# Cover Sheet for Analytical Method

# Pyriofenone in Water - MRID 49321801

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## II. MATERIALS

### A. Equipment

The equipment that was used is listed below:

- Balance, Analytical, Mettler Toledo XS204
- Volumetric flasks, glass: 10, 50 and 100 mL
- Bottles, amber glass with Teflon lined cap: 30, 60, and 120 mL
- Clear glass vials: 22 mL
- Volumetric glass pipette: various sizes
- Graduated Cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable micropipettes: various volumes
- Disposable Pasteur pipettes, glass
- Repeating Pipette, Eppendorf Stream
- HPLC vials, clear glass: 1.8 mL
- AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler

### B. Reagents and Standards

The following chemicals were used:

| Chemical | Grade | Manufacturer | Distributer | Part No: |
|---|---|---|---|---|
| Acetonitrile | Optima | Fisher | Fisher | A996-4 |
| Ammonium Formate (>99%) | Not Given | Alfa Aesar | VWR | AA14517-30 |
| Formic Acid (88%) | ACS | Fisher | Fisher | A118P-500 |
| Methanol | ChromAR | Macron | VWR | MK304110 |
| Water | HPLC | Macron | VWR | MK679510 |
| Water | HPLC | Fisher | Fisher | W5-4 |

Preparation of Reagent Solutions:

*Acetonitrile: HPLC grade water (50:50, v/v)*: Prepared by adding 250 mL of acetonitrile to 250 mL of HPLC grade water and mixing well.

*Mobile Phase A:*
*Methanol: formic acid (100:0.1, v/v)*: Prepared by adding 1 mL of formic acid to 1000 mL of Methanol and mixing well.

*Mobile Phase B:*
*HPLC grade water: methanol: formic acid (90:10:0.1, v/v/v) containing 0.01 M ammonium formate*: Prepared by dissolving 0.6 g of ammonium formate in 900

mL of HPLC grade water and adding 100 mL of methanol and 1 mL of formic acid. Mix well.

1. Reference Substance

The analytical reference standard was received in good condition on June 14, 2012 from Midwest Research Institute, Kansas City, MO. The certificate of analysis for the standard is in the archives at GPL. The following table contains detailed information for the analytical standard used in this study.

| Analytical Standard | CAS # | Batch # | Purity (%) | Expiration Date |
|---|---|---|---|---|
| IKF-309 | 688046-61-9 | 0608 | 99.19 | 09/23/2018 |

Upon receipt, the neat IKF-309 standard was stored in a freezer set to maintain ≤ -10 °C.

2. Preparation of Standard Solutions

The IKF-309 reference substance was used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

### a. Stock Solution

On March 10, 2014, 10.1 mg of IKF-309 reference standard was weighed directly into a 10-mL volumetric flask and diluted to 10 mL with acetonitrile. After correcting for purity, the stock solution contained 1.00 mg/mL IKF-309 (Solution A).

### b. Intermediate Solution

A 100-µL aliquot of Solution A was diluted to 100 mL with acetonitrile, resulting in a solution that contained 1.00 µg/mL IKF-309 (Solution B). Solution B was used to prepare the 100 ng/mL fortification solution.

### c. Fortification Solutions

A 5-mL aliquot of Solution B was diluted to 50 mL with acetonitrile, resulting in a solution that contained 100 ng/mL IKF-309 (Solution C). Further, a 5-mL aliquot of Solution C was diluted to 50 mL with acetonitrile, resulting in a 10 ng/mL IKF-309 solution (Solution D). Aliquots of Solution D were used to fortify at the LOQ level. Aliquots of Solution C were used to fortify at the 10x LOQ level. Solution C was used to prepare calibration standards. Solutions B, C, and D were given an expiration of three months. The fortification solutions and calibration standards were stored frozen (≤ -10 °C) when not in use.

### d. Calibration Standards

All calibration standards were diluted into acetonitrile: HPLC grade water (50:50, v/v). The calibration standards were given an expiration of three months. The calibration standards were prepared by diluting the solutions as listed in the table as follows into volumetric flasks:

| Initial Solution ID | Volume of Solution (mL) | Final Volume (mL) | Final Solution ID | Standard Concentration (ng/mL) |
|---|---|---|---|---|
| C | 1 | 100 | E | 1.00 |
| C | 0.8 | 100 | F | 0.800 |
| C | 0.4 | 100 | G | 0.400 |
| C | 0.2 | 100 | H | 0.200 |
| E | 1 | 10 | I | 0.100 |
| E | 0.8 | 10 | J | 0.0800 |
| E | 0.4 | 10 | K | 0.0400 |
| E | 0.2 | 10 | L | 0.0200 |
| E | 0.1 | 10 | M | 0.0100 |

## C. Safety and Health

Material Safety Data Sheets (MSDS) should be consulted anytime an analyst is to start work with an unfamiliar chemical. Proper personal protective equipment must be used during the execution of this method. Avoid breathing chemical vapor and avoid chemical contact with eyes and skin. MSDS for the chemicals used in this analysis are located in Appendix C. There are no procedural steps that require special precautions to avoid safety or health hazards.

# III. METHODS

## A. Principal of Analytical Method

The analysis of surface and drinking water was performed according to the reference method titled "IKF-309 Validation of Methodology for the Determination of Residues in Surface and Drinking Water" (contained in

Huntingdon Life Sciences Document Number: JSM0058). The limit of quantitation (LOQ) and limit of detection (LOD) were defined as 0.05 µg/L (ppb) and 0.02 µg/L (ppb) for IKF-309, respectively.

The method validations for both surface water and drinking water were performed on March 11, 2014. All samples for each validation were extracted in one analytical set. The two sets consisted of one reagent blank sample (HPLC grade water), two control samples, five LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number; the sample set designation and a sample number (e.g., 531ILV01-1).

Aliquots (10 mL) of control matrix water were fortified. An equal amount (10 mL) of acetonitrile was added to each sample. Samples were vialed and analyzed by LC-MS/MS.

## B. Analytical Procedure

### 1. Control Matrixes

### a. Drinking Water

The drinking water control matrix was obtained from the municipal supply at GPL on 03/11/2014. The sample was collected into a 4-L amber bottle and was kept refrigerated (~4 °C) until use.

### b. Surface Water

The surface water control matrix was obtained from the Fresno Irrigation District Canal "Herndon No. 39" at a point near the Gates Avenue Bridge on 02/25/2014. Sub-portions of this sample were taken and labeled as "Herndon39-140225" and were then transported by GPL personnel to BSK Laboratories in Fresno, California for non-GLP characterization. The non-GLP characterization results of the sample are presented below:

| Parameter | Found Value |
|---|---|
| pH | 7.3 |
| Dissolved Oxygen | 9.7 mg/L |
| Conductivity | 42 µmhos/cm |
| Alkalinity | 17 mg/L as $CaCO_3$ |
| Total Hardness | 12 mg/L as $CaCO_3$ |
| Total Residues | 31 mg/L |
| Total Organic Carbon | 1.6 mg/L |
| Dissolved Organic Carbon | 1.8 mg/L |

Sub-portions from the sample "Herndon39-140225-R" (refrigerated after collection) were used for the method validation.

2. Preparation of Samples

Sub-samples (10 mL) of the control water matrixes were measured into 22-mL clear glass vials.

3. Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.05 µg/L) or 10x the LOQ (0.5 µg/L). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 10-mL samples as follows:

| Fortification Level | Amount and Concentration of IKF-309 Spiking Solution Used |
|---|---|
| LOQ (0.05 µg/L) | 50 µL 10.0 ng/mL |
| 10x LOQ (0.5 µg/L) | 50 µL 100 ng/mL |

4. Extraction

After fortification, an aliquot (10 mL) of acetonitrile was added to each sample. Samples were shaken by hand for approximately 5 seconds. Samples were vialed and submitted for analysis by LC-MS/MS.

## C. Instrumentation

| | |
|---|---|
| Instrument: | AB Sciex API4000 LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler |

| | |
|---|---|
| HPLC Column: | Phenomenex Luna C8<br>150 x 2.00 mm, 5 µm (100 Å)<br>Part # 00F-4040-B0<br>Serial # 568517-6 |
| Guard Column: | NA |
| Data System: | Analyst Chromatography Data System version 1.5.2, AB Sciex |

Mobile Phases:

A) Methanol: Formic Acid (100: 0.1, v/v)
B) Methanol: HPLC grade water: formic acid (10:90:0.1, v/v/v) containing 0.01 M ammonium formate

| | |
|---|---|
| Flow Rate: | 0.2 mL/minute |
| Run Time: | 15.0 minutes |
| Injection Volume: | 20 µL |

Gradient Program:

| Time (minutes) | %A | %B |
|---|---|---|
| 0.0 | 70 | 30 |
| 6.0 | 100 | 0 |
| 10.0 | 100 | 0 |
| 11.0 | 70 | 30 |
| 15.0 | 70 | 30 |

Column Heater: NA

Retention Time:

IKF-309: 6.2 minutes

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

| **AB Sciex API-4000 Acquisition Parameters (ESI interface, MRM mode, Positive, Unit/Unit Resolution)** | | | |
|---|---|---|---|
| **IKF-309** | **Q1 (m/z)** | **Q3 (m/z)** | **Dwell (msec)** |
| Primary | 366.0 | 184.3 | 500 |
| Confirmatory | 366.0 | 209.3 | 500 |

| Parameter | Setting |
|---|---|
| **CUR:** | 20 |
| **GS1:** | 40 |
| **GS2:** | 40 |
| **IS:** | 4500 |
| **TEM:** | 450 |
| **CAD:** | 10 |
| **ihe:** | ON |
| **DP:** | 70 |
| **CE:** | 35 |
| **EP:** | 10 |
| **CXP:** | 10 |

The instrument parameters were optimized for analyte sensitivity and resolution prior to the chromatographic run. The exact parameters were documented with the data set.

## D. Potential Interferences

### 1. Matrix Interference

The detection technique is highly selective for this method. No interferences arising from co-eluting compounds from either water type were observed.

### 2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

### 3. Labware Interference

This method uses mostly disposable labware. No interferences from the labware use were observed.

## E. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS with monitoring of two ion transition pairs. As this method is highly selective, no additional confirmatory technique was used.

## F. Time Required for Analysis

One hour was required for one person to prepare an analysis set from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed overnight. An additional 0.5 hours was spent on data calculation and tabulation the following day. Due to the analysis time (15 minutes per sample), at most, two calendar days are needed to prepare an analysis set and to calculate and tabulate the data.

## G. Modification or Potential Problems

There were no modifications to the method. There were no potential problems encountered. However, the first analysis of the validation set for surface water showed an approximate 500% percent recovery of IKF-309 in one LOQ sample. The results of this analysis set are not reported. It was suspected that an error had been made during sample vialing. The set of samples and calibration standards were re-vialed and analyzed. The results of the second analysis set met all acceptance criteria and have been reported.

## H. Methods of Calculation

Analyst Chromatography Data System version 1.5.2, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations IKF-309 as ng/mL using the linear regression function with no weighting. The calibration was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient and other parameters of the standard curve were calculated. Calibration standards were injected every three to five sample injections as well as at the beginning and end of the injection sequence. Nine different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of IKF-309 detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as µg/L of residue found in samples was then calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL\ from\ curve) \times (Final\ Vol.\ in\ mL) \times 1\ \mu g \times 1000\ mL}{(Sample\ amount\ in\ mL) \times 1000\ ng \times 1\ Liter}$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{(Measured\ Concentration,\ \mu g/L) \times 100}{(Theoretical\ Concentration,\ \mu g/L\ added)}$$

An example calculation for drinking water for a IKF-309 laboratory fortification (primary ion) in set 531ILV02, sample 531ILV02-10 10x LOQ sample fortified at 0.500 µg/L, is as follows:

*standard curve equation:* $y = 3.62 \times 10^{5} (x) + (-561)$

*where* $x$ = *IKF-309 concentration in ng/mL and*

$y$ = *peak response* = *93423.0*

*IKF-309 concentration from the curve =0.260 ng/mL*

$$\mu g/L = \frac{(0.260\ ng/mL\ IKF\text{-}309) \times (20\ mL) \times 1 \mu g \times 1000\ mL}{(10\ mL) \times 1000\ ng \times 1\ Liter} = 0.520\ \mu g/L$$

$$\%\ recovery = \frac{0.520\ \mu g/L}{0.500\ \mu g/L}\ X\, 100 \quad = \quad 104\%$$

No detectable residues were measured in any control samples. Laboratory fortification samples were not corrected for reported control responses. Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

### I. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), coefficient of variation and 95% confidence interval for analyte recovery data. Linear regression analysis was applied to LC-MS/MS calibration curves for the determination of slope, y-intercept and correlation coefficient values.

### J. Chromatograms

Example chromatograms are presented in Appendix E of this report.

## Analysis of IKF-309 in Water by LC-MS/MS

Measure a 10 mL sub-sample of water into a 22-mL clear vial

↓

Fortify as necessary

↓

Add 10 mL of acetonitrile

↓

Shake by hand for approximately 5 seconds

↓

Load samples onto LC-MS/MS

*If further dilution is necessary,*
*dilute with Acetonitrile: HPLC grade water (50:50, v/v)*