

## I. ABSTRACT

Ishihara Sangyo Kaisha (ISK) analytical method for the determination of IKF-5411 and 4HP in soil (Document Number F-559) was successfully validated using High Performance Liquid Chromatography (HPLC) equipped with a MS/MS detector for the determination of IKF-5411 and 4HP in soil. The analytical method was validated to meet Environmental Protection Agency (EPA) Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods (EPA Draft Guideline for the Independent Laboratory Validation) requirements for independent laboratory validation.

One control soil sample was used in this study. The sample was collected from North Rose, New York, on May 17, 2007 for ISK study IB-2007-JLW-003-01-00.

The analysis of soil was performed according to ISK Residue Analytical Method for the determination of IKF-5411 and its metabolite 4HP in soil (Document Number: F-559). The limit of quantitation (LOQ) is defined as 0.01 µg/g (ppm) for both IKF-5411 and 4HP. The limit of detection (LOD) is defined at 0.01 ng/mL (0.005 µg/g) for both IKF-5411 and 4HP.

The method validation was performed on April 22, 2010. All validation samples were extracted in one analytical set. The set consisted of a reagent blank, two controls, five low laboratory fortification samples and five high laboratory fortification samples. Method validation samples were fortified at the LOQ (0.01 ppm) and 10x the LOQ (0.1 ppm).

## II. OBJECTIVE

ISK Biosciences Corporation contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to conduct an Independent Laboratory Validation. The objective of this study was to validate Ishihara Sangyo Kaisha's (ISK) analytical method for the determination of IKF-5411 and 4HP in soil (Document Number F-559). The purpose of this study was to demonstrate method ruggedness prior to the Sponsor's submission of the method to the EPA.

This study was conducted according to EPA's Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods and EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160.

## III. MATERIALS AND METHODS

### A. Test/Reference Substances

The analytical reference standards were received in good condition on March 24, 2010 from Midwest Research, Kansas City, MO. Copies of the certificates of analysis for both standards are in the archives at GPL. The following table contains detailed information on each analytical standard used in this study.

Analytical Standard	CAS #	Lot #	Purity (%)	Expiration Date
IKF-5411	NA	20100128	99.9	03/31/2013
4HP	NA	20091026	99.2	04/07/2015

Upon receipt, the neat IKF-5411 standard and the neat 4HP standard were stored in a freezer maintained at  $< 0^{\circ}\text{C}$ .

Characterization and stability of the reference standards were determined and are maintained by ISK Biosciences Corporation.

### B. Preparation of Standard Solutions

The IKF-5411 and 4HP reference substances were 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.

1. Stock Solutions

On April 20, 2010, 10.5 mg of IKF-5411 PAI was weighed directly into a 100-mL volumetric flask and diluted to 100 mL with acetonitrile. After correcting for purity, the stock solution contained 105 µg/mL IKF-5411 (Solution A).

On April 20, 2010, 10.5 mg of 4HP was weighed directly into a 100-mL volumetric flask and diluted to 100 mL with acetonitrile. After correcting for purity, the solution contained 104 µg/mL 4HP (Solution B).

An intermediate solution (Solution C) was prepared by aliquoting 10 mL of Solution A and 10 mL of solution B into a 100-mL volumetric flask and diluting to 100 mL with acetonitrile. Solution C contained 10.5 µg/mL IKF-5411 and 10.4 µg/mL 4HP.

2. Fortification Solutions

A 10-mL aliquot of Solution C was diluted to 52.5 mL with acetonitrile, resulting in a solution that contained 2.00 µg/mL IKF-5411 and 1.98 µg/mL 4HP (Solution D).

A 1-mL aliquot of Solution C was diluted to 105 mL with acetonitrile, resulting in a solution that contained 100 ng/mL of IKF-5411 and 99.0 ng/mL 4HP (Solution E).

Aliquots of Solution D and Solution E were used for fortification purposes. The fortification solutions were stored frozen (<0 °C) when not in use.

3. Chromatography Standards

A 2-mL aliquot of Solution E was diluted to 100 mL with acetonitrile:water (80:20, v/v), resulting in a solution that contained 2.00 ng/mL IKF-5411 and 1.98 ng/ml 4HP (Solution F). Solution F served as a liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) standard and was also used as an intermediate solution.

Additional LC/MS/MS standards were prepared by diluting Solution F with acetonitrile:water (80:20, v/v) as follows:

Volume of Solution F (mL)	Final Volume (mL)	Standard Concentration (ng/mL)	
		IKF-5411	4HP
10	50	0.400	0.369
5.0	50	0.200	0.198
1.0	50	0.0400	0.0369
0.5	50	0.0200	0.198
0.5	100	0.0100	0.00990

The LC/MS/MS standards were stored frozen (<0 °C) when not in use.

### C. Equipment

The equipment that was used is listed below:

- Balance, Analytical: Mettler model AB 204-S
- Volumetric flasks, glass: 10, 25, 50 and 100 mL
- Bottles, amber glass with Teflon lined cap: 50 mL and 100 mL
- Volumetric glass pipette: various sizes
- Graduated Cylinders: 10, 25, 50, 100, 250, 500 and 1000 mL
- Micropipet Drummond Wiretrol 100 µL disposable micropipets
- Eberbach, 2 Speed Shaker (Ann Arbor, MI, USA)
- Whatman Grade No. 5 Filter Paper
- Celite® 545
- Oasis HLB Vac RC cartridge, 60mg
- Adjustable micropipettor, 100–1000 µL, Lambda™ Single-Channel Pipettor (Corning, NY, USA)
- Disposable Pasteur pipettes, glass, various sizes
- HPLC vials, clear glass: 1.5 mL
- Sciex API5000 LC/MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu CBM-20A Controller, Shimadzu SIL-20AC XR Autosampler

### D. Chemicals and Reagents

The following chemicals were used:

- Acetonitrile, HPLC Grade, Acros #B0311290
- Hydrochloric Acid, Certified ACS, EMD #49287
- Water, Chrom AR®, Mallinckrodt #H36E29
- Methanol, Optima Grade, Fisher #A454-4
- Acetic Acid, Certified ACS, Fisher #082764

1. **Preparation of Reagent Solutions**

*6M Hydrochloric Acid Solution:* Prepared by slowly transferring 248 mL of hydrochloric acid into a storage bottle containing 252 mL of water and mixing well.

*Acetonitrile:Water (80:20, v/v):* Prepared by combining 3200 mL of acetonitrile and 800 mL of water in a 4-L storage container and mixing well.

*Acetic Acid:Acetonitrile (0.1:99.9, v/v):* Prepared by aliquoting 1 mL of acetic acid into 999 mL of acetonitrile and mixing well.

*Acetic Acid:Water (0.1:99.9, v/v):* Prepared by aliquoting 1 mL of acetic acid into 999 mL of water and mixing well.

*Acetonitrile:Water (20:80, v/v):* Prepared by combining 200 mL of acetonitrile and 800 mL of water and mixing well.

E. **Sample Receipt and Tracking**

One previously processed control soil sample was used in this study. The sample was collected from North Rose, New York, on May 17, 2007 for ISK study IB-2007-JLW-003-01-00. The soil sample was a 0-6 inch depth with a characterization of loam (37% sand, 44% silt and 19% clay) with organic matter at 4.5% and a pH of 5.0. The sample was received frozen and in good condition on June 12, 2007. Temperatures for the freezers ranged from -27 °C to -10 °C over the period the sample was stored (June 12, 2007 to April 22, 2010). Sample receipt and tracking documentation were maintained for chain-of-custody purposes

F. **Method of Analysis**

The analysis of soil was performed according to ISK Residue Analytical Method for the determination of IKF-5411 and its metabolite 4HP in soil (Document Number: F-559). The limit of quantitation (LOQ) is defined as 0.01 µg/g (ppm) for both IKF-5411 and 4HP. The limit of detection (LOD) is defined at 0.01 ng/mL (0.005 µg/g) for both IKF-5411 and 4HP.

G. **Method Validation Design**

The method validation was performed on April 22, 2010. All validation samples were extracted in one analytical set. The set consisted of a reagent blank, two controls, five low laboratory fortification samples and five high 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 and a sample number (e.g., 341MV01-1).

Method validation samples were fortified at the LOQ (0.01 ppm) or 10x the LOQ (0.1 ppm). Fortifications were performed using glass pipettes to directly fortify the 20-gram soil samples as follows:

<b>Fortification Level</b>	<b>Amount of Spiking Solution Used IKF-5411/4HP</b>
LOQ (0.01 ppm)	2 mL of a 100/99.0 ng/mL spiking solution
10x LOQ (0.1 ppm)	1 mL of a 2.00/1.98 µg/mL spiking solution

Samples were then analyzed according to ISK analytical method for the determination of IKF-5411 and 4HP in soil (Document Number F-559).

#### 1. Method Summary

For soil, approximately 20 grams of sub-sample was weighed into a 250-mL Nalgene<sup>®</sup> bottle. The sub-sample was extracted with 100 mL of acetonitrile:water (80:20, v/v) and 1 mL of 6 Normal hydrochloric acid. The sample was shaken for 30 minutes on a platform shaker. The mixture was vacuum filtered through Whatman 5 filter paper with a layer of Celite 545 on top. The filter cake was washed with 80 mL of acetonitrile:water (80:20, v/v) that first rinsed the 250-mL Nalgene<sup>®</sup> bottle. The filtrate and washing were combined and brought to a final volume of 200 mL with acetonitrile:water (80:20, v/v).

The extract was cleaned up using solid phase extraction (SPE). A SPE cartridge (OASIS HLB VAC RC, 60 mg) was placed onto a SPE vacuum manifold and conditioned using methanol (5 mL) followed by water (5 mL). A 1-mL aliquot of extract was combined with 10 mL of water, and this mixture was loaded onto the SPE cartridge. The sample solution was allowed to drain through the column, followed by a 4 mL rinse of acetonitrile:water (20:80, v/v) and both were discarded. IKF-5411 and 4HP were then eluted with 10 mL of acetonitrile:water (80:20, v/v). The eluate was collected and brought to a final volume of 50 mL with acetonitrile:water (80:20, v/v), and an aliquot was submitted for analysis on the LC/MS/MS.

2. Instrument Parameters: LC/MS/MS

Instrument: Sciex API5000 LC/MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu CBM-20A Controller, Shimadzu SIL-20AC XR Autosampler

HPLC Column: Acquity BEH C18  
2.1 x 5 mm, 1.7 µm (Waters)  
Lot #0176300741

Guard Column: Waters, Acquity UPLC® BEH C18 1.7 µm VanGuard™ Pre-column 2.1 x 5 mm  
P/N 186003975 (3pack)  
L/N 0172300751

Data System: Analyst Chromatography Data System version 1.5, AB Sciex.

Mobile Phase: Acetonitrile:water:acetic acid (70:30:0.1, v/v/v)  
Isocratic

Flow Rate: 500 µL/minute  
Run Time: 3.0 minutes  
Injection Volume: 4 µL

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

Interface: Turbo-Ion Spray (ESI)  
Polarity: Positive  
Scan Type: MRM with unit resolution

Ions Monitored:

IKF-5411: Q1 mass 360.2 (primary)  
Q3 mass 209.9 (primary)  
Q1 mass 360.2 (confirmatory)  
Q3 mass 124.9 (confirmatory)

4HP: Q1 mass 318.1 (primary)  
Q3 mass 125.0 (primary)  
Q1 mass 318.1 (confirmatory)  
Q3 mass 210.2 (confirmatory)

Retention Times:

IKF-5411 0.71 minutes  
4HP 0.39 minutes

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

## H. Quantitation Procedures

Analyst Chromatography Data System version 1.5, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations of IKF-5411 and 4HP as ng/mL using the linear regression function with 1/x weighting. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak 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 four to five sample injections as well as at the beginning and end of the injection sequence. Six different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of IKF-5411 and 4HP detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as µg/g of residue found in the samples was then calculated with Microsoft® Excel using the following equation:

$$\mu\text{g/g} = \frac{(\text{ng/mL from curve}) \times (\text{Final Vol. in mL}) \times (\text{Aliquot factor}) \times 1 \mu\text{g}}{(\text{sample amount in grams}) \times 1000 \text{ ng}}$$

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

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

An example calculation for an IKF-5411 laboratory fortification (primary ion) in set 341MV01, sample 341MV01-5 Low Spike at 0.00901 µg/g, is as follows:

$$\text{standard curve equation: } y = 9.7 \times 10^4 (x) + 82.9$$

where  $x$  = IKF-5411 concentration in ng/mL and

$$y = \text{peak response} = 1896.0$$

$$\text{IKF-5411 concentration from the curve} = 0.0187 \text{ ng/mL}$$

$$\text{ng/g} = \frac{(0.0187 \text{ ng/mL IKF-5411}) \times (50 \text{ mL}) \times (200 \text{ mL}) \times 1 \mu\text{g}}{(22.19 \text{ grams}) \times 1000 \text{ ng}}$$

$$\% \text{ recovery} = \frac{0.00843 \mu\text{g/g}}{0.00901 \mu\text{g/g}} \times 100 = 93.6\%$$



No detectable residues greater than the LOQ or the LOD were measured in any control samples. Laboratory fortification samples were not corrected for reported control responses.

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.