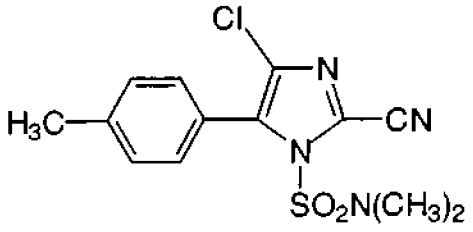
INTRODUCTION

A residue method for the determination of IKF-916 and 4 metabolites in soil 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 AND REFERENCE SUBSTANCES

- **IKF-916**

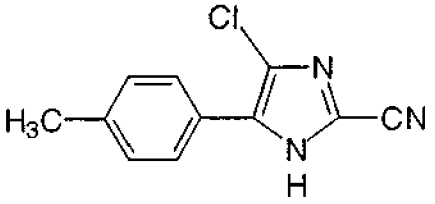


Chemical names: **(IUPAC-J and IUPAC)**
4-chloro-2-cyano-*N*,*N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide

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

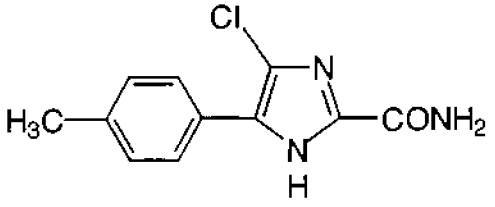
CAS number: 120116-88-3
Lot number: 9704-1
Purity: 99.1%

- **CCIM**



Chemical names: (IUPAC and IUPAC-J)
4-chloro-5-*p*-tolylimidazole-2-carbonitrile
(CA and CA-J)
4-chloro-5-(4-methylphenyl)-1*H*-imidazole-2-carbonitrile
CAS number: 120118-14-1
Lot number: 950308
Purity: 99.7%

- **CCIM-AM**

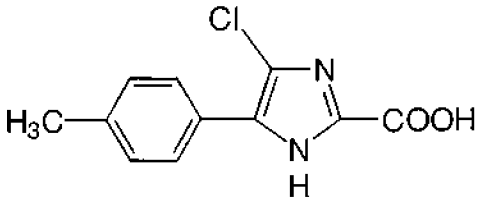


Chemical names: (IUPAC and IUPAC-J)
4-chloro-5-*p*-tolylimidazole-2-carboxamide

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

Lot number: 9804
Purity: 99.6%

- **CTCA**

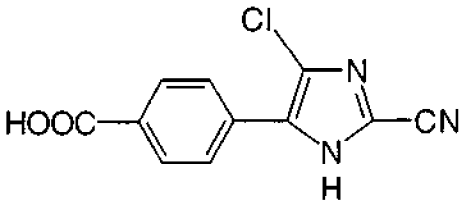


Chemical names: (IUPAC-J)
4-chloro-5-*p*-tolylimidazole-2-carboxylic acid

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

Lot number: 9804
Purity: 99.3%

- **CCBA**



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

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

Lot number: 9907

Purity: 99.2%

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

MATERIALS AND METHODS

SAMPLE PROCUREMENT AND RECEIPT

Soil from the states of New York and Washington were used to validate the method for IKF-916 and four metabolites. A 0-12-inch bulk sample and 18-24-inch core sample of control soil from each state were used. The samples were received from test sites at which ongoing IKF-916 soil dissipation studies are being conducted. The bulk 0-12-inch control soils were received and stored at ambient temperature. The 18-24-inch control samples were received and stored frozen.

SOIL CHARACTERIZATION

Table 1 contains results from soil characterization analyses for the 4 soils used in this method validation study. Rows 1 and 2 of Table 1 show characterization data of the 0-6" and 6-12" depths from New York. The 0-12-inch bulk soil used for validation in this study is a mixture of 0-6" and 6-12" depths. The percent organic matter of the 0-12-inch bulk New York soil would be in the range of 2.13 to 3.33%; the pH would be in the range of 5.8 to 6.1. The 18-24-inch depth from the New York site contained only 0.82% organic matter and the pH was 5.5. The 0-12-inch bulk control from Washington contained much less organic matter (0.38 to 0.60 %) as shown in rows 4 and 5 of Table 1. The pH range of the 0-12-inch bulk Washington soil is 6.5 to 6.8 based on the results from Table 1 for the 0-6" and 6-12" soil depths. The percent organic matter for the Washington 18-24-inch soil was only 0.22%; the pH was 7.4.

SAMPLE IDENTIFICATION

Control soil was used from the New York field site (Protocol 010229-0) and the Washington field site (Protocol 010227-0). At the time of receipt at

Ricerca, control samples were assigned a Ricerca identification code number consisting of the year and a consecutive number. The field protocol sample numbers and Ricerca code numbers are cross-referenced in the table below.

Control Soil	Field Study Protocol Sample Number	Ricerca Identification Code
NY 0-12-inch bulk	010229-030	99-0123
NY 18-24-inch	010229-031D	99-0151
WA 0-12-inch bulk	010227-030	99-0118
WA 18-24-inch	010227-031D	99-0149

At the time of analysis, each aliquot weighed for analysis was assigned a unique laboratory sample ID number consisting of the project number, month and day the sample was extracted. An integer was added usually (1,2) for controls, (3,4,5,6,7) for fortifications. The sample ID and corresponding sample description were recorded on Operations Form and Sample List (flowsheet).

ANALYTICAL PROCEDURE SUMMARY

Figure 1 contains a method flow diagram. A detailed analytical method is provided in Appendix B. A summary of the method is provided here.

Twenty grams of soil was extracted with 200 mL of 80/20 acetonitrile/0.1N HCl. The extract was filtered and brought to a volume of 250 mL. A 125 mL aliquot of the extract was buffered to pH 3, and then partitioned with 2% aqueous sodium sulfate and methylene chloride to isolate IKF-916 and CCIM. The organic phase was evaporated to dryness and taken through a Florisil™ cleanup. The eluate from the cleanup column was evaporated to dryness, and the residue was diluted with 50/50 acetonitrile/water for HPLC quantitation of IKF-916 and CCIM. A second 125 mL aliquot of the extract was concentrated, buffered to pH 3, and taken through a C_{18} solid phase cleanup to isolate CCBA, CTCA and CCIM-AM. All samples were quantitated by reversed-phase HPLC with UV absorbance detection at 280nm. A total of approximately 20 hours is required to complete analysis of a set of seven samples.

PROTOCOL DEVIATIONS

1) The protocol (page 8) stated that the soil samples would be stored frozen. However, the 0-12-inch bulk soil samples were stored at ambient

temperature. It is customary practice at Ricerca to store bulk soil samples used for method development/validation under ambient conditions since it is impractical to freeze/thaw large (approximately 25 pound) soil samples on a frequent basis.

2) CCIM lot number 950308 was used instead of lot number 9506.

There was no effect on the integrity of the study.

CALCULATION OF RESIDUES

Residues of IKF-916 and metabolites were quantitated 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: IKF-916, CCIM, CCIM-AM, CCBA, CTCA Soil Method Flow Diagram

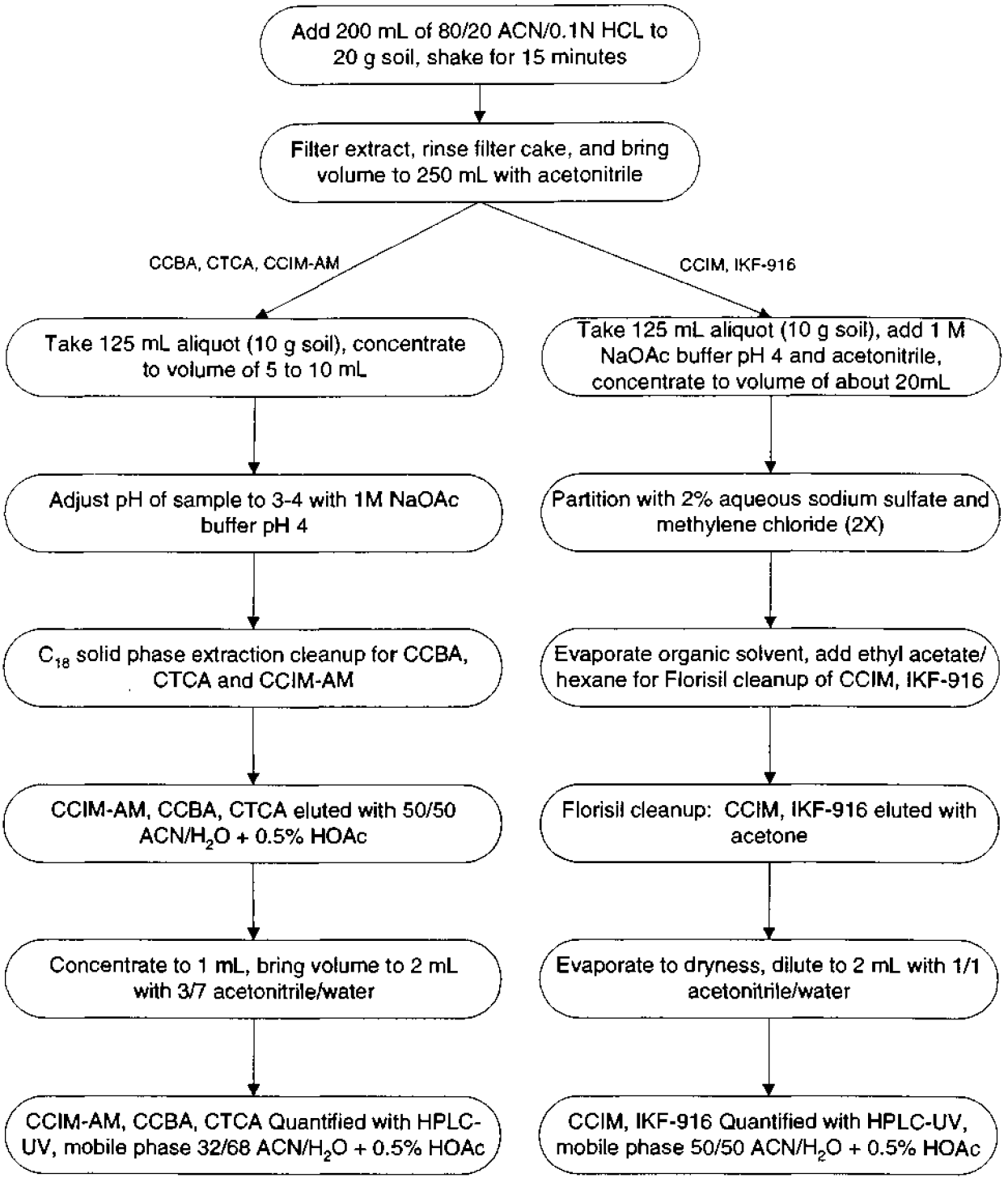


Table 1: Soil Characterization Data

Site	Depth (Inches)	Texture	Sand (%)	Silt (%)	Clay (%)	Organic Matter (%)	pH	CEC	Moisture At 1/3 Bar (%)	Bulk Density (gm/cc)
NY	0-6	Loamy Sand	78.0	16.4	5.6	3.33	6.1	6.11	10.6	1.30
NY	6-12	Sandy Loam	74.0	20.4	5.6	2.13	5.8	5.11	8.37	1.36
NY	18-24	Sandy Loam	72.0	22.4	5.6	0.82	5.5	1.90	6.47	1.39
WA	0-6	Loamy Sand	76.8	18.0	5.2	0.60	6.5	5.92	8.50	1.60
WA	6-12	Loamy Sand	80.8	14.0	5.2	0.38	6.8	6.11	7.13	1.53
WA	18-24	Loamy Sand	80.8	16.0	3.2	0.22	7.4	7.05	8.40	1.60

CEC = Cation Exchange Capacity (meq/100 g)

CHEMICALS

Acetic acid, glacial, Fisher HPLC Grade

Acetone, Fisher Optima Grade

Acetonitrile, Fisher HPLC Grade

CCBA Analytical Standard, Lot No. 9907-1, Purity 98.1%

CCIM Analytical Standard, Lot No. 950308, Purity 99%

CCIM-AM Analytical Standard, Lot No. 9804-1, Purity 98.7%

CTCA Analytical Standard, Lot No. 9804-1, Purity 99.3%

Diethyl ether, J. T. Baker 'Ultra Resi-Analyzed'

Ethyl acetate, Fisher HPLC Grade

Hexanes, Fisher HPLC Grade

Hydrochloric acid, Fisher Trace Metal Grade

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

Methylene chloride, Fisher Optima Grade

Sodium acetate trihydrate, HPLC grade

Sodium sulfate, anhydrous, Fisher ACS reagent

Water, Fisher HPLC Grade

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

REAGENTS

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

Extraction solvent: (80/20 acetonitrile/0.1N HCl (v/v)): Add 200 mL of 0.1N HCl to 800 mL of acetonitrile.

20/80 diethyl/hexanes (v/v): Add 20 mL diethyl ether to 80 mL hexanes.

10/90 acetonitrile/water + 0.5% acetic acid: add 0.5 mL glacial acetic acid to a mixture of 10 mL acetonitrile and 90 mL of water.

25/75 acetonitrile/water + 0.5% acetic acid: add 0.5 mL glacial acetic acid to a mixture of 25 mL acetonitrile and 75 mL of water.

50/50 acetonitrile/water + 0.5% acetic acid: add 0.5 mL glacial acetic acid to a mixture of 25 mL acetonitrile and 75 mL of water.

50/50 acetonitrile/water (v/v): Add 50 mL acetonitrile to 50 mL of water.

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

HPLC Mobile Phase: 32/68 acetonitrile/water + 0.5% acetic acid

Add 10 mL glacial acetic acid to a mixture of 640 mL acetonitrile and 1360 mL of water

HPLC Mobile Phase: 48/52 acetonitrile/water + 0.5% acetic acid

Add 10 mL glacial acetic acid to a mixture of 960 mL acetonitrile and 1040 mL of water

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

Add 10 mL glacial acetic acid to a mixture of 1 liter acetonitrile and 1 liter of water

0.1N hydrochloric Acid solution: Add 0.83 mL concentrated HCl to 100 mL using HPLC grade water.

Sodium acetate buffer, 1 M, pH 4: Add 24 mL of glacial acetic acid and 10 g of sodium acetate trihydrate to 500 mL of water.

2% sodium sulfate (w/v): Dissolve 20 g of Na_2SO_4 in 1000 mL of water.

EQUIPMENT

Analytical electronic balance with 0.1-mg readability

Büchner funnel, 90 mm

Centrifuge bottle with sealing cap, 250-mL polypropylene, Nalgene No. 3141-0250

Cotton, absorbent sanitary bulk

Filter paper, Whatman® 934-AH

Filters, syringe tip, PTFE, 0.2 µm – 1.0 µm, Acrodisc CR or equivalent

Graduated cylinder, 250 mL with ground joint and stopper

Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis.

Mechanical Shaker Box, Eberbach 2-speed or equivalent

PTFE stoppers, 24/40

Reservoirs for Sep-Pak cartridges, 20 mL, Supelco No. 57021

Rotary evaporators with heated water baths (capable of maintaining temperature in the range of ambient to 40 °C)

Round-bottom flasks, 100 mL and 250-mL, with 24/40 ground joint opening

Sep-Pak® Plus Florisil®cartridges, Waters Number WAT020525

Sep-Pak® tC_{18} Env ®cartridges, Waters Number WAT036800

Syringes, plastic disposable, 1 mL to 5 mL

Bakerbond ®C_{18} SPE cartridges, Baker No. 7020-07

Separatory funnel, 250-mL

Sonicator, Fisher FS-14 or equivalent

Top-loading electronic balance with 0.1-g readability

Vacuum manifold for solid phase extraction, Supelco

Vacuum pump

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

INSTRUMENTATION

High-performance liquid chromatograph (HPLC) with data system.

System 1

Waters Wisp 717 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

System 2

Waters Wisp 700 autosampler

Waters 600 E controller & quaternary pump

Applied Biosystems 785A Programmable Absorbance Detector

PE-Nelson Turbochrom chromatography data system

Eldex CH-150 column oven

Helium for degassing mobile phases

HPLC column: The column listed below is recommended.

Luna C_{18} (2), 150 mm × 3.0 mm, 3 µm particle size, Phenomenex No. 00F-4251-YO

HPLC guard column: Security Guard 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

Stock Standards

Weigh 0.0250 g of neat analytical standard for each analyte separately into a small vial. Quantitatively transfer the neat standard with rinses of acetonitrile into a Class A 250-mL volumetric flask. Dilute to the mark with acetonitrile to produce individual 100 µg/mL stock standards of IKF-916, CCIM, CCBA, CTCA and CCIM-AM. Sonication is required to completely dissolve the CCIM-AM and CTCA. The 100 µg/mL stock standards are stored in amber bottles and kept in freezers, except for CCIM-AM which is kept at room temperature. CCIM-AM will precipitate out of solution if kept in a freezer. If a stock CCIM-AM standard is cooled to freezer temperatures, it must be thoroughly sonicated before it is used again.

IKF-916/CCIM Mixed Standards

Prepare an IKF-916/CCIM working standard from the stock standards described above as follows. Transfer 10 mL of the 100-µg/mL IKF-916 standard, and 10 mL of the 100-µg/mL CCIM standard into a Class A 100-mL volumetric flask. Add 30 ml of acetonitrile to the volumetric, then bring volume to mark with water to produce a 10 µg/mL IKF-916/CCIM working standard.

Prepare IKF-916/CCIM calibration standards of concentration 0.025, 0.05, 0.10, 0.25, 0.50 and 1.00 µg/mL with 50/50 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/CCIM. Each calibration standard was prepared in a 30 mL amber vial.

Calibration Standard Concentration	Volume of Standard Used to Prepare	Volume of 50/50 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

NOTE: Recommended expiration interval for the working and calibration standards is one month. Working standard solutions are stored in freezers. It is recommended to sonicate working standards and calibration standards

briefly before withdrawing an aliquot. The working standard and calibration standards are used to make fortifications to soil.

CCBA/CTCA/CCIM-AM Mixed Standards

Prepare a CCBA/CTCA/CCIM-AM working standard from the stock standards described above as follows. Transfer 10 mL of the 100-µg/mL CCBA standard, 10 mL of the 100-µg/mL CTCA standard, and 10 mL of the 100-µg/mL CCIM-AM standard into a Class A 100-mL volumetric flask. Bring the volume to mark with water to produce a 10 µg/mL CCBA/CTCA/CCIM-AM working standard.

Prepare CCBA/CTCA/CCIM-AM 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 CCBA/CTCA/CCIM-AM. Each calibration standard was prepared in a 30 mL amber vial.

Calibration Standard Concentration	Volume of Standard Used to Prepare	Volume of 50/50 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

NOTE: Recommended expiration interval for the working and calibration standards is one month. Working standard solutions are stored in freezers. It is recommended to sonicate working standards and calibration standards briefly before withdrawing an aliquot. The working standard and calibration standards are used to make fortifications to soil.

EXTRACTION

1. Add 20 g of soil sample to a 250 mL Nalgene centrifuge bottle (fortifications are made at this point).

2. Immediately add 200 mL of 80/20 acetonitrile/0.1N HCl to the centrifuge bottle.

3. Shake the sample on a mechanical shaker for approximately 15 minutes.

4. Let soil particles settle in bottle for about 5 to 10 minutes.

5. Filter supernatant through 934-AH paper/Büchner funnel (90mm) into a 250mL graduate with a vacuum filter apparatus. Leave most of soil in the bottle.

6. Add 25 mL of extraction solvent to the bottle, swirl or shake bottle to rinse soil, filter.

7. Rinse centrifuge bottle with about 10 mL of extraction solvent from a squirt bottle, filter.

8. Bring volume to 250 mL with acetonitrile, invert graduate to mix sample.

9. Remove a 125 mL aliquot and transfer to a 500 mL flask for analysis of IKF-916 and CCIM **(Step 11)**.

10. Transfer the remainder to a 500-mL flask, for analysis of CCBA, CTCA and CCIM-AM **(Step 35)**.

ANALYSIS OF IKF-916 AND CCIM

11. Add 15 mL of sodium acetate buffer, pH 4, 1 molar to the sample.

12. Add 50 mL of acetonitrile to the sample.

13. Concentrate the solution to a volume of approximately 15 to 20 mL with a rotary evaporator at a bath temperature no greater than 40 °C and vacuum of about 27" Hg.

14. Transfer the solution from the flask to a 250-mL separatory funnel.

15. Add 50 mL of 2% sodium sulfate/water to the flask as a rinse; transfer to the separatory funnel.

16. Add 50 mL of methylene chloride to the flask as a rinse, transfer to the 250 mL separatory funnel.

17. Partition the sample by shaking the separatory funnel for approximately one minute.

18. After phase separation drain the lower(organic layer) through anhydrous sodium sulfate contained in a funnel plugged with cotton, and into a 250 mL flask.

19. Repeat partition with 50 mL of methylene chloride. **Analysis of IKF-916 and CCIM must be completed to this step on the same day the samples are extracted.**

20. Concentrate the solution to dryness in the 250-mL flask with a rotary evaporator at a bath temperature no greater than 40 °C and vacuum of about 27" Hg. Proceed to next step as soon as flask has cooled to room temperature (do not let sample sit 'dry' for very long).

21. Add 1 mL of ethyl acetate to the sample residue in the 250-mL flask, swirl to dissolve.

22. Add 20 mL of hexane to the sample.

23. Set up a solid phase extraction manifold with Waters Sep-Pak Florisil™ cartridges and 20 mL or 60 mL reservoirs (filtration tubes).

24. Add 10 mL of acetone to the reservoir; drain to the top of the packing to condition the Florisil™. Do not let column packing go dry during the procedure.

25. Add 10 mL of hexane to the reservoir; drain to the top of the packing to condition the Florisil™. Do not let column packing go dry during the procedure.

26. Transfer the sample solution to the SPE reservoir; drain to the top of the column packing.

27. Add 10 mL of 20/80 diethyl ether/hexane to the flask, swirl and transfer the solution to the SPE reservoir, drain the solution to the top of the column packing.

28. Place a 50-mL beaker or other suitable container under each column position in the solid phase extraction manifold.

29. Add 50 mL of acetone to the flask, swirl and transfer the solution to the SPE reservoir. Acetone may be added in 2 x 25 mL aliquots.

30. Drain the acetone through the column; collect the acetone eluate.

31. Transfer the solution to a 100 mL flask. Acetone may be collected and transferred twice, in 25 mL aliquots.

32. Concentrate the solution to dryness with a rotary evaporator at a bath temperature no greater than 40 °C and vacuum of about 27" Hg. Proceed to next step as soon as flask has cooled to room temperature (do not let sample sit 'dry' for very long).

33. Add 2 mL of 50/50 acetonitrile/water to the sample residue, swirl and/or sonicate the sample.

34. Transfer a sample aliquot into an autosampler vial for HPLC quantitation. Filter through a PTFE disk if necessary (up to analyst, depending on clarity of solution).

ANALYSIS OF CCBA, CTCA AND CCIM-AM

35. Concentrate the 125-mL of solution to a volume of approximately 5 to 10 mL with a rotary evaporator at a bath temperature no greater than 40 °C and vacuum of about 27" Hg.

36. Add 15 mL of sodium acetate buffer, pH 4, 1 molar to the sample in order to adjust pH of sample solution to approximately pH 3.

37. Set up a solid phase vacuum manifold with Waters tC_{18} Sep-Pak columns and 20-mL (or 60 mL) filtration reservoirs on top of the columns. Place a small plug of cotton in the reservoir; compress it with a pipet. *(Bakerbond C_{18} No. 7020-7 SPE columns (6 mL) may also be used. The cotton may be placed above the packing in the Baker SPE tube. When using C_{18} SPE Bakerbond 7020-7 with 6 mL reservoir, use a disposable pipet to draw solution down through the reservoir attached to the top of the column and into the body of the C_{18} column tube before draining the column. Otherwise, packing may go dry.)* Condition the C_{18} SPE columns with 5 mL of acetonitrile, then 5 mL of 1/9 acetonitrile/water + 0.5% acetic acid. Do not let packing go dry at any time during SPE cleanup.

38. Add the sample to the SPE reservoir.

39. Drain the sample solution through the C_{18} SPE tube by applying vacuum to the manifold. Do not exceed the recommended vacuum limit (20" Hg for the Supelco manifold). Drain until solution level is even with the cotton plug.

40. Add 15 mL of 25/75 acetonitrile/water + 0.5% acetic acid to the 500 mL flask, swirl and add the solution to the SPE reservoir.

41. Drain the 15 mL of 25/75 acetonitrile/water + 0.5% acetic acid through the C_{18} SPE column until the liquid level is even with the cotton plug.

42. Place a 30-mL beaker or other suitable container under each column position in the solid phase extraction manifold.

43. Add 30 mL of 50/50 acetonitrile/water + 0.5% acetic acid to the 500 mL flask, swirl, and add the solution to the SPE reservoir.

44. Drain the 30 mL of 50/50 acetonitrile/water + 0.5% acetic acid through the C_{18} SPE column completely, the packing may go dry at this step.

45. Remove the beaker or container holding the 50/50 acetonitrile/water + 0.5% acetic acid, this solution contains CCBA, CTCA and CCIM-AM.

46. Transfer the sample from the solid phase extraction step to a 250-mL flask.

47. Add 70 mL of acetonitrile to the sample. The acetonitrile should be added in two aliquots of 35 mL to the 50 mL beaker in which the sample was collected from the solid phase cleanup, then rinsed into the 250-mL flask.

48. Concentrate the solution to a volume of 5 to 10 mL with a rotary evaporator at a bath temperature no greater than 40 °C and vacuum of about 27" Hg.

49. Remove the flask from the rotary evaporator and add 10 mL of acetonitrile to the sample.

50. Continue evaporating the solvent in the water bath until about 1 to 2 mL of solution remains.

51. Elevate the flask out of the water bath, continue to evaporate solvent until 0.5 to 1.0 mL of solution remains.

52. Transfer the solution from the 250-mL flask to a 5 mL graduated centrifuge tube with a disposable pipette.

53. Bring volume to 2 mL with 30/70 acetonitrile/water as follows. Add approximately 1 mL of 30/70 acetonitrile/water to the flask. Swirl and transfer the solution to the centrifuge tube to bring the volume to nearly

2 mL. Take care to let solvent run down sides to bottom of flask, transfer as much of this solvent to the centrifuge tube as possible. Bring volume in centrifuge tube to the 2 mL mark with 30/70 acetonitrile/water.

54. Transfer a sample aliquot into an autosampler vial for HPLC quantitation. Samples may be filtered through PTFE disk filters if necessary (up to discretion of analyst).

HPLC QUANTITATION

Polar Metabolite Fraction (CCIM-AM, CCBA, CTCA)

Column:	Luna C_{18} (2), 150 x 3.0mm, 3µm particle size
Guard column:	Security Guard, C_{18}
Mobile phase:	32/68 acetonitrile/water + 0.5% acetic acid
Flow rate:	0.5 mL/min.
Injection volume:	100 µL
Column temperature:	40 °C
Detector:	Absorbance, Wavelength = 280 nm, range = 0.01 AUFS

CCIM/916 Fraction

Column:	Luna C_{18} (2) , 150 x 3.0mm, 3µm particle size
Guard column:	Security Guard, C_{18}
Mobile phase:	48/52 Acetonitrile/Water + 0.5% acetic acid
Flow rate:	0.6 mL/min.
Injection volume:	100 µL
Column temperature:	40 °C
Detector:	Absorbance, Wavelength=280 nm, range = 0.01 AUFS

NOTE: (HPLC conditions such as column type, mobile phase composition, flow rate, etc. may be modified at any time during this method validation study. Changes will be documented in each data set).

Instrument Calibration

Under the instrumental conditions described above the retention time of IKF-916 is approximately 17 minutes, and the retention time of CCIM is approximately 8 minutes (Luna C_{18} column).

Under the instrumental conditions described above the retention time of CCBA is approximately 8 minutes, the retention time of CTCA is

approximately 9 minutes, and the retention time of CCIM-AM is approximately 13 minutes (Luna C_{18} column).

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