1. Introduction

1.1 Objectives

The objective of this study was to perform an independent laboratory validation for the determination of penthiopyrad (MTF-753) and its metabolites 753-A-OH, 753-T-DO, PCA, DM-PCA and PAM in ground water and surface water. The methodology which was validated is described in the following document:

CEMAS report No. CEMR-3236 'Method validation for the determination of MTF-753 and its metabolites in drinking, ground and surface water' (Reference 1).

1.2 Study organisation

The location of the study was Eye Research Centre, Eye, Suffolk, IP23 7PX, Department of Environmental Analysis.

The signed protocol, a copy of the final report and the primary data pertaining to the study have been retained in the archives of Huntingdon Life Sciences.

1.3 Study timing

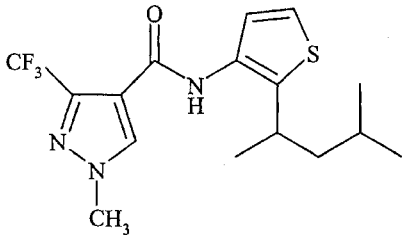
The protocol was signed by the Study Director and Huntingdon Life Sciences Management on 5 June 2009 and by the Study Monitor on 8 June 2009.

The study was undertaken at Huntingdon Life Sciences between 9 and 26 June 2009.

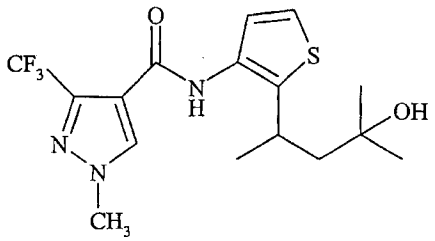
2. Materials

2.1 Test substances

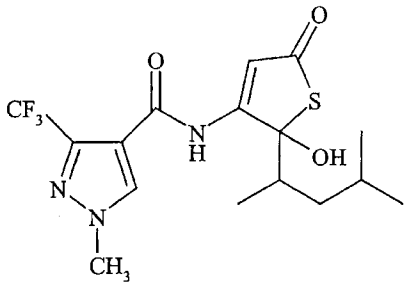
2.1.1 Penthiopyrad (MTF-753):

Name:	Penthiopyrad (MTF-753)
Chemical name (IUPAC):	(RS)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide
CAS registry number:	183675-82-3
Structure:	
Molecular formula:	$C_{16}H_{20}F_3N_3OS$
Molecular weight:	359.42 g/mole
Batch number:	2100111
Storage conditions:	Approximately +4°C / Dark
Supplier:	Mitsui Chemicals, Inc.
Purity:	99.8%
Appearance:	White solid
Expiry:	December 2010

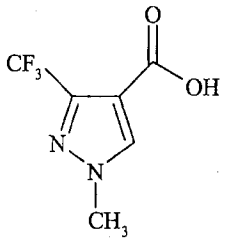
2.1.2 753-A-OH:

Name:	753-A-OH
Chemical name (IUPAC):	*N*-[2-(3-hydroxy-1,3-dimethylbutyl) thiophen-3-yl]-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
Structure:	
Molecular formula:	$C_{16}H_{20}F_3N_3O_2S$
Molecular weight:	375.4 g/mole
Batch number:	092-050824-1
Storage conditions:	Approximately +4°C / Dark
Supplier:	Mitsui Chemicals, Inc.
Purity:	100%
Appearance:	White powder
Expiry:	31 December 2010

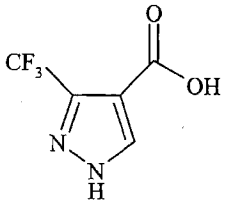
2.1.3 753-T-DO:

Name:	753-T-DO
Chemical name (IUPAC):	*N*-[5-hydroxy-5-(1,3-dimethylbutyl)-2-oxo-2,5-dihydrothiophen-4-yl]-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
Structure:	
Molecular formula:	$C_{16}H_{20}F_3N_3O_3S$
Molecular weight:	391.41 g/mole
Batch number:	188-004-42-2
Storage conditions:	Approximately +4°C / Dark
Supplier:	Mitsui Chemicals, Inc.
Purity:	99.84%
Appearance:	White powder
Expiry:	24 December 2011

2.1.4 PCA:

Name:	PCA
Chemical name (IUPAC):	1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
Structure:	
Molecular formula:	$C_6H_5F_3N_2O_2$
Molecular weight:	194.11 g/mole
Batch number:	053-001207-1
Storage conditions:	Approximately +4°C / Dark
Supplier:	Mitsui Chemicals, Inc.
Purity:	100%
Appearance:	White powder
Expiry:	31 December 2010

2.1.5 DM-PCA:

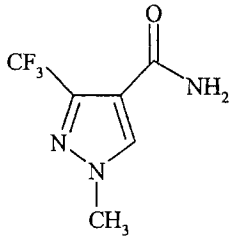
Name:	DM-PCA
Chemical name (IUPAC):	3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
Structure:	
Molecular formula:	$C_5H_3F_3N_2O_2$
Molecular weight:	180.09 g/mole
Batch number:	133-050713-1
Storage conditions:	Approximately +4°C / Dark
Supplier:	Mitsui Chemicals, Inc.
Purity:	99.71%
Appearance:	Off-white powder
Expiry:	December 2009

2.1.6 PAM:

Name: PAM

Chemical name (IUPAC): 1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide

Structure:



Molecular formula: $C_6H_6F_3N_3O$

Molecular weight: 193.13 g/mole

Batch number: 152-050805-1

Storage conditions: Approximately +4°C / Dark

Supplier: Mitsui Chemicals, Inc.

Purity: 100%

Appearance: White powder

Expiry: 31 December 2010

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

2.2 Untreated samples

Untreated ground water and surface water were obtained by the Environmental Analysis Department of Huntingdon Life Sciences for use in this study. Untreated samples were stored at approximately +4°C prior to use.

Matrix type	Analytical identification	Origin
Ground water	03/00/1241	Budby Pumping Station, Mansfield, England
Surface water	09/00/1032	Costessey Pits, Norwich, England

2.3 Reagents

A list of all reagents used is presented below:

Reagent	Grade	Product code	Manufacturer
Ethyl acetate [1]	HPLC	E/0906/17	Fisher Scientific
Methanol [1]	HPLC	M/4056/17	Fisher Scientific
Water (for sample processing) [2]	Ultra Pure (UP)	N/A	N/A (Elga water purifier)
Water (for mobile phase) [2]	HPLC	W/0106/17	Fisher Scientific
Formic acid [3]	Analytical	F/1900/PB08	Fisher Scientific
Ammonium acetate [3]	HPLC	A/3446/50	Fisher Scientific
Acetic acid [2]	Specified	A/0360/PB17	Fisher Scientific

[1] – identical to supplied method.
[2] – different to supplied method.
[3] – grade not specified in supplied method.

3. Procedures

3.1 Study Director's review of method

Prior to initiation of validation work, the Study Director reviewed the supplied methodology and was required to document any area's that were not clear and required interpretation or clarification. Step 6.4 of the method described in CEMAS report No. CEMR-3236 (SOP CEM-3329-004a) appeared unusual in the fact that the final sample composition for penthiopyrad (MTF-753), 753-A-OH and 753-T-DO (methanol:water 2:1 v:v) differed from the instrument calibration solution composition (methanol:water 1:1 v:v). A representative of the Study Monitor confirmed to the Study Director that the procedure described in the method was correct.

3.2 Modifications to the method

Minor modifications were made to the method and agreed to by the Study Monitor. A summary of the discussions between the Study Director and Study Monitor are presented in Appendix 3.

The method suggests that the calculation of the residue can be determined from the mean peak area of bracketing standards (CEMR-3236: Section 9 of SOP CEM-3329-004a). The usual practice of the independent laboratory is to determine residues from the linear regression derived from standards of various concentrations injected as part of the same analytical run. This was the procedure used for the method validation analysis.

Prior to commencement of the sample analysis, LC-MS/MS instrument investigations were performed to ensure acceptable performance could be achieved for all analytes. Initially the LC-MS/MS methodology presented in the CEMAS report No CEMR-3236 (SOP CEM-3329-004a) was followed as closely as possible; however, some modifications to the instrument conditions were required in order to obtain sufficient response, linearity and specificity. These modifications included an increase in the injection volume and alterations to the mobile phase gradient. All modifications were agreed to by the Study Monitor during the development of the LC-MS/MS instrument conditions for sample analysis (Appendix 3, entries for 10-17 June 2009).

The method suggests that all six analytes are to be included in the preparation of mixed standard solutions (CEMR-3236: Section 5 of SOP CEM-3329-004a). The independent laboratory was not in possession of the 753-T-DO standard at the commencement of the study; therefore separate batches of mixed standard solutions were prepared for the separate phases of work in the following combinations:

1) PCA, DM-PCA and PAM
2) Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO

Subsequently, the instrument calibration solutions were prepared in ranges appropriate to the LOD levels and the expected sample concentrations for each set of analytes. The following ranges were used:

1) PCA, DM-PCA and PAM: 0.5 to 20 ng/mL
2) Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO: 0.005 to 0.5 ng/mL

3.3 Preparation of test substance solutions

Weighed amounts (corrected for purity if necessary) of penthiopyrad (MTF-753), 753-A-OH, 753-T-DO, PCA, DM-PCA and PAM were dissolved in methanol to produce individual 1 mg/mL stock standard solutions.

For penthiopyrad (MTF-753), 753-A-OH and 753-T-DO, aliquots of the stock standard solutions were combined and progressively diluted with methanol to produce a series of fortification standard solutions in the range 100 µg/mL to 0.01 µg/mL.

For PCA, DM-PCA and PAM, aliquots of the stock standard solutions were combined and progressively diluted with methanol to produce a series of fortification standard solutions in the range 100 µg/mL to 0.1 µg/mL.

Aliquots of the mixed standard solutions were further diluted with methanol:water (50:50 v:v) to produce instrument calibration standard solutions.

3.4 Preparation of reagents

Final extract solution: methanol:water (50:50 v:v):

500 mL of methanol was mixed with 500 mL of UP water.

Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO mobile phase A: 0.01 M ammonium acetate:

1.93 g of ammonium acetate was dissolved in 2500 mL of HPLC water.

PCA, DM-PCA and PAM mobile phase A: 0.1% formic acid in water:

2.5 mL of formic acid was added to 2500 mL of HPLC water.

PCA, DM-PCA and PAM mobile phase B: 0.1% formic acid in methanol:

2.5 mL of formic acid was added to 2500 mL of methanol.

3.5 Procedure for penthiopyrad (MTF-753), 753-A-OH and 753-T-DO

1. Measure 50 mL of water into a 50 mL polypropylene tube.
2. Fortify control specimens for recovery determination at this stage and mix well.
3. Transfer a 0.5 mL aliquot of each sample to a HPLC vial.
4. Add 1 mL of methanol to each aliquot and mix well.

3.6 Procedure for PCA, DM-PCA and PAM

1. Measure 50 mL of water into a 250 mL separating funnel.
2. Fortify control specimens for recovery determination at this stage and mix well.
3. Add 1 mL of acetic acid to each sample and mix well.
4. Add 50 mL ethyl acetate and shake thoroughly, allow to separate.
5. Draw off the lower aqueous layer into a beaker and collect the ethyl acetate portion into a 250 mL round bottomed flask.
6. Repeat with a further 50 mL portion of ethyl acetate.
7. Evaporate the combined ethyl acetate using a rotary evaporator at approximately 40°C to approximately 2 mL.
8. Quantitativley transfer the extract to a 15 mL polypropylene tube using methanol.
9. Blow off the methanol under nitrogen at approximately 40°C. DO NOT blow down to dryness.
10. Add 2 mL of methanol and blow down as before (to remove all trace of ethyl acetate).
11. Blow down to a small volume and make to 1 mL with methanol and then make up to 2 mL with Ultra Pure water and transfer an aliquot into a HPLC vial.

3.7 Validation

Sub-samples of each untreated matrix type was fortified at known concentrations of the analytes, and analysed according to the following regime:

Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO:

2 untreated sub samples
5 untreated sub samples fortified at the LOQ (0.05 µg/L)
5 untreated sub samples fortified at 10 x LOQ (0.5 µg/L)

PCA, DM-PCA and PAM:

2 untreated sub samples
5 untreated sub samples fortified at the LOQ (0.05 µg/L)
5 untreated sub samples fortified at 10 x LOQ (0.5 µg/L)

These samples were then processed using the analytical methodology described in Section 3.5 or 3.6 as appropriate.

3.8 Sample final extract stability

Sample final extracts of each matrix type were fortified with the analytes at a concentration of 0.1 ng/mL for penthiopyrad (MTF-753), 753-A-OH and 753-T-DO and 10 ng/mL for PCA, DM-PCA and PAM. The concentrations of each analyte were quantified against equivalent calibration standards on day 0 and 7 days after storage at approximately –20°C in the dark. Control extracts were fortified at equivalent levels to act as procedural recovery samples for the day 7 samples.

3.9 LC-MS/MS analysis

The following LC-MS/MS conditions were developed to obtain sufficient analyte response and specificity using the independent laboratory LC-MS/MS equipment and were used for the analysis of the validation and sample final extract stability samples.

3.9.1 Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO

Instrument:	Quattro LC
Ionisation mode:	Negative electrospray (ESP-)
Source block temperature:	120°C
Desolvation temperature:	350°C
Nebuliser gas flow:	70 L/hr
Desolvation gas flow:	330 L/hr

Ion monitoring details:

Analyte	Ion transition	Collision energy (eV)	Cone (volts)
MTF-753	358.10>149.00	25	35
753-A-OH	374.10>149.04	30	40
753-T-DO	390.19>356.06	20	35

Column:	Phenomenex Synergi Polar-RP 4 µm (15 cm × 4.6 mm)
Column temperature:	40°C
Mobile phase A:	0.01 M ammonium acetate in water
Mobile phase B:	Methanol

Gradient:

Time (minutes)	A (%)	B (%)
0	35	65
7.5	15	85
8	35	65
10	35	65

(flow diverted to waste 0 – 1 minutes and 8 – 10 minutes)

Injection volume:	190µL
Flow rate:	1 mL/min with split flow post-column: MS:waste approximately 1:4 ratio (approximately 0.2 mL/min to MS)
Retention times:	Penthiopyrad (MTF-753): approximately 6.4 minutes 753-T-DO: approximately 6.0 minutes 753-A-OH: approximately 5.1 minutes

3.9.2 PCA and DM-PCA

Instrument:	Quattro LC
Ionisation mode:	Negative electrospray (ESP-)
Source block temperature:	120°C
Desolvation temperature:	350°C
Nebuliser gas flow:	70 L/hr
Desolvation gas flow:	330 L/hr

Ion monitoring details:

Analyte	Ion transition	Collision energy (eV)	Cone (volts)
PCA	193.1>109.0	25	15
DM-PCA	179.0>159.2	10	20

Column:	Phenomenex Synergi Polar-RP 4 µm (15 cm × 4.6 mm)
Column temperature:	40°C
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in methanol

Gradient:

Time (minutes)	A (%)	B (%)
0	70	30
7.5	15	85
8	70	30
10	70	30

(flow diverted to waste 0 – 1 minutes and 7.5 – 10 minutes)

Injection volume:	190µL
Flow rate:	1 mL/min with split flow post-column: MS:waste approximately 1:4 ratio (approximately 0.2 mL/min to MS)
Retention times:	PCA: approximately 5.8 minutes DM-PCA: approximately 4.6 minutes

3.9.3 PAM

Instrument:	Quattro LC
Ionisation mode:	Positive electrospray (ESP+)
Source block temperature:	120°C
Desolvation temperature:	350°C
Nebuliser gas flow:	70 L/hr
Desolvation gas flow:	330 L/hr

Ion monitoring details:

Analyte	Ion transition	Collision energy (eV)	Cone (volts)
PAM	194.1>174.1	15	15

Column:	Phenomenex Synergi Polar-RP 4 µm (15 cm × 4.6 mm)
Column temperature:	40°C
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in methanol

Gradient:

Time (minutes)	A (%)	B (%)
0	70	30
7.5	15	85
8	70	30
10	70	30

(flow diverted to waste 0 – 1 minutes and 7.5 – 10 minutes)

Injection volume:	190µL
Flow rate:	1 mL/min with split flow post-column: MS:waste approximately 1:4 ratio (approximately 0.2 mL/min to MS)
Retention time:	PAM: approximately 4.3 minutes

4. Calculation of results

Samples were quantified using the following equation:

$$\text{Residue found } (\mu g/L) = x \times \frac{1}{M}$$

Where x (residue concentration in final solution) was calculated using the linear regression

$$y = m x + c \qquad \text{where } x \text{ (concentration in ng/mL)} = \frac{y - c}{m}$$

y = peak area
m = slope
c = intercept
M = matrix concentration (mL/mL)
D = dilution factor

Example calculation of penthiopyrad (MTF-753) detected in ground water fortified at 0.05 µg/L (sample identification 03/00/1241 F0.05 µg/L A).

Linear regression $y = m x + c$

$$46.785 = 2888.74 x + 4.02995$$

Where:
y = 46.785
m = 2888.74
c = 4.02995

Therefore, concentration (x) $= \frac{46.785 - 4.02995}{2888.74} = 0.0148$ ng/mL

Matrix concentration = 0.333 mL/mL

Penthiopyrad (MTF-753) detected $= \frac{0.0148 \text{ ng/mL}}{0.333 \text{ mL/mL}} = 0.0444$ ng/mL = 0.0444 µg/L

Recovery $= \frac{0.0444 \text{ µg/L}}{0.05 \text{ µg/L}} \times 100\ \% = 89\ \%$

Note: for sample final extract stability samples the concentration of the analyte in the final extract was determined by direct comparison with the equivalent calibration standard solution.

Appendix 3 Summary of communications between the ILV Study Director and the Study Monitor

Dates of communications	Stage of ILV study	Discussion details	Outcome of discussion
14 May 2009	Review of method prior to study start	Clarification was required for step 6.4 of the method (see Section 3.1 for further details).	Study Monitor representative confirmed step 6.4 of the method as correct.
10 June 2009	Instrument response investigation (PCA, DM-PCA and PAM)	Initial instrument response investigation results sent to Study Monitor with request to increase injection volume in order to obtain sufficient response.	Study Monitor agreed to increase in injection volume.
15 June 2009	PCA, DM-PCA and PAM validation results	Validation results sent to Study Monitor with notification of the requirement to modify the LC gradient in order to separate analytes and interferences and thus obtain acceptable recovery data.	Study Monitor confirmed that the results were acceptable.
17 June 2009	Instrument response investigation (penthiopyrad (MTF-753), 753-A-OH and 753-T-DO)	Initial instrument response investigation results sent to Study Monitor with request to: 1) increase injection volume in order to obtain sufficient response and 2) delay diverter valve until 8 minutes to ensure penthiopyrad (MTF-753) has completely eluted.	Study Monitor agreed to proposed modifications.
19 June 2009	Penthiopyrad (MTF-753), 753-A-OH and 753-T-DO validation results	Validation results sent to Study Monitor with notification of the occasional result >110%. ILV Study Director also commented on the observation that there were also results >110% in the original method validation.	Study Monitor queried whether the ILV Study Director could offer possible explanations for the occasional high results. ILV Study Director responded with a comment on the slight inaccuracy in the assumption of the final solution volume being 1.5 mL. In reality, addition of 1 mL methanol to 0.5 mL water will result in slightly less than 1.5 mL. Further investigations were performed by the ILV Study Director into final solvent composition effects and matrix enhancement effects, however, no further explanation for the high results could be offered.
03 July 2009	Sample final extract stability	Results sent to Study Monitor. ILV Study Director acknowledged lower than expected results for PAM; however, stability in final extract had been demonstrated.	Study Monitor confirmed that the results were acceptable.