SUMMARY

An analytical method, employing solid phase extraction (SPE) and liquid chromatography with tandem mass spectroscopy detection (LC-MS/MS), for the determination and confirmation of IKF-5411, 3-MTCAM, IBA and PPA in untreated surface water and drinking water was validated at Smithers Viscient (ESG) Ltd.

Characteristics of the surface water is shown below.

Water type	Source	pH	DOC (ppm)	Total hardness (mg/L as $CaCO_3$)	Conductivity (µS/cm)
Surface	Oak Beck	8.0	7.9	107	290

DOC = dissolved organic carbon

The method involved cleanup of the water samples with mixed mode anion exchange (MAX) SPE, followed by evaporation and reconstitution, prior to determination of IKF-5411, 3-MTCAM, IBA and PPA by LC-MS/MS. The LC-MS/MS analysis was performed twice on each sample, using different conditions, in order to successfully detect each analyte.

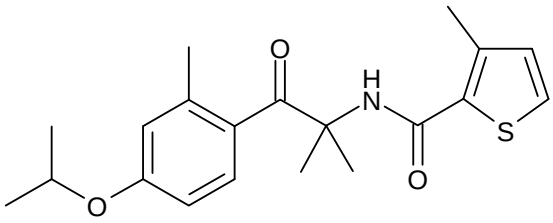
Control samples of surface water and drinking water were fortified with IKF-5411, 3-MTCAM, IBA and PPA at 0.05 and 0.5 µg/L, in quintuplicate and analysed. Recovery of IKF-5411, 3-MTCAM, IBA and PPA was determined and the validity of the analytical procedure was assessed.

Response of the LC-MS/MS system to IKF-5411, 3-MTCAM, IBA and PPA was linear over the range 0.2 to 10 ng/mL. The coefficient of determination (r) for each calibration line was ≥ 0.99.

Control extracts of both surface water and drinking water did not contain any components equivalent to above 30% of the limit of quantification (LOQ) and these did not interfere with the analysis of IKF-5411, 3-MTCAM, IBA and PPA.
The method was considered specific for IKF-5411, 3-MTCAM, IBA and PPA.

INTRODUCTION

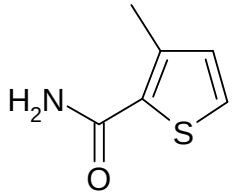
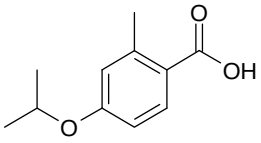
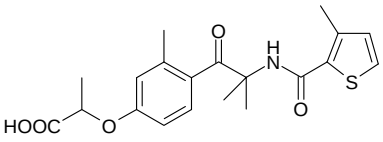
The test substance, IKF-5411, is a novel fungicide currently undergoing development and can be described as follows.



Chemical Name (IUPAC): *N*-[1,1-dimethyl-2-[2-methyl-4-(1-methylethoxy) phenyl]-2-oxoethyl]-3-methyl-2-thiophenecarboxamide (CA)

Molecular Weight: 359.48

3-MTCAM, IBA and PPA are metabolites of IKF-5411 and can be described as below:

3-MTCAM	3-methyl-2-thiophenecarboxamide	
IBA	2-methyl-4-(2-propyloxy)benzoic acid	
PPA	2-[3-methyl-4-[2-methyl-2-(3-methylthiophene-2-carboxamido)propanoyl] phenoxy]propanoic acid	

The study was undertaken to comply with the known data requirements and study guidelines stated on the front cover of the report.

OBJECTIVES

The objective of this study was to develop and validate an analytical method, based upon liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), for measuring residues of IKF-5411 and its metabolites 3-MTCAM, IBA and PPA in surface and drinking water.

PROCEDURES

Protocol Adherence

The study was performed in accordance with the protocol and seven amendments. Any deviations that were noted in study raw data are described in Appendix 6.

Analytical Standards

Analytical grade IKF-5411, 3-MTCAM, IBA and PPA were supplied by the Sponsor. Certificates of Analysis including purity, expiry date and batch number were supplied for each standard.

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

The date of receipt and expiry date, plus batch number and purity details are recorded in the table below, along with the allocated unique identification number.
The analytical standards were stored frozen at <-10°C (nominally -20°C). The Certificates of Analysis are presented in Appendix 1.

Analytical Standard	Unique ID Number	Date Received	Supplier Batch Number	Chemical Purity	Expiry Date
IKF-5411	ESTS 171/11	26 August 2011	20100128	99.9%	31 March 2013
3-MTCAM	ESTS 206/11	28 October 2011	10124091	99.7%	18 September 2016
IBA	ESTS 207/11	28 October 2011	080-013-46-1	99.9%	18 September 2016
PPA	ESTS 208/11	28 October 2011	281-110620-1	99.7%	18 September 2016

Receipt and Storage of Study Samples

Control samples of surface water were sourced locally by Smithers Viscient (ESG) Ltd. from Oak Beck, Harrogate and the drinking water used was Smithers Viscient (ESG) Ltd. tap water.

The surface water and drinking water were both assigned unique identification numbers on receipt, in accordance with departmental Standard Operating Procedures (SOPs), and were stored under refrigeration (2 to 8°C).

Water Characterisation
Full 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)
Surface	Oak Beck	8.0	7.9	107	290

DOC = dissolved organic carbon

Preparation of Study Samples
Control samples of surface water and drinking water did not require any preparation prior to analysis.

Analytical Procedures
Analytical procedure CLE 8261479-01V was developed by Smithers Viscient (ESG) Ltd. All procedures were carried out in compliance with departmental SOPs.

A copy of the analytical procedure is presented in Appendix 3.

During the course of the study a number of draft analytical procedures were developed and attempts were made to validate these. All methods have one or more analytes achieving criteria, however it was desired to have a single analytical procedure and therefore these validations were not reported. A summary of the validation attempts conducted within this summary are detailed in Table 17.

Validation Procedure
The analytical method was validated by fortifying aliquots of untreated control surface water and drinking water with known amounts of IKF-5411, 3-MTCAM, IBA and PPA (in quintuplicate).Fortified and control samples were then subjected to cleanup using Oasis MAX (mixed mode anion exchange) SPE and concentration by evaporation and re-constitution.

Validation included consideration of the criteria in the following sections.

Linearity
In order to establish linearity of response of the analytical chromatographic system to IKF-5411, 3-MTCAM, IBA and PPA eight standard solutions of increasing concentration were prepared over the range 0.2 to 10 ng/mL for validation of surface water and drinking water. The lowest concentration was equivalent to less than 30% of a sample extract at the limit of quantification (LOQ) and the highest concentration was equivalent to greater than 120% of the highest level analysed.

Single determinations at each concentration were made and injected into the chromatograph in random order and concentration/response curves were prepared.

Specificity / Selectivity
The ability of the method to distinguish between IKF-5411, 3-MTCAM, IBA and PPA 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. The overall relative standard deviation (RSD) and RSD at each validation level were determined and these were considered acceptable it they were ≤ 20%.

Recovery
Recovery of IFK-5411, 3-MTCAM, IBA and PPA from control surface water and drinking water fortified at 0.05 (LOQ) and 0.5 µg/L (10 ×LOQ) was determined in quintuplicate.

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

Mean recoveries of IFK-5411, 3-MTCAM, IBA and PPA 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 IFK-5411, 3-MTCAM, IBA and PPA was obtained and a relative standard deviation of ≤ 20% was achieved. For this analytical method, the limit of quantification for IFK-5411, 3-MTCAM, IBA and PPA was proposed to be 0.05 µg/L in both surface water and drinking water.

Limit of Detection (LOD)
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 LC-MS/MS using a second ion transition. The same criteria for precision and recovery (see above) were used to assess this confirmatory method.

Matrix Effect Assessment
Matrix effects were assessed by fortifying final extracts of control water which had been taken through the analytical procedure. These were measured against a non-matrix matched calibration line and determined as a function of expected measured concentration.

Analytical Procedure

Procedure Title	IKF-5411, 3-MTCAM, IBA and PPA: A Method for the Determination of Residues in Surface Water and Drinking Water
Procedure Code	CLE 8261479-01V
Issue Date	18 December 2012
Page Number	1 of 44

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 residues of IKF-5411, 3-MTCAM, IBA and PPA in surface and drinking water. Residues of IKF-5411, 3-MTCAM, IBA and PPA in surface and drinking water are analysed by SPE cleanup and concentration. IKF-5411, 3-MTCAM, IBA and PPA are quantified by UPLC using MS-MS detection. The limit of quantification for this procedure is 0.05 µg/L.

Summary of the Procedure
The method of analysis comprises the following stages:

1. Subjection of 20 mL of sample to Oasis MAX (mixed mode anion exchange) SPE cleanup.
2. Evaporation and re-constitution.
3. Quantification by UPLC/MS-MS using matrix matched calibration.

All procedures will be carried out in compliance with departmental SOP.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware
- UPLC system: Waters Acquity
- MS-MS system: Applied Biosystems API5000
- UPLC Column: Acquity UPLC® C18 BEH 1.7 µm 2.1 x 100 mm
- UPLC Column: Acquity UPLC® C18 BEH 1.7 µm 2.1 x 50 mm
- Waters Oasis MAX 3 cc, 60 mg SPE cartridges
- 50 mL Polypropylene Centrifuge Tubes
- Various laboratory glassware and volumetric flasks
- Various pipettes
- Short form pipettes

With the exception of the analytical column equivalent equipment may be used

Materials

• Acetonitrile (MeCN)	Rathburn, HPLC grade
• Water	Rathburn, HPLC grade
• Methanol (MeOH)	Rathburn, HPLC grade
• 2-Propanol	Rathburn, HPLC grade
• Glacial Acetic Acid	Merck, 100%
• Ammonium hydroxide	Sigma-Aldrich, 28-30%
• Sodium hydroxide solution	Sigma-Aldrich, 50%

Equivalent or better grade reagents/solvents may be used.

Reagents and Solutions [1a/b, 4d]
Acetonitrile:water (25:75 v/v)
Mix together 250 mL of acetonitrile and 750 mL of water.

MAX SPE Conditioning Solution- 5M Sodium Hydroxide Solution
In a 100 mL volumetric flask already containing approximately 60 mL of water add 26 mL of 50% NaOH and mix. Whilst swirling add water to dilute to the 100 mL mark and mix well. Transfer to an appropriately sized plastic container.

MAX SPE Wash Solution- 5% Ammonia in water
In a 100 mL volumetric flask already containing approximately 60 mL of water add 17.9 mL of 28-30% (assume 28%) ammonium hydroxide and mix. Whilst swirling add water to dilute to the 100 mL mark and mix well. Transfer to an appropriately sized glass container.

MAX SPE Elution Solution- 4% Acetic acid in methanol
In a 250 mL volumetric flask already containing approximately 200 mL of water add 10 mL of glacial acetic acid and mix. Whilst swirling add water to dilute to the 250 mL mark and mix well. Transfer to an appropriately sized glass container.

Mobile Phase A1- 0.1% Acetic Acid in Water
Mix together 1 mL of glacial acetic acid and 1000 mL of water. Sonicate to degas.

Mobile Phase B1- 0.1% Acetic Acid in Methanol
Mix together 1 mL of glacial acetic acid and 1000 mL of methanol. Sonicate to degas.

Mobile Phase A2- 0.2% Acetic Acid in Water
Mix together 2 mL of glacial acetic acid and 1000 mL of water. Sonicate to degas.

Mobile Phase B2- 0.2% Acetic Acid in Acetonitrile
Mix together 2 mL of glacial acetic acid and 1000 mL of methanol. Sonicate to degas.

Weak and Strong Wash- Water: MeOH: MeCN: 2-Propanol (1:1:1:1 v/v/v/v)
Mix 250 mL water with 250 mL methanol, 250 mL acetonitrile and 250 mL of 2-propanol. Sonicate to degas.

Preparation can be scaled up or down as appropriate.

Standard Solution Preparation [1a/b, 4b/4d]

Prepare analytical standard stock solutions separately for each compound, corrected for purity, in MeCN using volumetric flasks. The amount weighed must be at or slightly above the desired weight so that the solvent volume can be corrected to ensure that these stock solutions and their subsequent dilutions are whole units, (1000 µg/mL for example). Stock solutions are given a nominal expiry date of one month when stored refrigerated. Recommend weighing nominal 10 mg of standard into 10 mL glass volumetric flasks.

If a correlation is required prepare stock solutions in duplicate. If an acceptable correlation is achieved use one of the duplicate stocks to prepare both fortification and calibration solutions.

Standard Correlation

Standard correlation is achieved by analysis of each of the duplicate stock solutions. It is recommended the two solutions are diluted to a concentration near the mid-calibration point. Inject each solution alternately in a run sequence at least 5 times each using the relevant instrument conditions. Correlation is calculated using chromatographic peak areas. Standard acceptance criteria is correlation of $\pm$ 2%.

Calibration Standards [1a/b, 4b]

Prepare appropriate mixed calibration standards in acetonitrile: water (25:75 v/v) to cover the range 0 to 10 ng/mL, with a lowest calibration level being 0.2 ng/mL. Calibration standards may be stored refrigerated for up to one month from the preparation of the relevant stock solutions. See the table below for the recommended preparations. Use volumetric flasks.

Preparation of calibration standards:

Standard (ng/mL)	Analyte	Volume of Standard (mL)	Final Volume (mL)	Final concentration/ID (ng/mL)
1000000	IKF-5411	0.1	10*	10000#
1000000	3-MTCAM	0.1		
1000000	PPA	0.1		
1000000	IBA	0.1		
10000	Mixed	0.1	10	100
100	Mixed	1.0	10	10
100	Mixed	0.8	10	8
100	Mixed	0.5	10	5
100	Mixed	0.2	10	2.0
100	Mixed	0.1	10	1.0
10	Mixed	0.5	10	0.5
10	Mixed	0.4	10	0.4
10	Mixed	0.2	10	0.2

*Combine the aliquots of the four different analyte stocks into one 10 mL glass volumetric flask.
Mixed analyte standard

QC Fortification Solutions [1a/b, 4b]

Assuming a sample volume of 20 mL and a fortification volume of 100 µL, the following fortification solution preparations (using MeCN) are recommended for LOQ (0.05 µg/L) and 10xLOQ (0.5 µg/L). Use volumetric flasks.

Preparation of QC fortification solutions:

Standard (ng/mL)	Analyte	Volume of Standard (mL)	Final Volume (mL)	Final concentration/ID (ng/mL)
1000000	IKF-5411	0.1	10*	10000#
1000000	3-MTCAM	0.1		
1000000	PPA	0.1		
1000000	IBA	0.1		
10000	Mixed	0.1	10	100
100	Mixed	1	10	10

*Combine the aliquots of the four different analyte stocks into one 10 mL glass volumetric flask.
Mixed analyte standard

PROCEDURES

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.

Notes:
After each addition of reagent/sample allow the meniscus to reach level with the top of the sorbent before adding the next reagent. Do not allow the cartridges to run dry unless otherwise stated.

It is recommended that approximately 1 mL of sample is added to the cartridge before the reservoir is fitted (step 3) so that the cartridge is constantly wetted.

Analysis of surface and drinking water [1a/b, 4a]

1. Measure 20 mL of surface or drinking water sample into a 50 mL polypropylene centrifuge tube. Untreated control samples may be fortified at this point for the determination of procedural recovery.
2. Condition an Oasis MAX 3 cc, 60 mg SPE cartridge with 2 mL of Methanol followed by 1 mL of 5M Sodium Hydroxide and 2 mL of water. Discard the eluates.
3. Fit an appropriately sized reservoir to the cartridge and load the sample onto the cartridge at a rate of approximately 1 mL per minute. Discard the eluate.
4. Wash the cartridge with 2 mL of water followed by 1 mL of 5% ammonia in water and 1 mL of water. Dry the cartridge under vacuum for approximately two minutes. Discard the eluates.
5. Fit a 10 mL glass TurboVap tube under the cartridge and elute the sample with 5 mL of 4% acetic acid in methanol.
6. Evaporate the sample using a TurboVap set at 40°C until a small amount of residual water is present.
7. Add 0.5 mL of acetonitrile to the tube to re-constitute. Aid re-constitution by brief sonication and vortex mixing. Transfer to a 2 mL glass volumetric flask
8. Add 1.5 mL of water to the tube, sonicate and vortex mix briefly and transfer to the same 2 mL glass volumetric flask. Dilute to the 2 mL mark with water, cap and mix well.
9. The samples are now ready for UPLC/MS-MS analysis.

Note: IKF-5411, 3-MTCAM and PPA are analysed using a different set of conditions from IBA including differences in the column used and the mobile phases. Both methods are described below.

All extracts derived during or from this procedure may be stored refrigerated if storage is required.

Conditions for UPLC Analysis with Mass spectrometry Detection (IKF-5411, 3-MTCAM and PPA only)

UPLC Conditions

UPLC column#	Acquity UPLC® C18 BEH 1.7 µm 2.1 x 100 mm
In line filter (Acquity)	Supplier: Waters Part n/o 700002775
Column oven temperature#	Nominal 40°C
Autosampler temperature	Nominal 5°C
Mobile phase A1#	0.1% acetic acid in water
Mobile phase B1#	0.1% acetic acid in methanol
Flow rate#	0.6 mL/min to 0.8 mL/min (see table below)
Gradient settings:	See table below

Time (minutes)	Flow rate (mL/min)	A1 (%)	B1 (%)
0.00	0.6	95	5
0.50	0.6	95	5
3.00	0.6	5	95
3.50	0.8	2	98
4.50	0.8	2	98
4.51	0.8	95	5
5.50	0.6	95	5

Switching Valve times	0 – 0.1 mins – To waste 0.1 – 5 mins – To MS
Slave pump solvent	MeOH: water (1:1 v/v)
Wash solvent 1#	MeCN: MeOH: water: 2-propanol (1:1:1:1 v/v/v/v)
Weak Wash/Volume	(4990 µL)
Wash solvent 2#	MeCN: MeOH: water: 2-propanol (1:1:1:1 v/v/v/v)
Strong Wash/Volume	(4990 µL)
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)	40 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)	650°C
Q1 Resolution	Unit
Q3 Resolution	Unit
Interface Heater Status	Off
Analysis time	5.0 minutes (± 1 minute)

Compound name	Ions monitored (± 0.5 Da)	Dwell time (ms)	Declustering Potential (DP) Volts	Collision Energy (CE) Volts	Collision Cell Exit Potential (CXP) Volts
IKF-5411_Quant	360.2→210.1	75	60	15	14
IKF-5411_Conf	360.2→182.1	75	60	23	12
PPA_Quant	390.2→210.1	75	80	16	15
PPA_Quant	390.2→182.1	75	80	23	15
3-MTCAM_Quant	142.0→125.0	75	70	23	16
3-MTCAM_Conf	142.0→99.0	75	70	23	12

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

Conditions for UPLC Analysis with Mass spectrometry Detection (IBA only)

UPLC Conditions

UPLC column#	Acquity UPLC® C18 BEH 1.7 µm 2.1 x 50 mm
In line filter (Acquity)	Supplier: Waters Part n/o 700002775
Column oven temperature#	Nominal 40°C
Autosampler temperature	Nominal 5°C
Mobile phase A2#	0.2% acetic acid in water
Mobile phase B2#	0.2% acetic acid in acetonitrile
Flow rate#	0.6 mL/min to 0.8 mL/min (see table below)
Gradient settings:	See table below

Time (minutes)	Flow rate (mL/min)	A2 (%)	B2 (%)
0.0	0.6	55	45
0.2	0.6	55	45
1.1	0.6	10	90
1.2	0.8	2	98
1.5	0.8	2	98
1.6	0.8	55	45
1.8	0.6	55	45

Switching Valve times	0 – 0.3 mins – To waste 0.3 – 1.1 mins – To MS
Slave pump solvent	MeOH: water (1:1 v/v)
Wash solvent 1#	MeCN: MeOH: water: 2-propanol (1:1:1:1 v/v/v/v)
Weak Wash/Volume	(4990 µL)
Wash solvent 2#	MeCN: MeOH: water: 2-propanol (1:1:1:1 v/v/v/v)
Strong Wash/Volume	(4990 µL)
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)	30 µL (this may vary depending on instrument performance)

Mass Spectrometer Parameters API 5000

Mode of operation#	Turbo IonSpray (negative 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)	-4500 V
Nebuliser Current (NC)	3.0 µA
Temperature (TEM)	650°C
Q1 Resolution	Unit
Q3 Resolution	Unit
Interface Heater Status	On
Analysis time	1.8 minutes (± 1 minute)

Compound name	Ions monitored (± 0.5 Da)	Dwell time (ms)	Declustering Potential (DP) Volts	Collision Energy (CE) Volts	Collision Cell Exit Potential (CXP) Volts
IBA_Quant	193.1→106.0	100	-100	-31	-12
IBA_Conf	193.1→149.1	100	-105	-20	-9

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

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

The presence of IKF-5411, 3-MTCAM, IBA and PPA in a sample is confirmed if the resulting peaks arising from the test sample have the same chromatographic retention times as the appropriate standard. All peak measurements and calculations are performed using a data system (Analyst 1.5.1).
Residues of IKF-5411, 3-MTCAM, IBA and PPA are determined by the interpolation of the peak area of IKF-5411, 3-MTCAM, IBA and PPA, from the standard regression equation, as follows:
The calibration line is determined by plotting the responses from the calibration solutions (R) against the of amount test substance 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 substance (A) in sample extracts are calculated from their response using the equation:
Concentration of extract A (ng/mL) = (peak area – intercept)/slope
The use of weighted least squares regression (1/x) for the calculation of intercept and slope is recommended for calibration lines with a range in excess of 100. Standard linear regression and weighted least squares regression (1/x) calculations are performed using suitable validated software.
The residue of IKF-5411, 3-MTCAM, IBA and PPA in each test sample is calculated as follows. Note µg/L = ng/mL.

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

Where the final volume includes dilution steps, if applicable.
Procedural recovery data from fortified samples are calculated via the following equation:

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

Where:-
A = concentration found in fortified sample (µg/L)
C = concentration (or interference) found in control sample (µg/L)
S = concentration added to fortified sample (µg/L)

Linearity of Response
The linearity of response of the UHPLC system should be determined with at least six different concentration matrix matched standard solutions across the range 0 to 10 ng/mL, with the lowest calibration level being 0.2 ng/mL.

METHOD CRITERIA

Analysis by UPLC/MS-MS will be considered successful only if the following criteria are met.

- A procedural recovery of 70 to 110% will be obtained for each batch of analysis
- Control sample contains a concentration ≤ 30% of the limit of quantification
- At least 6 calibration standards will be used in the determination of each calibration line
- For 1/x weighing a coefficient of determination (r^2) for each calibration line will be ≥ 0.98
- All test samples will be within the appropriate calibration standards range

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 shied 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)

REVISION HISTORY

CLE 8261479-02D
Due to the need to use matrix matched calibration (due to significant matrix effects) the method has been altered to describe the procedure for include matrix matched calibration where CLE 8261479-01D employed pure solvent standard calibration.

CLE 8261479-03D
As the response generated from the confirmation ions was insufficient to quantify the LOQ samples for some the analytes, the method has been altered to include an SPE procedure where the samples can be concentrated so as to generate a higher response. All mean recoveries were within the acceptable range of 70 to 110%. Precision of the method was acceptable (relative standard deviation, RSD ≤ 20%) for IKF-5411, 3-MTCAM, IBA and PPA, in both surface water and drinking water.

Control extracts of surface water and drinking water did not contain any components equivalent to above 30% of the limit of quantification (LOQ) and these did not interfere with the analysis of IKF-5411, 3-MTCAM, IBA and PPA. The method was considered specific for IKF-5411, 3-MTCAM, IBA and PPA

Results for mean recovery and precision supported a limit of quantification (LOQ) of 0.05µg / L in surface and drinking water for IKF-5411, 3-MTCAM, IBA and PPA.

Appendix 6
Protocol Deviations

All validations in water were performed using non-matrix matched calibration lines. Matrix assessments were performed on all analytes in both water types and in both transitions. All assessments showed matrix effects not to be significant (<20%) with the exception of PPA. This deviates from guideline SANCO825/00 rev 8.1 cited in the protocol which states:

Calibration should be generated using standards prepared in blank matrix extracts (matrix matched standards) for all sample materials included in the corresponding validation study. Only, if experiments clearly demonstrate that matrix effects are not significant (i.e. <20 %), calibration with standards in solvent may be used.

The water tested for the matrix assessment was not representative of it at the time of validation, due to it being in storage for approximately five months. It is therefore argued that the validation was correctly performed at the time and the use of matrix-matched standards were unnecessary. This had no impact on the integrity of the results reported.