypropylene centrifuge tubes as the sampling container, and the whole sample must be analysed to account for adsorption onto the tube surface. Tolfenpyrad may adsorb differently to other container types. Tolfenpyrad has only been validated using polypropylene tubes.

18. DEVIATIONS FROM THIS METHOD

Any minor practical changes to this written procedure, which may be necessary, will be recorded as a file note or on the appropriate proforma.

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19. GENERAL HANDLING CONTROL CATEGORIES

CATEGORY		CONTROL
Main	Division	Name and Specification
1		GLOVES a Disposable latex b Disposable nitrile c Rubber gloves d Specific type for 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 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)

Appendix 6 Protocol Deviations

The following deviations from the protocol occurred during this study

Protocol section	Deviation
Test System - Receipt and Storage of Study Samples	<p>Under the protocol Section Receipt and Storage of Study Samples it states the following: <i>"Control samples of water will be obtained locally by Covance. Details of the samples and the supplier will be recorded in the raw data.</i></p> <p><i>The samples will be given a unique Covance number on receipt, in accordance with departmental Standard Operating Procedures (SOP), and will be stored refrigerated (2 to 8°C, nominally 4°C)".</i></p> <p>The control samples were sourced as follows:</p> <p>Control sample of surface water (Oak Beck, Harrogate) was sourced locally by Covance and the distilled water was commercially sourced from Rathburns, UK.</p> <p>The deviation, relates to the sourcing of the distilled water and booking in of the sample to assign a unique identification.</p> <p>The distilled water was sourced commercially from Rathburns, UK and the sample was not booked in and given a unique number. The distilled water was however identified in the raw data by the Lot number of the bottled water (11C28TA). The distilled water used directly from the laboratory store for which the storage conditions were room temperature (10 to 30°C).</p>
Experimental Procedures	<p>Although not specifically required according to the EU guidance documents stated on the front cover of the protocol, an analytical LOD will be estimated for water. The limit of detection is defined as the lowest concentration of the test compound measurable in water extracts, calculated from the lowest concentration of the calibration standards. The LOD should be 30% of the LOQ or lower, and its signal to noise ratio (S/N) should be greater than three.</p> <p>The LOD could not be calculated against the lowest calibration standard of 0.001 µg/L as this would have been equivalent to 40% of the LOQ when calculated as an equivalent sample injection.</p> <p>The procedure for calculation was modified to calculate from baseline noise which is a more true representation of LOD.</p> <p>The limit of detection was defined as 3 times baseline noise measurable in each control water matrix extract, estimated from the lowest concentration of the calibration standards.</p>
