1 OBJECTIVE

The objective of this study was to validate the residue analytical method for the determination of IKI-3106 and its metabolites NK-1375 and NSY-137 in soil.

2 CONDUCT OF STUDY

The study was conducted at Ishihara Sangyo Kaisha, Ltd., Central Research Institute, Safety Science Research Laboratory, Environmental Sciences Group, 3-1, 2-Chome, Nishi-shibukawa Kusatsu-shi, Shiga-ken, 525-0025 Japan. The experimental start and termination dates were April 26, 2011 and June 6, 2011, respectively.

3 MATERIAL AND METHOD

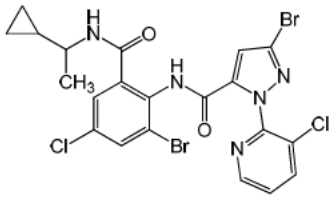
3.1 Analytical standards

3.1.1 IKI-3106

Product name: IKI-3106

Chemical name: 3-bromo-*N*-[2-bromo-4-chloro-6-[[(1-cyclopropylethyl)amino] carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide

Structure:



Molecular weight: 602.11

Lot No.: 0804

Purity: 99 %

3.1.2 NK-1375

Product name: NK-1375

Chemical name: 3-bromo-2-((2-bromo-4H-pyrazolo[1,5-d]pyrido[3,2-b] [1,4]oxazin-4-ylidene)amino)-5-chloro-N-(1-cyclopropylethyl) benzamide

Structure:

Molecular weight: 565.65

Lot No.: 20100825

Purity: 98.0 %

3.1.3 NSY-137

Product name: NSY-137

Chemical name: 8-bromo-2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3-(1-cyclopropylethyl)quinazoline-4(3H)-one

Structure:

Molecular weight: 565.65

Lot No.: 20100917

Purity: 95.9 %

3.2 Test soil

Two Japanese soils of different type were used in the study. Soil properties are shown in table below.

	Unit	Ibaraki	Kochi
pH (H_2O)		6.7	6.6
pH (KCl)		6.2	5.5
Caly <2 µm	% w/w	23.8	18.9
Silt 2-50 µm	% w/w	27.8	24.5
Sand 50-2000 µm	% w/w	38.5	56.8
Cation Exchange Capacity	meq/100g	33.2	13.3
Maximum Water Holding Capacity	g/100g	96.0	59.4
Organic Carbon	% w/w	4.1	1.4

Note: These information were based on the data of soil collected in same place in past time.

3.3 Reagents and apparatus

All reagents were of analytical, HPLC, or LC/MS/MS grade.

REAGENTS & APPARATUS	SUPPLIER	NOTE
Purified water	Millipore	MILLIPORE UF
Acetonitrile	Wako Pure Chemical	
Methanol	Nacalai Tesque	
Acetic acid	Wako Pure Chemical	
Celite	Nacalai Tesque	545RVS
Erlenmeyer flask with ground glass stopper	Iwaki	200 mL
Volumetric flasks	Iwaki	various sizes
Volumetric pipettes	Iwaki	various sizes
Measuring cylinder	Iwaki	various sizes
Glass funnel	Iwaki	
µL-Pipettes	Nichiryo	various sizes
SPE Cartridge	Waters	OASIS® HLB VAC RC (60mg)
SPE manifold	Waters	
HPLC vial	Waters	LC/MS certificated
Analytical balance	A&D	HA-202M
Laboratory balance	UX4200S	Shimadzu
Reciprocal shaker	TAIYO	SR-2
Oven	ISUZU SEISAKUSHO	MODEL 2-2132

3.4 Standard solutions

3.4.1 Stock solutions

Each 5.0 mg of IKI-3106, NK-1375 and NSY-137 was weighed into separate 500 mL-Volumetric flask. Acetonitrile was added to make stock standard solutions with a concentration of 10 μg/mL.

3.4.2 Fortification solutions

The stock solutions of 10 μg/mL were diluted using acetonitrile to obtain standard solutions with a concentration of 1 μg/mL.

The standard solutions of 1 μg/mL were diluted using acetonitrile to obtain fortification solutions with a concentration of 100 ng/mL.

3.4.3 Calibration solutions

Calibration solutions, over the concentration range 0.025 to 10 ng/mL of IKI-3106, NK-1375 and NSY-137, were prepared by serial dilution of the fortification solutions in acetonitrile.

Note: NK-1375 and NSY-137 were prepared separately.

3.5 Fortification

To demonstrate the validity of the method used, untreated soils were fortified with the following levels for the IKI-3106, NK-1375 and NSY-137.

0.005 mg/kg	1 mL of the fortification solution (100 ng/mL) was added to 20 g (dry mass) soil.
0.2 mg/kg	0.4 mL of the stock solution (10 μg/mL) was added to 20 g (dry mass) soil.

Note: NK-1375 and NSY-137 were separately added to the individual untreated soil.

3.6 Analytical method

3.6.1 Extraction

20 g (dry mass) of the untreated soil sample was weighed into a 200 mL Erlenmeyer flask with ground glass stopper. 100 mL of acetonitrile:water (80:20, v/v) and 1 mL of 6 mol/L hydrochloric acid were added to the soil sample. The sample was shaken for 30 minutes using a reciprocal shaker. The mixture was filtered through a Celite 545. The filter cake was washed with 80 mL of acetonitrile:water (80:20, v/v). The filtrate and washings were combined and then filled up to 200 mL with acetonitrile:water (80:20, v/v).

3.6.2 Sample clean up on 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). 2 mL of the extract and 10 mL of water were mixed and transferred into the SPE cartridge. The aqueous sample solution was sucked through the column followed by 4 mL of acetonitrile:water (20:80, v/v). All eluates were discarded. IKI-3106, NK-1375 and NSY-137 were eluted with 10 mL of acetonitrile. The eluate was collected and then filled up to 10 mL with acetonitrile.

Note: The eluate samples were stored in refrigerator (4°C) overnight before quantitation

3.6.3 Quantitation

Quantitation of the IKI-3106, NK-1375 and NSY-137 concentration was performed by LC/MS/MS using the external standard method. The calibration standards at seven concentrations (0.025, 0.05, 0.1, 0.2, 1, 2 and 10 ng/mL) were used for construction of a calibration curve. The calibration curve was constructed by plotting the peak areas against the concentration of calibration standards. From the calibration curve, the concentration of IKI-3106, NK-1375 and NSY-137 in the injected solution was determined and the residue of IKI-3106, NK-1375 and NSY-137 in soil sample was calculated.

3.7 LC/MS/MS conditions

3.7.1 HPLC

Instrument:	ACQUITY UPLC System (Waters)
Column:	BEH C18 2.1×50 mm, 1.7 μm (Waters)
Guard column:	VanGuard BEH C18 2.1×5 mm, 1.7 μm (Waters)
Column temp.:	40°C
Mobile phase:	Acetonitrile:Water:Acetic acid (70:30:0.1, v/v/v)
Flow rate:	0.4 mL/min
Injection volume:	4 μL
Retention time:	0.68 min (IKI-3106) 0.92 min (NK-1375) 1.95 min (NSY-137)

3.7.2 MS/MS

Instrument:	API4000QTRAP (AB sciex)
Ionization mode:	ESI
Scan mode:	MRM
Mass resolution	Q1;unit, Q3;low
Heater gas temp.:	600 °C
Ion voltage:	5000 V
Gas flow settings:	Gas1;50, Gas2;90, CUR;15, CAD;11

3.7.3 Primary method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKI-3106	Pos. $[M+H]^+$	601.8	283.8	27	66	10	20
NK-1375	Pos. $[M+H]^+$	565.8	497.9	23	81	10	12
NSY-137	Pos. $[M+H]^+$	565.8	498.1	27	61	10	14

3.7.4 Confirmatory method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKI-3106	Pos. $[M+H]^+$	601.8	177.0	73	66	10	28
NK-1375	Pos. $[M+H]^+$	565.8	265.8	33	81	10	44
NSY-137	Pos. $[M+H]^+$	565.8	404.9	55	61	10	10

3.8 Calculation

The residue of IKI-3106, NK-1375 and NSY-137 in soil was calculated according to equation 1.

$$R = \frac{X \times V_F \times D}{W \times 1000} \qquad (1)$$

Where

R = Residue of IKI-3106, NK-1375 and NSY-137 in soil sample [mg/kg]

X = Concentration of injected solution [ng/mL]

V_F = Final Volume [10 mL]

D = Dilution Factor [if applicable]

W = Aliquot of sample [0.2 g]

1000 = Conversion factor from ng to μg

The recovery of IKI-3106, NK-1375 and NSY-137 in soil was calculated according to equation 2.

$$Rec = \frac{R \times 100}{F} \qquad (2)$$

Where

Rec = Recovery of IKI-3106, NK-1375 and NSY-137 [%]

R = Residue of IKI-3106, NK-1375 and NSY-137 in soil sample [mg/kg]

F = Fortification level [mg/kg]