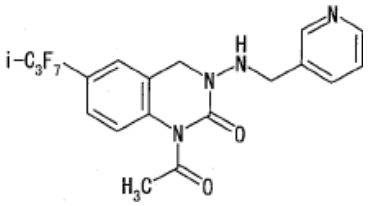
3.0 MATERIALS AND METHODS

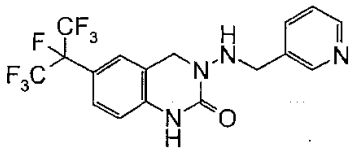
3.1 Test Substances

The reference analytical standards (test substances) used for this study were:

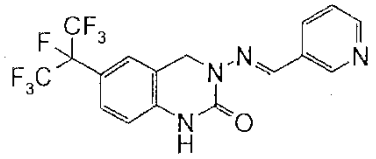
Pyrifluquinazon:

Common Name:	Pyrifluquinazon
Code Name:	NNI-0101
Chemical Name (CAS):	1-acetyl-3,4-dihydro-3-[(3-pyridinylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-2(1*H*)-quinazolinone
(IUPAC):	1-acetyl-1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one
Structure:	
CAS Number:	337458-27-2
Source:	Nihon Nohyaku Co Ltd.
Purity:	99.9%
Lot Number:	4FZ0017P
Receipt Date:	1 Mar 2013
Expiration Date:	3 Apr 2013
Storage:	2-8 °C

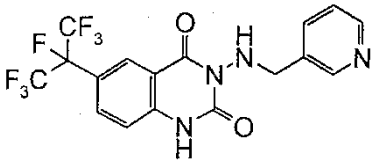
NNI-0101-1H, (IV-01):

Common Designation:	NNI-0101-1H, pyrifluquinazon metabolite
Code Name:	NNI-0101-1H
Chemical Name:	1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	98.7%
Lot Number:	4FZ6404P
Receipt Date:	1 Mar 2013
Expiration Date:	19 Aug 2017
Storage:	2-8 °C

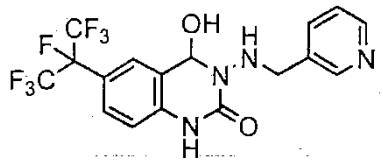
NNI-0101-1H-imino, (IV-02):

Common Designation:	NNI-0101-1H-imino, pyrifluquinazon metabolite
Code Name:	NNI-0101-1H-imino
Chemical Name:	1,2,3,4-tetrahydro-3-[(3-pyridylmethylene)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	99.3%
Lot Number:	4FZ6304P
Receipt Date:	1 Mar 2013
Expiration Date:	13 Oct 2020
Storage:	2-8 °C

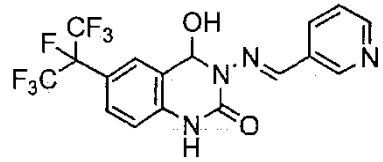
NNI-0101-1H-4-oxo, (IV-15):

Common Designation:	NNI-0101-1H-4-oxo, pyrifluquinazon metabolite
Code Name:	NNI-0101-1H-4-oxo
Chemical Name:	1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2,4-dione
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	99.5%
Lot Number:	4FZ0301S
Receipt Date:	1 Mar 2013
Expiration Date:	9 Oct 2020
Storage:	2-8 °C

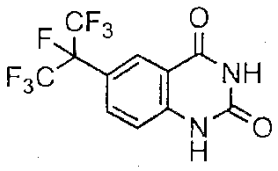
NNI-0101-1H-4-OH, (IV-27):

Common Designation:	NNI-0101-1H-4-OH, pyrifluquinazon metabolite
Code Name:	NNI-0101-1H-4-OH
Chemical Name:	1,2,3,4-tetrahydro-4-hydroxy-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	91.5%
Lot Number:	4FZ0901S
Receipt Date:	1 Mar 2013
Expiration Date:	22 Oct 2020
Storage:	2-8 °C

NNI-0101-1H-imino-4-OH, (IV-28):

Common Designation:	NNI-0101-1H-imino-4-OH, pyrifluquinazon metabolite
Code Name:	NNI-0101-1H-imino-4-OH
Chemical Name:	4-hydroxy-3-[(pyridin-3-ylmethylene)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-3,4-dihydro-1*H*-quinazolin-2-one
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	96.9%
Lot Number:	5FZ1301S
Receipt Date:	1 Mar 2013
Expiration Date:	3 Nov 2013
Storage:	2-8 °C

NNI-0101-quinazolinedione, (IV-203):

Common Designation:	NNI-0101-quinazolinedione, pyrifluquinazon metabolite
Code Name:	NNI-0101-quinazolinedione
Chemical Name:	1,2,3,4-tetrahydro-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl]quinazolin-2,4-dione
Structure:	
CAS Number:	Not Available
Source:	Nihon Nohyaku Co Ltd.
Purity:	99.7%
Lot Number:	7FZ0602S
Receipt Date:	1 Mar 2013
Expiration Date:	23 Mar 2015
Storage:	2-8 °C

The pyrifluquinazon and metabolite standards were supplied by the Sponsor. Information pertaining to the characterization and stability of the test substance is archived by the Sponsor. The Certificates of Analysis are included in Appendix 2.

3.2 Test Systems

In this study, the analytical method was validated on the following matrix: soil. This matrix was chosen as representative of the matrix for which the method was designed.

The control sample of soil used in the study was provided by the laboratory. The control soil sample was obtained from AGVISE Laboratories, Northwood, ND. The sample remained in frozen storage until removed for subsampling and analysis.

The soil specimen was GLP characterized by AGVISE Laboratories; details of the characterization results are as follows:

Sample ID	Sand (%)	Silt (%)	Clay (%)	USDA Textural Class	Disturbed Bulk Density (g/cm^2)	Percent Moisture @ 1/3 bar	Organic Matter (%)	Soil pH[a]
RMN 0-6”	81	8	11	Loamy sand	1.14	11.3	1.7	6.0

[a] Soil pH was measured in a 1:1 water/soil pH.

The samples were assigned unique identification by the laboratory. Additional designations such as "control" and "fortified control," as appropriate, were also assigned by the laboratory.

3.3 Equipment

Equipment used is the same as that specified in the analytical method, except as follows:

Equipment Description	Product ID	Supplier
Analytical Balances	Mettler XP205 Analytical Balance Mettler BB2440 Analytical Balance	Mettler Instrument Corp (Hightstown, NJ)
Labware	Sorvall RC-5B SuperSpeed Centrifuge	DuPont Instruments (Wilmington, DE)
	Beckman GS-6R Centrifuge Beckman GP Centrifuge	Beckman (Palo Alto, CA)
	Platform shaker	Eberbach Corp. (Ann Arbor, MI)
	Kimble-Chase 50-mL glass conical tubes Graduated mixing cylinder Glass wool National Scientific 2-mL HPLC Snap-It glass vials National Scientific Snap-It caps, PTFE/Sil Septa	Fisher Scientific (Pittsburgh, PA)
Pipettors	Gilson 3-1000 µL	Gilson (Middleton, WI)

3.4 Reagents and Standards

Reagents and standards used were of equivalent grade as that specified in the analytical method.

3.5 Principles of the Analytical Method

The residue analytical method described in Morse Laboratories, LLC, Analytical Method# Meth-203 entitled "Determination of Pyrifluquinazon and Relevant Metabolites in Soil" was used for the analyses in this study. See Appendix 1 for the complete text of the method as conducted at ABC Laboratories, Inc. The following is a summary of that method:

Pyrifluquinazon and its metabolite residues were extracted from the sample with neutral extraction solution (acetonitrile and sodium ascorbate buffer (pH7)). The sample was centrifuged and the supernatant was collected in a graduated mixing cylinder. The sample was extracted a second time, centrifuged and the extracts combined in the same mixing cylinder. The sample was brought to final volume with neutral extraction solution, mixed well and an aliquot, diluted in water, was taken for SPE cleanup. The sample was loaded onto the SPE cartridge, rinsed with water and 5:95 acetonitrile:water. The sample was eluted from the SPE cartridge with acetonitrile and allowed to go dry under vacuum. The sample was brought to final volume with methanol to make a 50:50 mixture and submitted for HPLC analysis.

The purified extract was analyzed by reversed-phase HPLC using conventional LC/MS/MS. Detection of the analytes was by turbo ion spray mass spectrometry/mass spectrometry (TIS-MS/MS) in the positive ion mode for quantitative analysis for all analytes except IV-203 which required negative ion mode.

3.6 Modifications, Interpretations, and Critical Steps

The analytical method was run exactly as written except as follows:

Section 8 Sample Extraction. A platform shaker run at high speed was substituted for a wrist-action shaker. Based upon its mode of action, the platform shaker was deemed equivalent to the wrist-action shaker.

Section 10.1 Operating Conditions (HPLC). When utilizing an Agilent 1100 HPLC system, with an Applied Biosystems/Sciex API 4000 LC-MS/MS for quantitation, it may be necessary to increase the column flow rate as well as adjust the gradient ratios between aqueous and organic to maintain a reproducible elution profile on the HPLC column.

3.7 Instrumentation

The quantitative analysis of pyrifluquinazon and its metabolites was performed using an Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system. The system parameters are shown in the tables below. Peak area was used for quantitation.

Typical HPLC Conditions (positive and negative mode):

System:	Applied Biosystems/Sciex API 4000 LC-MS/MS; Agilent 1100 Column Compartment, Well Plate Autosampler, Thermostat Control, Vacuum Degasser, Binary Pump, and Handheld Controller; Valco Divert Valve. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software.
Column:	100 mm × 2 mm, Phenomenex Luna C18(2)-HST analytical column with 2.5 µm particle size
Column Temperature:	40 °C
Injection Volume:	10 µL
Autosampler Temperature:	23 °C
Flow Rate:	350 µL/min
Mobile Phase:	A: 0.1% Formic Acid in Water B: Acetonitrile

Mobile Phase Conditions:

Time	%A	%B	Flow (µL/min)
0.00	90	10	350
0.50	90	10	350
1.00	75	25	350
3.00	55	45	350
8.50	40	60	350
9.00	40	60	350
9.10	0	100	350
11.00	0	100	350
11.01	90	10	350
16.00	90	10	350

Retention Times:	Pyrifluquinazon	~9.5 minutes
	IV-01	~8.4 minutes
	IV-02	~9.7 minutes
	IV-15	~8.0 minutes
	IV-27	~7.6 minutes
	IV-28	~9.3 minutes
	IV-203	~10.4 minutes (switched to negative mode @ 10 mins)
Total Run Time:	~16 minutes	

The detection method utilized was LC-MS/MS employing turbo ion spray (TIS) interface in the positive mode, except IV-203 which required negative ion mode, on a triple quadrupole instrument. The instrument was tuned by infusing the analytes into a TIS source, then creating a tune file to maximize the response of each analyte using the TIS source. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for each analyte are shown in the table below:

Typical MS Conditions:

System: Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system

Analytes Monitored	Ions Monitored (AMU)	Declustering Potential (volts)	Collision Energy (volts)	Dwell Time (seconds)	EP (volts)	CXP (volts)	Acquisition Timing (minutes)
Pyrifluquinazon	465 → 423[a]	135	30	0.050	10	16	2-3
	465 → 92[b]	135	63	0.050		16	
IV-01	423 → 107[a]	145	39	0.050	10	15	2-3
	423 → 93[b]	145	74	0.050		15	
IV-02	421 → 104.8[a]	160	61	0.050	10	5	2-3
	421 → 77.6[b]	160	92	0.050		5	
IV-15	437 → 93[a]	160	60	0.050	10	4	2-3
	437 → 107[b]	160	47	0.050		4	
IV-27	439 → 421[a]	140	26	0.050	10	12	2-3
	439 → 106.9[b]	140	41	0.050		12	
IV-28	437 → 104.8[a]	160	57	0.050	10	15	2-3
	437 → 91.7[b]	160	60	0.050		5	
IV-203	329 → 309[a]	--	-33	0.020	-10	-20	1-2
	329 → 240[b]		-50	0.020		-20	

[a] Transition ion used for quantitation
[b] Transition ion used for confirmation.

Additional detector settings are shown in the table below:

Parameter	Setting	Parameter	Setting
Acquisition Mode:	MRM	Acquisition Mode:	MRM
Ionization Mode:	positive (+)	Ionization Mode:	negative (-)
Source Temp.:	650 °C	Source Temp.:	650 °C
Nebulizer (GS1):	40	Nebulizer (GS1):	40
Auxillary Gas (GS2):	40	Auxillary Gas (GS2):	40
Curtain Gas:	20	Curtain Gas:	20
CAD Gas:	12	CAD Gas:	12
Ion Spray Voltage:	5500	Ion Spray Voltage:	-4500

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis for all analytes except IV-203 which required negative ion mode. Single transition chromatograms for each analyte were integrated and the peak areas used for quantitation. Quantitation was performed using a single transition for each analyte.

For each analytical run, a five-point standard curve was prepared by injecting constant volumes of standard solutions of a mixture of all seven analytes. Constant volume

injections were used for sample extracts as well. A curve check standard was typically injected every 3-4 sample injections.

3.8 Calculations

Calculations were performed using Analyst 1.5.1 software to create a standard curve based on linear regression. Linear regression was monitored to support the response linearity of the mass spectrometer detector. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) to demonstrate that a linear relationship exists between analyte concentration and peak response.

The equation used for the least squares fit is: (1/X weighting used)

$$y = mx + b$$

where:

y	=	peak response
m	=	slope
x	=	ng/mL found for peak of interest
b	=	y-intercept

Equations

The calculations for ppm found and percent recovery (for fortified samples) were:

1. The amount of analyte (in ppm) found in the sample was calculated according to the following equation:

$$\text{ppm Found} = \text{ng/mL found} \times \frac{\text{mL FV} \times \text{Aliquot Factor} \times \text{HPLC dil.factor}}{\text{g samp. wt.} \times 1000}$$

$$\text{Aliquot Factor} = \frac{\text{Extraction Volume (mL)}}{\text{Aliquot Volume (mL)}}$$

where:

ng/mL found	=	ng/mL of analyte found as determined by the analysis
mL FV	=	volume of final extract submitted to instrumentation (2 mL)
Extraction Vol. mL	=	volume of extraction solution (250 mL)
Aliquot Vol. mL	=	volume of extract taken through the procedure (2.0 mL)
HPLC dil. factor	=	dilution of sample extract required to produce an analyte response bracketed by standards. No dilution = HPLC dilution factor of 1

g samp. wt. = amount of sample taken through the extraction process (10 g)

2. Percent recovery of fortified samples (procedural fortifications) was determined using the following equation:

$$\% \text{ Recovery} = \frac{\text{ppm Found in Fortified Sample}}{\text{ppm Added}} \times 100$$