

Cover Sheet for Analytical Method

Pyriofenone in Soil - MRID 49256127

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SCHEDULE OF EVENTS

Study Initiation Date: March 17, 2011
Experimental Start Date: March 24, 2011
Experimental Termination Date: March 30, 2011

RETENTION OF DATA

Upon completion of the study, the complete study file including all original raw data was submitted to the Ricerca Biosciences, LLC Corporate Archives for storage.

CONDUCT OF THE STUDY

The study was conducted at the Ricerca Biosciences, LLC AgChem Product Development Department Laboratories according to the Ricerca Biosciences, LLC protocol "Independent Laboratory Validation (ILV) of the Residue Analytical Method for Detection of IKF-309 in Soil (RCC Study #B18843)," Document Number 027336-0.

INTRODUCTION

IKF-309 is under development as an agricultural crop protection agent. As part of the registration, scientists at RCC, Ltd. developed an analytical method for detection of IKF-309 in agricultural soil. This report describes the Independent Laboratory Validation (ILV) of this method.

OBJECTIVE/PURPOSE

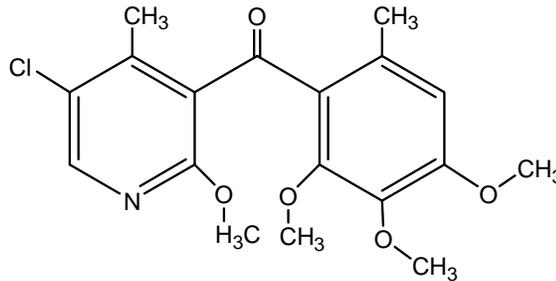
The purpose of this study was to perform an independent laboratory validation of the method of analysis for detection of IKF-309 in agricultural soil.

TEST SUBSTANCE

The sponsor supplied the test substance, IKF-309. The certificate of analysis is included in Appendix A.

The standard and the stock solutions made from them were stored under freezer conditions (~ -20 °C). Solubility and stability data for the reference substances, expiration and storage conditions were the responsibility of the Sponsor. Information concerning the test substance including purity is provided below:

- **IKF-309**



| | |
|--------------------|--|
| Common Name: | IKF-309 |
| Chemical Name: | (5-Chloro-2-methoxy-4-methyl-3-pyridinyl) (2,3,4-trimethoxy-6-methylphenyl)methanone (CA) |
| CAS No.: | 688046-61-9 |
| Molecular Formula: | C ₁₈ H ₂₀ ClNO ₅ |
| Batch Number: | 0608 |
| Purity: | 99.19% |
| Expiration Date: | 09/2013 (per Sponsor) |
| Storage: | Frozen (~ -20°C) |

TEST SYSTEM

The test system (North Dakota Soil IB-2010-JLW-004-01-030) was supplied by the Sponsor and stored at ~ -20 0°C (in a freezer) until analysis. The soil parameters are summarized in the table below. The certificate of analysis is included in Appendix A.

| Parameters | | |
|---|------------|------|
| Percent Sand | 67 | |
| Percent Silt | 15 | |
| Percent Clay | 18 | |
| USDA Textural Class (Hydrometer method) | Sandy loam | |
| Bulk Density (disturbed) gm/cc | 1.16 | |
| Cation exchange capacity (meq/100g) | 18.6 | |
| % Moisture at 1/3 Bar | 24.4 | |
| % Moisture at 15 Bar | 12.3 | |
| % Organic Matter –Walkley Black | 2.5 | |
| pH in 1:1 soil:water ratio | 7.3 | |
| Base Saturation Data | | |
| Cation | Percent | ppm |
| Calcium | 69.5 | 2587 |
| Magnesium | 15.9 | 355 |
| Sodium | 0.4 | 17 |
| Potassium | 3.0 | 220 |
| Hydrogen | 11.2 | 21 |

The water content of the soil was determined prior to the study. Three aliquots of the wet soil were weighed into aluminum weighing dishes and dried over the weekend at approximately 100 °C after which the soil samples were re-weighed and the soil moisture determined to be 21.9%. 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 weight soil for moisture determination (10 g)

Therefore, $X = (10 \times 10)/7.81 = 12.8 \text{ g}$

ANALYTICAL METHODOLOGY

EXTRACTION

Samples were extracted according to the method provided by the Sponsor:

1. Approximately 12.8 g of soil samples were weighed into 50-mL polypropylene centrifuge tubes and fortified.
2. 37.2 mL of acetonitrile (ACN)/HPLC water/hydrochloric acid (HCl) (800/200/5) was added to the soil sample to give an extraction volume of 40 mL.
3. The samples were shaken using a laboratory shaker for 30 minutes.
4. The samples were centrifuged for 10 minutes at ~3000 rpm.
5. An aliquot of 4 mL of the extract was removed and mixed with 20 mL of HPLC water.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge was placed on an SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge was loaded with the sample from step # 5 and the cartridge was sucked with vacuum to dryness. The eluent was discarded.
3. IKF-309 was eluted with 5 mL of acetonitrile. The fraction was collected into a 10-mL volumetric flask and the cartridge was sucked with vacuum to dryness.
4. The volumetric flask was filled to the mark with HPLC water and the sample was mixed.

5. Samples fortified with 0.001 mg/kg IKF-309 and control soil samples were directly analyzed by LC-MS/MS without dilution. Samples fortified with 0.1 mg/kg IKF-309 were diluted 20x using acetonitrile/HPLC water (1:1) before LC-MS/MS analysis.

LC-MS/MS ANALYSIS

Separation of the analyte from soil matrix was achieved by high performance liquid chromatography (HPLC). Quantitative LC-MS/MS analysis of IKF-309 in the samples utilized a highly specific and sensitive MRM (Multiple Reaction Monitoring) method. IKF-309 precursor ion (m/z 366.00) was monitored in Q1 and the fragment ions were monitored in Q3 (m/z 184.30, primary method and m/z 209.30, confirmatory method). The analyte was identified by the coincidence of its retention time with the calibrant standards, and quantitated by integration of the peak area relative to the calibration curve.

The following are the LC-MS/MS parameters used.

HPLC: Two Shimadzu LC20-AD pumps and a Shimadzu SIL-HTA Controller/Autosampler

Column: Inertsil ODS-3, 50 mm x 2.1 mm x 3µ particle size

Injection Volume: 10 µL

Solvent System:

Solvent A = HPLC water/acetonitrile/formic acid (95:5:0.1%)

Solvent B = HPLC water/acetonitrile/formic acid (5:95:0.1%)

Solvent Program:

| Time (minutes) | Flow Rate (mL/min) | %A | %B |
|-----------------------|---------------------------|-----------|-----------|
| 0.00 | 0.30 | 50 | 50 |
| 2.00 | 0.30 | 0 | 100 |
| 2.50 | 0.30 | 0 | 100 |
| 2.60 | 0.30 | 50 | 50 |
| 4.50 | 0.30 | 50 | 50 |

Mass Spectrometer: SCIEX API 4000

The mass spectrometer was calibrated every four weeks per SOP 04-C023-03.

Mass Spectrometer settings:

| | |
|-------------------------------------|-------------|
| Scan Type: | MRM |
| Polarity: | Positive |
| Ion Source: | Turbo Spray |
| Resolution Q1 | Unit |
| Resolution Q3 | Unit |
| Ion Source Gas 1 (GS1): | 20.0 psi |
| Ion Source Gas 2 (GS2): | 25.0 psi |
| Curtain Gas (CUR): | 25.0 psi |
| Collision Gas (CAD): | 6.0 psi |
| IonSpray Voltage (IS): | 3500 V |
| Temperature (TEM): | 550 °C |
| Declustering Potential (DP): | 75.0 V |
| Entrance Potential (EP): | 10.0 V |
| Collision Gas Exit Potential (CXP): | 17.0 V |

Period 1 settings:

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell Time (msec) | Collision Energy (CE) |
|---------------|---------------|-------------------|-----------------------|
| 366.00 | 184.30 | 200.00 | 32 V |
| 366.00 | 209.30 | 200.00 | 37 V |

Retention Time: ~3.9 minutes

METHODS OF CALCULATION

Recoveries

The recoveries of IKF-309 from fortified samples were calculated relative to the linearity curve generated with each set:

Linear regression formula from calibration curve $y = mx + b$

$$\text{ng/mL IKF-309} = \frac{y - b}{m}$$

Where y = Sample peak area

b = Calibration intercept

m = Calibration slope

$$\text{Sample Concentration (ng/mL)} = \frac{\text{Sample peak area} - \text{intercept}}{\text{Slope}}$$

ppm IKF-309 =

$$\frac{\text{Sample Conc. (ng/mL)} \times \text{Final Vol. (mL)} \times \text{Initial Extract Vol. (mL)} \times 0.001 \mu\text{g/ng}}{\text{Aliquot vol. (mL)} \times 10 \text{ grams}}$$

where $\mu\text{g/g}$ is equivalent to mg/kg and ppm.

Percent Recovery =

$$\frac{\text{Conc. of IKF - 309 Fortified Sample (ppm)} - \text{Conc. of Control (ppm)}}{\text{IKF - 309 Fortification Level (ppm)}} \times 100$$

An example calculation for the recovery of IKF-309 (0.001 ppm fortification) from soil (sample no. 027336-**Soil 0.001 mg/kg A**) is shown below:

The calibration curve equation was $y = 43400x + (-467)$ ($r = 0.9993$)

$$\text{ng/mL IKF-309} = \frac{3620 - (-467)}{43400} = 0.0942 \text{ ng/mL}^*$$

* - slight differences from reported data due to rounded numbers are used for calculation.

Calculation of the ppm IKF-309 in soil was calculated as shown:

$$\text{ppm IKF-309} = \frac{0.0942 \text{ ng/mL} \times 10 \text{ mL} \times 40 \text{ mL} \times 0.001}{4 \text{ mL} \times 10 \text{ grams}} = 0.000942 \text{ ppm}$$

The percent recovery of IKF-309 equals 0.000942 ppm minus control (0 ppm) IKF-309, divided by the 0.001 ppm fortification times 100 equals the percent recovery for this sample at 94.2%.

$$\text{Percent Recovery} = \frac{0.000942 \text{ ppm} - 0 \text{ ppm}}{0.001 \text{ ppm}} \times 100 = 94.2\%$$

SIGNIFICANT OBSERVATION

PTFE syringe filters (Fisher Scientific, 0.45 μm , 13 mm, lot #ROMA82407) removed a portion of IKF-309 from the samples, that is, 15-33% of the 0.001 ppm samples and 12-22% of the 0.1 ppm samples after 20x dilution (primary method, product ion: $m/z = 184.3$). Therefore, to analyze soil samples following the RCC method B18843, it may be necessary to remove the syringe filtration step from the method unless quantitative recovery of IKF-309 through syringe filters can be demonstrated.