Cover Sheet for Analytical Method

Pyriofenone in Water - MRID 49256133

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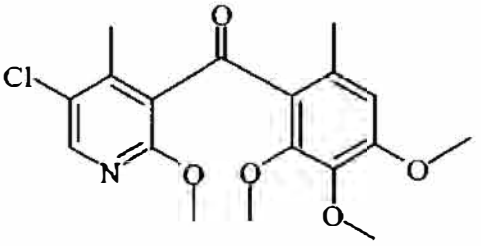
2. Materials

2.1 Analytical Standard 1 – IKF-309

Chemical name: (5-chloro-2-methoxy-4-methyl-3-pyridinyl) (2,3,4-trimethoxy-6-methylphenyl) methanone (CAS)
(5-chloro-2-methoxy-4-methyl-3-pyridyl)(2,3,4-trimethoxy-6-methylphenyl)ketone (IUPAC)

Structural formula:



Purity:	99.19%
Expiry date:	September 2013
Batch number:	0608
Appearance:	White powder
Storage conditions:	Frozen (approximately -20°C, in the dark)

A Certificate of Analysis is presented in Appendix 1.

2.2 Control matrices

The surface water sample was obtained from a local source (Costessey Pits No. 1) and the drinking water from a source within the Environmental Analysis Department. Upon receipt the water samples were allocated unique Huntingdon Life Sciences, Environmental Analysis Department identification numbers and stored at approximately +4°C prior to use.

The surface water was characterised in a separate study and is presented in the following table:

Parameter	Found Value
pH	8.11
Dissolved Oxygen	11.11 mgO_2/L at 19.9°C
Conductivity	543 µS/cm at 6.0°C
Alkalinity	260 mg/L as $CaCO_3$
Total Hardness	376 mg/L as $CaCO_3$
Total Organic Carbon	5.804 mgC/L
Dissolved Organic Carbon	5.660 mgC/L

3. Methods

3.1 Validation

Sub-samples of surface and drinking water were fortified with known concentrations of the test substance and analysed according to the following regime:

2 sub-samples of untreated sample matrix
5 sub-samples of untreated sample matrix fortified at the LOQ (0.05 µg/L)
5 sub-samples of untreated sample matrix fortified at 0.5 µg/L

These samples were then analysed using the analytical methodology, with each sample injected onto the chromatographic system once.

3.2 Final extract stability

An experiment was set up to demonstrate the stability of the analyte under the typical storage conditions of the final extracts if they are not quantified immediately after preparation. Processed control extracts, fortified with IKF-309 were stored at approximately -20°C in the dark (i.e. in a freezer).

Aliquots of each of the control sample extracts were fortified with IKF-309 at a concentration of 1 ng analyte/mL of final extract. The concentration of analytes in the stored extracts was determined at day 0 and after 6 days. The concentration of the analytes in freshly fortified control extracts was also determined at that time.

3.3 Matrix effects

Any possible sample matrix effects were investigated by the comparison of the instrument response to the analytes in the fortified final extract samples with the response of the analytes in solvent based calibration standard solutions prepared at the same time.

3.4 Analytical method

Samples were diluted with acetonitrile. Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

The analytical method is presented in Appendix 3.

The standard solutions used in this study were also used in other GLP studies being performed for the same Sponsor. The use of these standard solutions is fully traceable to the other studies and copies of the standard solution preparation are included in the raw data package for these studies.

A stock standard solution of IKF-309 was prepared by dissolving an accurately weighed amount of the analyte in a suitable volume of acetonitrile, correcting for purity as appropriate. The stock solution was further diluted with acetonitrile to produce fortification solutions at 10 ng/mL and 100 ng/mL concentrations.

The instrument calibration solutions, over the concentration range 0.01 ng/mL to 1 ng/mL, were prepared by serial dilution of the fortification solution in acetonitrile:water (50:50 v:v), as detailed below:

Standard solution used (ng/mL)	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100	0.1	10	1
100	0.08	10	0.8
100	0.04	10	0.4
100	0.02	10	0.2
1	1	10	0.1
1	0.8	10	0.08
1	0.4	10	0.04
1	0.2	10	0.02
1	0.1	10	0.01

3.5 Calculation of results for validation samples

Test samples were quantified using the following equation:

$$\text{Residue found (mg/kg)} = x \times \frac{1}{M} \times D$$

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

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

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

Example calculation of IKF-309 detected in drinking water fortified at 0.05 µg/mL (09/00/9375 F0.05 D, analysis batch 3). The primary data for this sample is presented in Table 8, Appendix 2.

Linear regression $y = m\,x + c$

$$67.745 = 2162.92x + 13.5074$$

where $y = 67.745$
$m = 2162.92$
$c = 13.5074$

$$\text{Therefore, concentration of IKF-309 } (x) = \frac{67.745 - 13.5074}{2162.92} = 0.02508 \text{ ng/mL}$$

Matrix concentration = 0.5 mL matrix/mL final extract
Dilution factor = 1

$$\text{IKF-309 detected (µg/L)} = \frac{0.02508 \text{ ng/mL} \times 1}{0.5 \text{ mL/mL}} = 0.0502 \text{ ng/mL} = 0.0502 \text{ µg/L}$$

$$\text{Recovery (\%)} = \frac{0.0502 \text{ µg/L} \times 100}{0.05 \text{ µg/L}} = 100\%$$

Appendix 3 Analytical Method

DETERMINATION OF RESIDUES OF IKF-309 IN WATER

1. General principle

Samples are diluted with acetonitrile. Quantitation is performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

2. Apparatus, glassware etc

Balances (various ranges)
Volumetric flasks (various sizes)
Syringes (various sizes)
Volumetric pipettes (various sizes)
Measuring cylinders (various sizes)
Glass scintillation vials (20 mL)

3. Materials	**Typical Grade**
Ammonium formate	AR
Acetonitrile	HPLC
Formic acid	AR
Methanol	HPLC
Water	HPLC

4. Preparation of reagents

Preparation of acetonitrile:water (50:50 v:v):

Acetonitrile (250 mL) is added to water (250 mL) and mixed thoroughly.

Preparation of mobile phase A - water:methanol:formic acid (90:10:0.1 v:v:v) containing 0.01M ammonium formate:

Methanol (100 mL), ammonium formate (0.6 g) and formic acid (1 mL) are added to HPLC water (900 mL) and mixed thoroughly prior to use.

Preparation of mobile phase B - methanol:formic acid (100:0.1 v:v):

Methanol (1000 mL) is mixed thoroughly with formic acid (1 mL).

Note: variable quantities of the above may be prepared by adjusting the constituent quantities accordingly.

5. Analytical standard solutions

An appropriate amount of the test substance (corrected for purity) is accurately weighed and dissolved in acetonitrile to give a stock standard solution. Appropriate dilutions of the stock standard solutions are made with acetonitrile to give fortification standard solutions.

The fortification solutions are progressively diluted with acetonitrile:water (50:50 v:v) to produce a series of instrument calibration solutions in the range 0.01 to 1 ng/mL.

6. Procedure

6.1 Transfer a sub-sample (10 mL) into a scintillation vial.
6.2 Add fortification solutions at this stage if required.
6.3 Add an aliquot (10 mL) of acetonitrile.
6.4 Perform any further dilutions using acetonitrile:water (50:50 v:v), as required.
6.5 Quantify the samples by the use of LC-MS/MS.

7. LC-MS/MS conditions

Instrument:	Quattro LC / Varian 1200 / Acquity TQD
Ionisation mode:	Positive electrospray
Ion monitoring details:	MRM *m/z* 366>184 (quantitation) MRM *m/z* 366>209 (confirmation)
Column:	Phenomenex Luna C_8 (15 cm × 2 mm)
Mobile phase A:	Methanol:water (10:90 v:v) + 0.01M ammonium formate + 0.1% formic acid
Mobile phase B:	Methanol + 0.1% formic acid

Gradient:

Time (minutes)	A (%)	B (%)
0	30	70
6	0	100
10	0	100
11	30	70
15	30	70

Injection volume:	20 µl
Flow rate:	0.2 mL/min
Retention time:	Approximately 5.5 minutes
LOD:	0.01 ng/mL (≡ 0.02 µg/L in sample matrix)
LOQ:	0.05 µg/L