Cover Sheet for Analytical Method

Pyriofenone in Soil - MRID 49256126

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# 2 PURPOSE

The purpose of the study was to validate a residue analytical method for the determination of IKF-309 in agricultural soil.

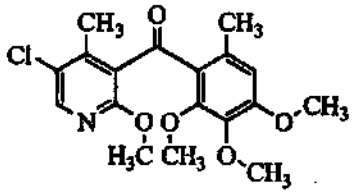
For IKF-309 in agricultural soil a limit of quantification (LOQ) of 0.001 mg/kg and a working range from 0.001 mg/kg to 0.300 mg/kg were successfully validated.

# 3 MATERIALS AND METHODS

## 3.1 TEST ITEM

### 3.1.1 IKF-309

The test item will be used as reference item (analytical standard) and was provided by the Sponsor. All information concerning the test item was provided by the Sponsor.

| | |
|---|---|
| Product name: | IKF-309 |
| Chemical name: | (5-chloro-2-methoxy-4-methyl-3-pyridinyl) (2,3,4-trimethoxy-6-methylphenyl)methanone (CA) |
| CAS registry no.: | 688046-61-9 |
| Chemical structure: |  |
| Molecular formula: | $C_{18}H_{20}ClNO_5$ |
| Molecular mass: | 365.8 g/mol |
| Batch number: | 0608 |
| Purity: | 99.19% (w/w) |
| Expiry date: | September 21, 2008 |
| Storage conditions: | Keep frozen (approximately -20°C) |

## 3.2 TEST SYSTEMS

The test systems (four agricultural soils) were selected by the Sponsor in accordance with the quoted guidelines. The following agricultural soils were used:

| Action | Agricultural soil from (location) | Country |
|---|---|---|
| Full validation | Worcester | United Kingdom |
| Cross validation | Muhlhouse | France |
| | Stolpe | Germany |
| | Asti | Italy |

### 3.2.1 Sample preparation

The soil specimens were sieved through a 2 mm sieve and kept at approximately –20°C (in a deep freezer).

The water content of each sample was determined prior to analysis. For this procedure, 10.0 g of the wet soil was weighed into a glass container and dried over night at approximately 100–120°C after which the soil sample was re-weighed and the soil moisture determined. The residues in treated samples were measured on dry soil weight basis. The calculation used for initial weight for analysis corresponding to 10 g dry weight is shown below:

$X = (a \times c) / b$

x = weight of wet soil corresponding to 10 g dry soil
a = weight of dry soil for analysis (10 g)
b = weight of dry soil after drying
c = initial weight of wet soil for moisture determination (10 g)

### 3.2.2 Soil characteristics

The soil parameters were determined by AgroLab Swiss GmbH; Oberfeld 3;6037 Root; Switzerland.

| Parameters | Worcester (United Kingdom) |
|---|---|
| pH-Value ($CaCl_2$) | 6.57 |
| Organic carbon (%) | 1.90 |
| Cation exchange capacity (mmol/100 g soil) | 22.63 |
| Water Holding Capacity, pF 1.0 (%) | 55.65 |
| Soil classification (USDA) | clay loam |
| < 2 µm (%) | 34.17 |
| 2 µm - 50 µm (%) | 31.08 |
| 50 µm - 2000 µm (%) | 34.75 |

| Parameters | Muhlhouse (France) |
|---|---|
| pH-Value ($CaCl_2$) | 6.98 |
| Organic carbon (%) | 1.66 |
| Cation exchange capacity (mmol/100 g soil) | 16.28 |
| Water Holding Capacity, pF 1.0 (%) | 58.61 |
| Soil classification (USDA) | silt loam |
| < 2 µm (%) | 22.22 |
| 2 µm - 50 µm (%) | 51.97 |
| 50 µm - 2000 µm (%) | 25.81 |

| Parameters | Asti (Italy) |
|---|---|
| pH-Value ($CaCl_2$) | 7.39 |
| Organic carbon (%) | 1.06 |
| Cation exchange capacity (mmol/100 g soil) | 15.15 |
| Water Holding Capacity, pF 1.0 (%) | 67.84 |
| Soil classification (USDA) | loam |
| < 2 µm (%) | 24.98 |
| 2 µm - 50 µm (%) | 47.25 |
| 50 µm - 2000 µm (%) | 27.78 |

Determination of soil from Stolpe is still running

| Parameters | Stolpe (Germany) |
|---|---|
| pH-Value ($CaCl_2$) | 5.00 |
| Organic carbon (%) | 0.59 |
| Cation exchange capacity (mmol/100 g soil) | 5.12 |
| Water Holding Capacity, pF 1.0 (%) | 30.75 |
| Soil classification (USDA) | sand |
| < 2 µm (%) | 3.78 |
| 2 µm - 50 µm (%) | 4.14 |
| 50 µm - 2000 µm (%) | 92.08 |

## 3.3 REAGENTS AND APPARATUS

All reagents were of analytical, residue analytical or HPLC grade.

| REAGENTS & APPARATUS | SUPPLIER | ARTICLE NO. |
|---|---|---|
| Acetonitrile | Baker | 9017 |
| Methanol | Baker | 8402 |
| Hydrochloric acid (32%) | Baker | 6070 |
| Formic acid (98%) | Baker | 6037 |
| Water (ELGA ultra purified) | RCC | |
| SPE Cartridge: Oasis HLB VAC RC (60 mg) | Waters | 186000381 |
| Volumetric pipettes | various sizes | |
| Measuring pipettes | various sizes | |
| Measuring cylinders | various sizes | |
| Volumetric flasks | various sizes | |
| Glass flasks with screw tops | various sizes | |
| Centrifuge tubes (50 mL, plastic) | Sarstedt | 62.548.004 |
| Test tubes | various sizes | |
| Beakers | various sizes | |
| SPE manifold | Waters | 7208-00 |
| Pasteur pipettes | Volac | various sizes |
| µL-Pipettes | Gilson | various sizes |
| Syringes (2 mL) | Becton Dickinson | BD Discardit II 300928 |
| 0.45 µm PTFE filter | BGB | F 2504-2 |
| HPLC vials (2 mL) | BGB | 080401 |
| Screw tops for HPLC vials | BGB | 11030201 |
| Centrifuge | Heraeus (Kendro) | Cryofuge 6000i |
| Laboratory shaker | B. Braun | Certomat U |
| Ultra sonic bath | Branson | 2510 |
| Analytical balance | Mettler | UMT-2 |
| Laboratory balance | Satorius | L2200P |
| Oven (drying cupboard) | Memmert | |

### 3.3.1 PREPARATIONS

Acetonitrile / water / hydrochloric acid (800+200+5):
Acetonitrile (800 mL), ELGA water (200 mL) and hydrochloric acid (32%) were mixed.

Acetonitrile / water (1+1):
Acetonitrile (500 mL) and ELGA water (500 mL) were mixed.

HPLC wash solvent 1 [water / methanol / formic acid (80+20+0.5)]:
ELGA water (80 mL), methanol (20 mL) and formic acid (0.5 mL) were mixed.

HPLC wash solvent 2 [water / acetonitrile / methanol (10+45+45)]:
ELGA water (10 mL), acetonitrile (45 mL) and methanol (45 mL) were mixed.

HPLC eluent A: [water / acetonitrile / formic acid (95+5+0.1)]:
ELGA water (950 mL), acetonitrile (50 mL) and formic acid (1 mL) were mixed.

HPLC eluent B: [water / acetonitrile / formic acid (5+95+0.1)]:
ELGA water (50 mL), acetonitrile (950 mL) and formic acid (1 mL) were mixed.

## 3.4 STANDARD SOLUTIONS

### 3.4.1 STOCK SOLUTION

The test item (13.51 mg of a 99.19% analytical standard) was weighed into a 20 mL sealable test tube. Acetonitrile (13.40 mL) was added and the mixture was treated for approximately 5 minutes in an ultra sonic bath to obtain a stock solution of 1000 µg/mL IKF-309 (100%).

The solution was kept at approximately –20°C (in a deep freezer).

### 3.4.2 FORTIFICATION SOLUTIONS

A defined volume (1000 µL) of the stock solution of 1000 µg/mL IKF-309 was diluted to 10 mL using acetonitrile to obtain a solution with a concentration of 100 µg/mL IKF-309.

A defined volume (1000 µL) of the solution of 100 µg/mL IKF-309 was diluted to 10 mL using acetonitrile to obtain a fortification solution with a concentration of 10 µg/mL IKF-309.

A defined volume (1000 µL) of the fortification solution of 10 µg/mL IKF-309 was diluted to 10 mL using acetonitrile to obtain a solution with a concentration of 1 µg/mL IKF-309.

A defined volume (1000 µL) of the solution of 1 µg/mL IKF-309 was diluted to 10 mL using acetonitrile to obtain a further fortification solution with a concentration of 0.1 µg/mL IKF-309.

This solutions were kept at approximately 4°C (in a refrigerator).

### 3.4.3 CALIBRATION SOLUTIONS

For example:

A defined volume (500 µL) of the fortification solution of 0.1 µg/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 5 ng/mL IKF-309.

A defined volume (200 µL) of the fortification solution of 0.1 µg/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 2 ng/mL IKF-309.

A defined volume (100 µL) of the fortification solution of 0.1 µg/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 1 ng/mL IKF-309.

A defined volume (1000 µL) of the calibration solution of 5 ng/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 0.5 ng/mL IKF-309.

A defined volume (1000 µL) of the calibration solution of 2 ng/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 0.2 ng/mL IKF-309.

A defined volume (1000 µL) of the calibration solution of 1 ng/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 0.1 ng/mL IKF-309.

A defined volume (1000 µL) of the calibration solution of 0.5 ng/mL IKF-309 was diluted to 10 mL using acetonitrile/ ELGA water (1+1) to obtain a calibration solution with a concentration of 0.05 ng/mL IKF-309.

These solutions were kept at approximately 4°C (in a refrigerator).

Quantification was performed using a HPLC-MS/MS.

IKF-309 calibration standards were injected concurrently with IKF-309 specimen injections for the determination of the retention time and for preparing the standard calibration curve. Each analytical run was started and ended with a IKF-309 calibration standard injection. A maximum of two specimen injections were made between IKF-309 calibration standard injections.

## 3.5 FORTIFICATION

To demonstrate the validity of the method used, untreated specimens were fortified with different amounts of IKF-309.

**Fortification levels for soil:**

0.001 mg/kg: 100 µL of the fortification solution (0.1 µg/mL*) were added to 10 g (dry mass) soil.

0.300 mg/kg: 300 µL of the fortification solution (10 µg/mL*) were added to 10 g (dry mass) soil.

*: 10 µg/mL and 0.1 µg/mL fortification solution dissolved in acetonitrile, see section 3.4.2.

## 3.6 ANALYTICAL METHOD

### 3.6.1 Extraction

Before the preparation of samples, the water content of the soils were determined as described in section 3.2.1. Ten gram dry weight equivalent of the untreated soil sample was weighed into a 50 mL centrifuge tube with screw top. The specimen was fortified at this stage of the procedure as described in section 3.5. A defined volume of acetonitrile / water / hydrochloric acid (800+200+5) was added to the soil sample to give a extraction volume of 40 mL.

| Agricultural soil from (location) | Weight of wet soil, corresponding to 10.0 g dry mass | Acetonitrile / water / hydrochloric acid (800+200+5) added to a final volume of 40 mL |
|---|---|---|
| Worcester, United Kingdom | 12.25 g | 37.75 mL |
| Muhlhouse, France | 11.88 g | 38.12 mL |
| Stolpe, Germany | 10.55 g | 39.45 mL |
| Asti, Italy | 10.85 g | 39.15 mL |

The specimen was shaken for approximately 30 minutes using a laboratory shaker at approximately 150 rpm. Afterwards the mixture was centrifuged for 10 minutes at 3000 rpm.

### 3.6.2 Sample clean up on SPE

A SPE cartridge (Oasis HLB VAC, 60 mg) was placed onto a SPE vacuum manifold and conditioned using methanol (5 mL) followed by ELGA water (5 mL). An aliquot of 4 mL of the clear extract from section 3.6.1 (corresponding to 1.0 g dry soil) was transferred into a 50 mL centrifuge tube. ELGA water (20 mL) was added and the sample was mixed. The aqueous sample solution was transferred into the SPE cartridge and sucked through the column. After there, the cartridge was sucked to dryness for a few seconds. All elutes were discarded. IKF-309 was eluted with acetonitrile (5 mL). The fraction was collected into a 10 mL volumetric flask and the cartridge was sucked to dryness for a few seconds. The volumetric flask was filled up to the mark with ELGA water and the sample was mixed.

### 3.6.3 PREPARATION FOR THE HPLC-MS/MS-ANALYSIS

An aliquot of the extract from section 3.6.2 was filtered through a 0.45 µm PTFE filter into a 2 mL HPLC vial and capped immediately for injection. Soil samples, fortified with 0.3 mg/kg IKF-309 were 1+19 diluted with acetonitrile / ELGA water (1+1). The concentration of IKF-309 in soil specimen was determined after HPLC separation using a MS/MS detector.

## 3.7 HPLC-MS/MS CONDITIONS

### 3.7.1 Part A: HPLC

**Instrumentation:**

| | |
|---|---|
| Autosampler: | CTC PAL |
| Pumps: | High pressure gradient system consisting of 2 Shimadzu LC-10AD pumps and a Shimadzu SCL System Controller |

**Injector Washing Procedure:**

| | |
|---|---|
| Wash Solvent 1: | 80 vol. water + 20 vol. methanol + 0.5 vol. formic acid |
| Wash Solvent 2: | 10 vol. water + 45 vol. acetonitrile + 45 vol. methanol |
| Wash Sequence: | 1 x pre clean with sample<br>2 x syringe and 2 x injection port with each wash solvent |

**Injection Volume:** 10 µL (typical value)

**Mobile Phase:**

| | |
|---|---|
| Eluent A: | 95 vol. water + 5 vol. acetonitrile + 0.1 vol. formic acid |
| Eluent B: | 5 vol. water + 95 vol. acetonitrile + 0.1 vol. formic acid |

| Time [minutes] | 0 | 2.0 | 2.5 | 2.6 | 4.0 |
|---|---|---|---|---|---|
| A [%] | 50 | 0 | 0 | 50 | 50 |
| B [%] | 50 | 100 | 100 | 50 | 50 |
| Flow [µL/minutes] | 300 | 300 | 300 | 300 | 300 |

**Column:**
Stationary Phase: GL Sciences; Inertsil ODS-3; Particle size: 3 µm
Dimension: 2.1 mm x 50 mm
Backpressure: approximately 70 bar at t = 0 minutes

**Flow split to MS:**
no

**Divert Valve setting:**
LC-flow to MS: 1.7 minutes – 3.1 minutes
Back-up flow: 50 µL / minute of Eluent B

**Retention Time** (typical value):
IKF-309: 2.3 minutes

### 3.7.2 Part B: API-MS/MS

**Instrumentation:**
Mass spectrometer: API 4000; triple stage quadrupole mass spectrometer MDS Sciex, Toronto, Canada;
Ionization mode: Pneumatically and thermally assisted electrospray ionization (ESI) Ion Source: Sciex Turbo-V-Source
Gases: Nebulizer Gas ('Gas1'): Air; Heater Gas ('Gas2'): Air; Curtain Gas: Nitrogen; Collision Gas: Nitrogen
Software: Analyst (Applied Biosystems/MDS Sciex)

**MS Parameters:**
Ion Source: Heater Gas Temperature: 550°C; Spray Voltage: 3500 V, Interface heater: on
Sprayer position: Vertical: 2.3 mm, Lateral/Right: 7.8 mm
Gas flow settings: Gas 1: 20, Gas2: 25; CUR: 25; CAD: 6 (settings in arb. units)
Scan Mode: Multiple Reaction Monitoring (MRM)
Mass Resolution: Q1: unit resolution; Q3: unit resolution
Ion energies: IE1= 1.0 eV; IE3 = -0.5 eV
Multiplier settings: DF: -100 V, CEM: 2000 V

**Primary method:**

| Analyte | Ion Polarity | Precursor Ion | Product Ion | Collision. Energie (eV) | Dwell Time (ms) |
|---|---|---|---|---|---|
| IKF-309 | pos./$[M+H]^+$ | *m/z* 366.0 | *m/z* 184.3 | 32 | 200 |

**Confirmatory method:**

| Analyte | Ion Polarity | Precursor Ion | Product Ion | Collision. Energie (eV) | Dwell Time (ms) |
|---|---|---|---|---|---|
| IKF-309 | pos./$[M+H]^+$ | *m/z* 366.0 | *m/z* 209.3 | 37 | 200 |

**Ion Path Parameters:**

| Analyte | DP (V) | EP(V) | IQ1(V) | ST(V) | IQ2(V) | CXP (V) |
|---|---|---|---|---|---|---|
| IKF-309 | 75 | 10 | -11 | -17 | -20 | 17 |

Abbreviatons: NEB: nebulizer gas; CUR: curtain gas; CAD: collision gas; DP: declustering potential; FP: focusing potential; EP: entrance potential; IQ1: focusing (interquadrupol) lens; ST: prefilter (stubbies); CXP: collision cell exit potential; CE: collision energy; IE1: ion energy at Q1; IE3: ion energy at Q3; DF: deflector potential; CEM: electron multiplier voltage

## 3.8 EVALUATION OF RESULTS

### 3.8.1 Concentration of residues

Injected specimens were quantified by peak area with reference to the calibration curve. The latter was obtained by correlation of the peak area of the analytical standards with their corresponding concentration in ng/mL. The calibration was performed using standards in the range of 0.05 - 5.0 ng/mL (corresponding to 0.5 µg/kg – 50.0 µg/kg IKF-309 in a 10 g soil sample).

The correlation was performed using a least squares fit of a linear function (equation 1).

Regression Model: y = a + b * x, weighting 1 / y

where

*y* = Peak area of injected specimen [counts]

*x* = IKF-309 in injected specimen [ng/mL]

*a* = y-axis intercept

*b* = Slope

The residue of IKF-309 in the specimen is calculated according to equation 2.

$$R = \frac{X \cdot V_F \cdot D}{W \cdot 1000} \qquad (2)$$

where

R = Residue of IKF-309 in specimen material [mg/kg]

X = Concentration of injected specimen [ng/mL] calculated from equation 1

$V_F$ = Final volume [10 mL]

D = Dilution Factor [if applicable]

W = Aliquot of specimen [1.0 g (dry mass)]

1000 = Conversion factor from ng to µg

The recovery of IKF-309 in the specimen is calculated according to equation 3.

$$Rec = \frac{R \cdot 100}{F} \qquad (3)$$

where

Rec = Recovery of IKF-309 [%]

R = Residue of IKF-309 in specimen material [mg/kg]

F = Fortification level [mg/kg]