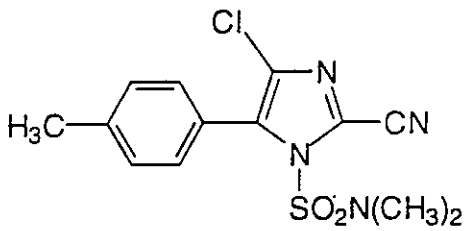
INTRODUCTION

A residue method for the determination of IKF-916 in drinking and surface water was developed and evaluated in this study. Method performance was discussed in terms of EU method parameters including accuracy, precision, limit of quantitation, specificity and linearity.

TEST/REFERENCE SUBSTANCE

The structure, CAS registry number, and chemical name for IKF-916 are listed below.



Chemical names: 4-chloro-2-cyano-*N*,*N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide (IUPAC-J and IUPAC)

4-chloro-2-cyano-*N*,*N*-dimethyl-5-(4-methylphenyl)-1*H*-imidazole-1-sulfonamide (CA-J and CA)

CAS No.: 120116-88-3
Lot Number: 9704-1
Purity: 99.1% (Date of Certificate: July 15, 1997)

OBJECTIVE

The objective of this study was to develop and evaluate an analytical method for the determination of IKF-916 residues in drinking and surface water. Specific criteria under evaluation were method accuracy, precision (repeatability), limit of quantitation, specificity and linearity. The proposed method limit of quantitation was 0.1 ppb, and was to be achieved by demonstrating the successful recovery of IKF-916 from 0.1-ppb fortifications.

MATERIALS AND METHODS

SAMPLE PROCUREMENT AND RECEIPT

Control pond water was obtained from a pond located in Leroy Township, OH. Control tap water was obtained from a laboratory at the Ricerca, Inc. site. The pond water was maintained in a cooler with ice prior to analysis.

The characteristics of the pond water sample are:

pH: 7.03
total hardness: 79.3 mg/L
DOC: 13 mg/L

SAMPLE IDENTIFICATION

At the time of receipt at Ricerca (pond water) or at the time of use (tap water), control samples were assigned a Ricerca laboratory code number consisting of the year and a consecutive number. Sample identification numbers are listed below.

Sample Matrix	Control Sample Identification Number
Pond Water	99-0496
Tap Water	99-0497

At the time of analysis, each aliquot of water was assigned a unique sample ID.

The sample ID and corresponding sample description were recorded on Operations Form and Sample List (flowsheet).

Appendix D contains sample history information (dates of extraction and analysis).

Analytical Procedure Summary

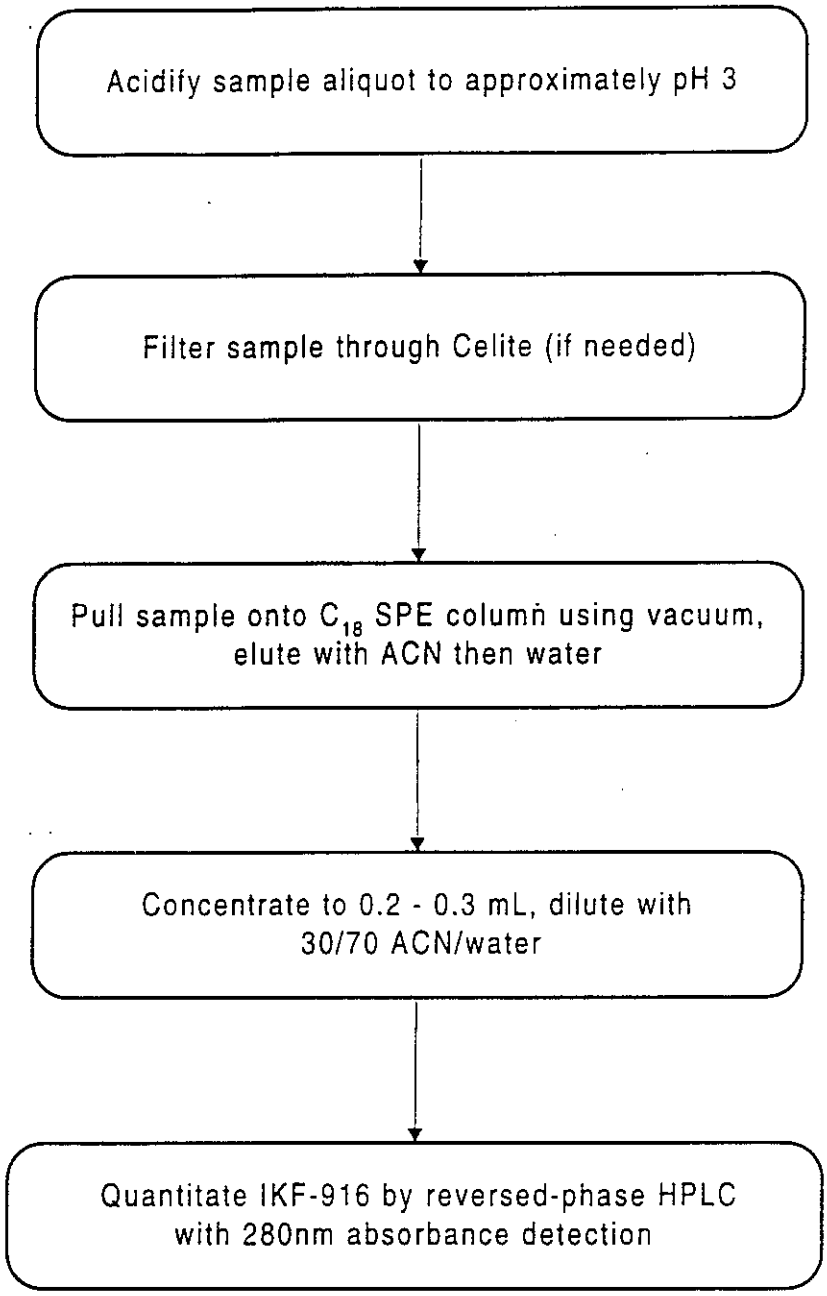
A detailed analytical method is provided in Appendix B. A summary of the method is provided here.

An aliquot of the water sample was acidified to approximately pH 3. For pond water samples, the sample was filtered through a bed of Celite. The sample was pulled onto a C_{18} solid phase extraction column using vacuum. The column was eluted with acetonitrile followed by water. The acetonitrile/water was evaporated to 0.2 – 0.3 mL, and the sample residue was diluted with 30/70 acetonitrile/water for HPLC quantitation. All samples were quantified by reversed-phase HPLC with UV absorbance detection at 280nm. Figure 1 contains a method flow diagram. Preparation of a set of 12 samples for HPLC analysis required approximately 8 hours.

CALCULATION OF RESIDUES

IKF-916 residues were quantified using linear multi-point calibration curves generated from the injection of IKF-916 external standards. Detailed sample calculations are found in Appendix C.

Figure 1: Flow Diagram for IKF-916 Water Method

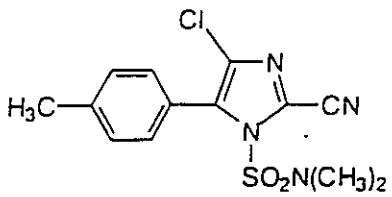


INTRODUCTION

This protocol covers the development of an analytical procedure for the analysis of IKF-916 and its metabolites (CCIM, CCIM-AM, CTCA and CCBA) in drinking (tap or ground) water and surface (river or pond) water. The development of a method for water will be necessary for European Registration, and provides justification for the test system.

TEST AND REFERENCE SUBSTANCES

- IKF-916



Chemical names:

(IUPAC-J and IUPAC)

4-chloro-2-cyano-*N*,*N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide

(CA-J and CA)

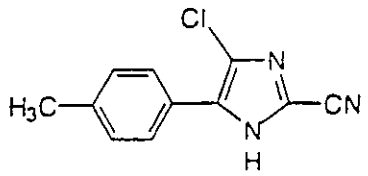
4-chloro-2-cyano-*N*,*N*-dimethyl-5-(4-methylphenyl)-1*H*-imidazole-1-sulfonamide

CAS No.: 120116-88-3

Lot Number: 9704-1

Purity: 99.1%

- CCIM



Chemical names:

4-chloro-5-*p*-tolylimidazole-2-carbonitrile (IUPAC and IUPAC-J)

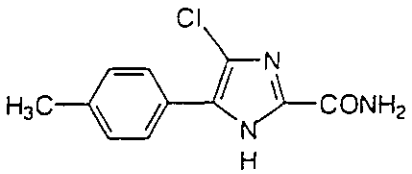
4-chloro-5-(4-methylphenyl)-1*H*-imidazole-2-carbonitrile (CA and CA-J)

CAS No.: 120118-14-1

Lot Number: 9506

Purity: 99.7%

- CCIM-AM



Chemical names:

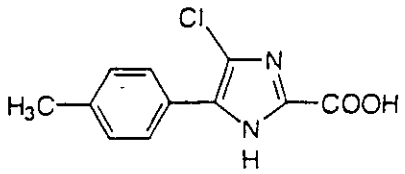
4-chloro-5-*p*-tolylimidazole-2-carboxamide (IUPAC and IUPAC-J)

4-chloro-5-(4-methylphenyl)-1*H*-imidazole-2-carboxamide (CA and CA-J)

Lot Number: 9804

Purity: 98.7%

- CTCA



Chemical names:

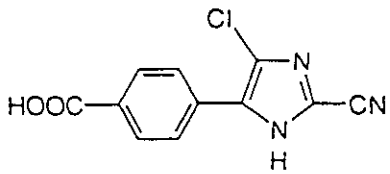
4-chloro-5-*p*-tolylimidazole-2-carboxylic acid (IUPAC-J)

4-chloro-5-(4-methylphenyl)-1*H*-imidazole-2-carboxylic acid (CA-J)

Lot Number: 9804

Purity: 99.3%

- CCBA



Chemical names:

4-(4-chloro-2-cyanoimidazol-5-yl)benzoic acid (IUPAC-J)

4-(4-chloro-2-cyano-1*H*-imidazol-5-yl)benzoic acid (CA-J)

Lot Number: 9907

Purity: 98.1%

The stability, characterization, retention and disposal of the test and reference substances are the responsibility of the Sponsor.

APPENDIX B

Detailed Analytical Method

CHEMICALS

Acetic acid, glacial, Fisher TraceMetal Grade

Acetonitrile, Burdick & Jackson, High Purity

Celite 545®, Fisher Scientific, #C212-500

Hydrochloric acid, concentrated, Fisher ACS certified PLUS

IKF-916 Analytical Standard, Lot No. 9704-1, Purity 99.1%

Water, Burdick & Jackson, High Purity

(Chemicals with equivalent purity from alternate sources may be substituted.)

REAGENTS

(Quantities may be adjusted proportionately to make more or less reagent)

30/70 acetonitrile/water (v/v): add 30 mL of acetonitrile to 70 mL of water

0.5 N Hydrochloric Acid solution: dilute 4.13 mL concentrated HCl to 100 mL using HPLC water

HPLC Mobile Phase: 50/50 acetonitrile/water + 0.5% acetic acid

To prepare, add 10 mL glacial acetic acid to a mixture of 1 liter acetonitrile and 1 liter water

EQUIPMENT

Analytical electronic balance with 0.1-mg readability

Büchner funnels, 110 mm

Disposable syringes, 3-mL, B-D* Brand #390585

Extraction columns, BAKERBOND spe ™ C_{18}, 6 mL, 1000 mg per column, J. T. Baker, #7020-07

Filter paper, Whatman® 934-AH, 90 mm

Filters, syringe tip, 0.45 µm PTFE, Gelman Acrodisc #4422 (13 mm) or #4219 (25 mm)

Glassware: Assorted beakers, Erlenmeyer flasks with side-arms, graduated cylinder, culture tubes, graduated centrifuge tubes, pipets, volumetric flasks, etc.

pH paper, colorpHast® pH 0-14, EM-Reagents

Reservoirs for sep-pak cartridges, 20 mL, Supelco #57021

Stir bars

Turbo Vap® LV Evaporator, Zymark

Vacuum manifold for solid phase extraction, Supelco

Visiprep Large Volume Sampler for 6 mL SPE tubes, Supelco, #57275 modified with adapters for easy connect/disconnect (female Luer adapter #58721, male Luer to female 1/4-28, PEEK adapter #55072)

NOTE: Appropriate substitution for certain items are left to the analyst's discretion.

INSTRUMENTATION

High-performance liquid chromatograph (HPLC) with data system Model 620 quaternary pump

Waters Wisp 714 autosampler

Waters MS-600 controller and quaternary pump

Waters model 486 absorbance detector

PE-Nelson Turbochrom chromatography data system

Systec Model CH-1448 temperature controller

Systec Goldenfoil heating element

Helium for degassing mobile phases

HPLC column: The column listed below is recommended.

Ultracarb 3 ODS (20), 150 mm x 3.2 mm, Phenomenex No. 00F-0205-RO

HPLC guard column: SecurityGuard cartridge, C_{18} (ODS, Octadecyl), 4 mm L × 3 mm ID, Phenomenex No. AJO-4287

(Alternate HPLC Systems and/or columns may be used.)

STANDARD PREPARATION

Weigh 0.0500 g of IKF-916 analytical standard into a small vial. Quantitatively transfer the neat standard with rinses of acetonitrile into a Class A 100-mL volumetric flask. Dilute to the mark with acetonitrile to produce a stock standard with a concentration of 500 µg/mL IKF-916. Transfer 20 mL of the stock standard into a Class A 100-mL volumetric flask. Dilute to the mark with acetonitrile to produce a 100-µg/mL IKF-916 standard solution.

Prepare working standards from the stock standard described above as follows. Transfer 10 mL of the 100-µg/mL standard into a Class A 100-mL volumetric flask. Dilute to the mark with acetonitrile to produce a 10-µg/mL standard. Transfer 10 mL of the 10-µg/mL standard to a Class A 100-mL volumetric flask. Dilute to the mark with acetonitrile to produce a 1-µg/mL fortification standard. Transfer 20 mL of the 1-µg/mL fortification standard to a Class A 100-mL volumetric flask. Dilute to the mark with acetonitrile to produce a 0.2-µg/mL fortification standard.

Prepare IKF-916 calibration standards of concentration 0.025, 0.05, 0.10, 0.25, 0.50 and 1.00 µg/mL with 30/70 acetonitrile/water (see Reagents).

The following table shows volumes of standards and sample dilution solvent needed to prepare 20 mL of each calibration standard for IKF-916. Each calibration standard was prepared in a 30 mL amber vial.

Calibration Standard Concentration	Volume of Standard Used to Prepare	Volume of 30/70 ACN/H_2O
1.00 µg/mL	2.0 mL of 10 µg/mL*	18.0 mL
0.50µg/mL	1.0 mL of 10 µg/mL*	19.0 mL
0.25 µg/mL	0.5 mL of 10 µg/mL*	19.5 mL
0.10 µg/mL	2.0 mL of 1.00 µg/mL	18.0 mL
0.05 µg/mL	2.0 mL of 0.50 µg/mL	18.0 mL
0.025 µg/mL	2.0 mL of 0.25 µg/mL	18.0 mL

*Prepare a 10 µg/mL working standard as follows. Transfer 10 mL of the 100 µg/mL standard solution prepared above to a 100 mL class A volumetric flask. Dilute to the mark with 20 mL of acetonitrile and 70 mL of water.

NOTE: Recommended expiration interval for the calibration standards is one month. All standard solutions are stored in freezers.

SAMPLE EXTRACTION

1. Measure a 1 liter aliquot of the water sample into a beaker. Add a stir bar.
2. Add HCl to reach pH 3. Use a dilute HCl solution (0.5 N). Stir while adding the acid. Use pH paper to determine the pH.
3. Fortify with IKF-916.
4. For dirty samples (pond or river water): Add 1 scoop (≈ 7 grams) of Celite 545® to the beaker of sample and stir. Prepare a bed of Celite (15 – 20 grams) on a glass fiber filter paper in a Büchner funnel. Filter sample through the bed of Celite into a side-arm flask.
5. Setup the solid phase extraction manifold.
6. Condition BAKERBOND C_{18} columns with 5 mL acetonitrile then 5 mL water.
7. Pull sample onto column using Visiprep Large Volume Sampler.
8. Rinse sample container with 10 mL HPLC water. Pull onto column.
9. Elute column with 10 mL acetonitrile into a small beaker or vial using vacuum. Stop the solvent front at the top of the packing.
10. Elute with 3 mL water into the same container as the previous step. Allow column to pull dry.
11. Quantitatively transfer column eluate to a culture tube with rinses of acetonitrile.
12. Concentrate sample to approximately 3 mL in a TurboVap at 38°C using additions of acetonitrile to drive off the water.
13. Transfer to 10 mL graduated centrifuge tube with acetonitrile. Concentrate to approximately 0.2 – 0.3 mL.

14. Dilute with 30/70 acetonitrile/water (v/v). Filter through 0.45 µm PTFE filter. Load into amber HPLC vials.

HPLC QUANTITATION

Instrument Setup

Analytical column: Ultracarb 3 ODS 20; 150 mm × 3.2 mm
Guard column: SecurityGuard cartridge, C_{18} (ODS, Octadecyl), 4 mm L × 3 mm ID
Mobile Phase: 50/50 acetonitrile/water (v/v) + 0.5% acetic acid
Flow Rate: 0.5 mL/min
Column Temp: 40 ° C
Injection Volume: 100 µL

Detection: UV absorbance
Detector: Waters
Wavelength: 280 nm
Sensitivity: 0.01 AUFS

Instrument Calibration

Under the instrumental conditions described above the approximate retention time of IKF-916 is approximately 14 minutes.

The normal range of calibration standards is listed below.

ID	Concentration
LEV1	0.025 µg/mL
LEV2	0.05 µg/mL
LEV3	0.10 µg/mL
LEV4	0.25 µg/mL
LEV5	0.50 µg/mL
LEV6	1.00 µg/mL

SAMPLE CALCULATIONS

Residues of IKF-916 were calculated by electronic integration of the detector signal. The analog voltage measured by the absorbance detector was digitized in a PE-Nelson 900 Series interface and sent to the data system. The computer data system used to integrate the signal and calculate results was Perkin-Elmer Turbochrom Client Server version 6.1. Recovery calculations were performed using functions in Microsoft Excel ™.

The equation used to quantitate residues is shown below.

$$\text{Gross residue ppb} = \frac{(D - I)}{S} * \frac{V}{W} * \frac{1000mL}{L}$$

Where: S = Slope (µV-sec/µg/mL)
D = Detector Response,(Peak Area, µV-sec)
I = Intercept (µV -sec)
V = Volume for HPLC or Multiplier (mL)
W = Sample Aliquot Volume (mL)

For pond water sample PWLOQ1 (see Figure C-10, page 54), the residue was calculated as follows:

$$\text{Gross Residue ppb} = \frac{46076 - (-572.581867)}{463454.800624} * \frac{1}{1000} * 1000$$

$$= 0.1007\ ppb$$

$$= 0.101\ ppb\ (rounded)$$

$$\%\ Recovery = \frac{(ppb\ found - ppb\ control) * 100}{ppb\ added}$$

$$\%\ \text{Recovery} = \frac{(0.101 - 0) * 100}{0.1} = 101\ \%$$

The calibration curve coefficients were calculated by the Turbochrom data system using the peak area responses of the IKF-916 external calibration standards injected with the samples.

The limit of detection (LOD) was 0.025 ppb. The LOD was based on the residue equivalent to the lowest level calibration standard injected (0.025 µg/mL), and a concentration factor of 1. For water samples, 1000 mL aliquots of sample were diluted in 1 mL of solvent for HPLC analysis. The LOD is then calculated as:

$$0.025\ \text{ppb} = 0.025\ \mu\text{g/mL} \times \frac{1\ \text{mL}}{1000\ \text{mL}} \times \frac{1000\ \text{mL}}{\text{L}}$$

Peaks at the retention time of IKF-916 in control samples with area counts equal to or greater than the area of the 0.025 µg/mL calibration standard were

considered to be detections, and the residues were quantified. No peaks were found in any control sample which met this criteria.