EXECUTIVE SUMMARY

The purpose of the study was to demonstrate Method I-605, entitled "Validation of the Residue Analytical Method for the Determination of IKI-3106 and Its Metabolites NK-1375 and NSY-137 in soil," [**1**] could be performed successfully at an outside facility with no prior method experience. The study was conducted in compliance with USA EPA Good Laboratory Practice Standards, US EPA test guidelines OCSPP 850.6100, OPPTS 860.1340, OPPTS 850.7100, and EC Guidelines SANCO/825/00 rev 8.1 (Nov. 16, 2010).

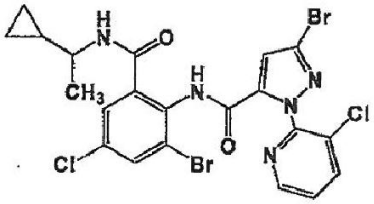
The analytical method was successfully implemented at ADPEN during the first trial for a loamy sand soil sample, and IKI-3106 and NK-1375 were recovered within acceptable limits.

Briefly, residues of IKI-3106 and NK-1375 are extracted with acetonitrile/water and hydrochloric acid. Sample extract was taken through a solid-phase extraction (SPE) cleanup and residues were determined by LC-MS/MS. The analytical method has a limit of quantitation (LOQ) is 0.005 ppm (mg/kg) for the analysis of IKI-3106 and NK-1375 in soil. The method limit of detection (LOD) in soil was estimated at 0.00125 ppm, or approximately 25% of the LOQ.

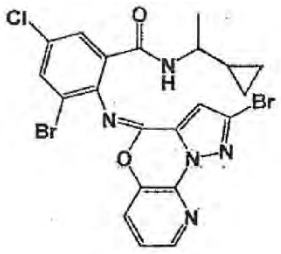
For validation, untreated soil samples were fortified with IKI-3106 and NK-1375, and analyzed according to the established method validation guidelines. The analytical set consisted of a method blank, two controls, five replicates fortified with IKI-3106 and NK-1375 at the method LOQ, and five replicates fortified at a higher level (0.20 ppm). Apparent residues of IKI-3106 and NK-1375 were below the LOD in the control soil sample.

The method validation was successfully completed during the first trial for both primary and confirmatory methods of IKI-3106 and NK-1375 in soil matrices after modifications to the LC-MS/MS conditions as presented in the analytical method. Suppression of the instrument response was found to be 50% or greater using the instrument conditions listed in the method and initial recovery results were not within the acceptable range of 70–120%. Sample extracts were reanalyzed using a gradient elution, which lengthened the elution time and minimized matrix suppression.

Substance 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
Empirical Formula: $C_{21}H_{17}Br_2Cl_2N_5O_2$
Molecular Weight: 602.1 g/mol
Lot Number: 20110113
Purity: 99.18% (w/w)
Storage Conditions: Keep frozen
Expiration Date: January 24, 2016
Molecular Structure:



Substance Name: NK-1375
Chemical Name: 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-*d*]pyrido[3,2-*b*][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl)benzamide
Empirical Formula: $C_{21}H_{16}Br_2ClN_5O_2$
Molecular Weight: 565.65 g/mol
Lot Number: 233-004-22-3
Purity: 98.6%
Storage Conditions: Keep frozen. Dark.
Expiration Date: July 25, 2015
Molecular Structure:



3.0 STUDY DESIGN

The residue analytical method for the determination of IKI-3106 and NK-1375 in soil was validated at a limit of quantitation (LOQ) of 0.005 mg/kg. The analytical set consisted of a method blank, two unfortified matrix control samples, five matrix control samples fortified at the LOQ (0.005 ppm) and five matrix control samples fortified at 40 times the LOQ (0.2 mg/kg) for each of the analytes. An additional workorder was analyzed to test that the matrix was free of inteferences at the retention times of the analytes.

4.0 PROCEDURE – METHOD SYNOPSIS

Method Number I-605 is used to determine residues of IKI-3106 and NK-1375 in soil as investigated for this validation study. The following is a brief summary of the procedure:

Residues of IKI-3106 and NK-1375 are extracted from soil by adding acetonitrile/water (80:20, v/v) and 6 M hydrochloric acid (1 mL) using a 250-mL amber bottle. The sample was shaken for 30 minutes and filtered through Celite 545. The filter cake was washed with acetonitrile/water (80:20, v/v; 80 mL). The filtrate and washings were combined and brought to volume with acetonitrile/water (80:20, v/v). A SPE cartridge was placed onto a SPE vacuum manifold and conditioned using methanol (5 mL) followed by water (5 mL). A 2-mL aliquot of the extract and 10 mL water were mixed and transferred into the SPE cartridge. The aqueous sample solution was sucked through the column followed by 4 mL acetonitrile/water (20:80, v/v). All eluates were discarded, and the analytes were eluted with 10 mL acetonitrile. The eluate was collected and then brought to a volume of 10 mL using acetonitrile. A 1-mL aliquot of the sample was vialed into a 2-mL autosampler vial and stored at 4 °C before analysis. Quantitation of IKI-3106 and NK-1375 was performed by LC-MS/MS. A flow diagram of the analytical method is presented in Appendix D.

5.0 TIME REQUIRED FOR ANALYSIS

The methodology is normally performed with a batch of 13 samples. One skilled person can complete one batch of samples in eight working hours.

6.0 LIMIT OF QUANTITATION AND DETECTION

The limit of quantitation (LOQ) for residues of IKI-3106 and NK-1375 in soil is 0.005 mg/kg for each analyte. The limit of detection (LOD) is 0.00125 mg/kg for each analyte.

7.0 CALIBRATION, CALCULATIONS, AND STATISTICS

Quantitation of IKI-3106 and NK-1375 was performed by LC-MS/MS using the external standard method. Calibration standards at eight concentrations (0.025, 0.05, 0.1, 0.2, 1, 2, 5, and 10 ng/mL) were used for construction of the calibration curve. A calibration curve for each analyte was generated by plotting the detector's response (peak area) versus amount of standard injected. The data system derived an equation for the fit of the standard curve, and this equation was used to calculate intercept and slope of the linear regression curve.

Peak integration and quantitation were performed using the primary and confirmatory ions using MassHunter data system. A confirmatory ion transition provided in the method can be used as a confirmatory ion or as an alternate means of calculating residues should interferences be found with the primary quantitation ion transition.

Recovery results were calculated for each set of samples within the Laboratory Information Management System (LIMS) and reported in Microsoft® Excel, which are presented in Appendix A. Statistical treatment of the data included calculation of averages and standard deviations. No calculations were made with rounded numbers.

a) Calibration curve: Solving for x: $\frac{y - b}{m}$

Where, m = slope
b = y-intercept
x = Amount found (ng)
y = Peak area

b) Amount of sample in ected mg $\frac{\text{in ection si e}}{\text{final olume m}}$ sample weight (g) $\frac{1\ \text{m}}{1000}$ $\frac{1000\ \text{mg}}{1\ \text{g}}$

c) esidue found (ppm) $\frac{\text{ng found}}{\text{Amount of sample in ected mg}}$

d) eco e ry $\frac{(\text{e sidue in Sample ppm}) - \text{e sidue in ontr ol Sample ppm}}{\text{Amount fortified (ppm)}}$ 100

As an example, lab code 13073101-Recovery1-1 for IKI-3106 (primary, *m/z* 602 → 284) on QC-13073101B.

a) Calibration curve: y 393556 566058 - 2 489630

Solving for x: $\frac{404 \quad 2\ 489630}{393556\ 566058}$ 0 001033 ng

b) Amount of sample in ected (g) $\frac{10}{1000\ \text{m}}$ 20 00 g $\frac{1\ \text{m}}{1000}$ $\frac{1000\ \text{mg}}{1\ \text{g}}$ 0 2000 mg

c) esidue found ( ) $\frac{0\ 001033\ \text{ng}}{0\ 20000\ \text{mg}}$ 0 0 5164 ng/mg

d) eco e ry ( ) $\frac{0\ 005164\ \text{ppm} - 0\ 000266\ \text{ppm}}{0\ 005\ \text{ppm}}$ 100 98

TABLE 5. Typical LC-MS/MS Instrument Parameters

Instrument:	Agilent 1290 UHPLC
Detector:	Agilent 6490 Series QQQ
Processing software:	MassHunter Software Acq. B.04.01
Column:	Zorbax Eclipse Plus C_{18}; 1.8 µ, 2.1 × 50 mm
Injection:	10 µL
Column Temperature:	40 °C

Mobile Phase:	A = 0.1% acetic acid in HPLC water B = 0.1% acetic acid in acetonitrile		
Gradient:	**Time (minute)**	**Composition (%)**	
		A	**B**
	0.00	30.0	70.0
	3.00	30.0	70.0
Flow Rate:	0.4 mL/minute		

Analytes	**Expected Retention Times (minutes)**	**Transitions (*m/z*):**
		Quantitation ion
IKI-3106	2.78	601.8 → 283.8
IKI-3106 C	2.78	601.8 → 176.9
NK-1375	3.60	565.8 → 497.8
NK-1375 C	3.60	565.8 → 265.7
Ionization Mode:	Positive ion; Electrospray (ESI) at 150 °C	

Appendix B: Recommendations for ISK Analytical Method I-605

The following recommendations should be considered for inclusion in analytical method I-605:

1. In Section 3.3 and 3.7, a general statement should be made regarding equivalent apparatus and instrumentation.

2. Recommend adding in Section 3.4.1 the addition of 0.05% acetic acid in ACN to stabilize NK-1375 stock standard solution.

3. In Section 3.6.3, an additional calibration standard is recommended between the 2 and 10 ng/mL standards. A 5 ng/mL standard was used in this validation study.

4. In Section 3.6.1, the thickness of the Celite 545 bed is not given and should be specified.

5. Recommend inclusion of alternate LC gradient conditions in Section 3.7 to lengthen the elution time and minimize matrix suppression.

Appendix D: Flow Diagram of Analytical Method I-605