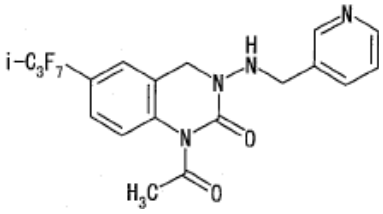
3.0 MATERIALS AND METHODS

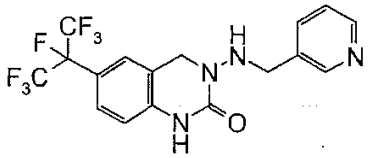
3.1 Test Substances

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

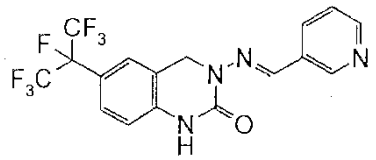
Pyrifluquinazon:

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

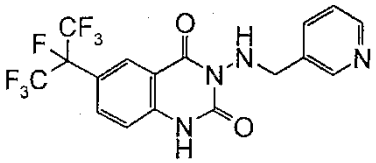
NNI-0101-1H, (IV-01):

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

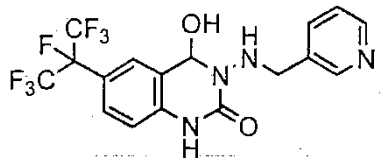
NNI-0101-1H-imino, (IV-02):

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

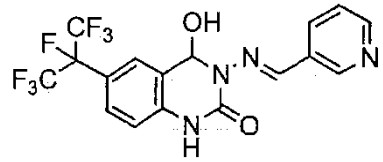
NNI-0101-1H-4-oxo, (IV-15):

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

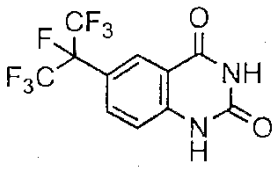
NNI-0101-1H-4-OH, (IV-27):

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 matrices: ground, surface, and drinking water. These matrices were chosen as representative of the matrices for which the method was designed.

Control samples of ground, surface, and drinking water used in the study were provided by the laboratory. The control drinking water sample was obtained locally, and the control ground and surface water samples were obtained from Joann Grant, Truxton, MO for use in this study. The sample, once received, remained in refrigerated storage until removed for subsampling and analysis.

The water specimens were GLP characterized by ABC Laboratories. Details of the characterization results are as follows:

Specimen (Date of Collection/ Characterization)	Conductivity (µS)	Alkalinity (mg/L)[a]	Total Hardness (mg/L)[a]	DO (mg/L)	pH	Dissolved Organic Carbon (ppb)	Total Organic Carbon (ppb)
Ground water (28 Feb 13, 01 & 28 Mar 13)	842	278	432	7.84	7.27	ND	0.13
Drinking water (27 Feb 13, 01 & 28 Mar 13)	492	92	146	9.64	8.81	ND	0.95
Surface water (28 Feb 13, 01 & 28 Mar 13)	98.3	16	32	9.42	7.52	2.52	3.43

[a] Calculated value of endpoint as $CaCO_3$

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 Balance	Mettler XP205 Analytical Balance	Mettler Instrument Corp (Hightstown, NJ)
Labware	OA-SYS N-Evap nitrogen blow down	Organomation Associates, Inc (Berlin, MA)
	Ultrasonicator Branson Model 2210	VWR International (Brisbane, CA)
	Kimble-Chase 50- mL glass conical tubes Graduated cylinders (various sizes) Disposable serological pipets, glass (various sizes) Disposable Pasteur pipets, glass (5¾ inch) 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 Analytical Procedure LMS/0075-01R entitled, "Analytical Method for the Determination of Pyrifluquinazon and Metabolites in Water" 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 methylene chloride. Following partitioning, the mixture is allowed to settle and the methylene chloride (lower layer) was collected in a 50-mL conical glass tube. The aqueous layer remaining in the separatory funnel is extracted again with methylene chloride and the mixture is combined with the original extract in the 50-mL tube. The extraction is performed a third time with methylene chloride and mixture is combined with the original extract in the 50-mL tube. The combined aliquots are evaporated to just dryness and brought to final volume with methanol 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, Critical Steps, and Deviations

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

Section 10.1 Operating Conditions (HPLC). The drinking and surface water injection volume was decreased from 10 µL to 5 µL for IV-203 only. Due to the sensitivity and signal to noise ratio achieved using a Waters Acquity HPLC system, with an Applied Biosystems/Sciex API 5000 LC-MS/MS, it may be necessary to prepare dilutions of the samples and decrease the linearity curve range. As a result of diluting the surface water samples, it was not necessary to quantitate using a matrix matched linearity curve.

It would be helpful to include a more detailed description for the preparation of the IV-02 standard. It was necessary to prepare this standard at a 200 µg/mL concentration in 50:50 acetone:methanol.

One deviation occurred during the course of the analysis where the controls were not determined to be free of interferences prior to the initiation of the ILV. However, the controls were determined to be free of interferences at the retention times of the target analytes during the course of the study.

3.7 Instrumentation

The quantitative analysis of pyrifluquinazon and its metabolites was performed using an Applied BioSystems/MDS Sciex API 5000 LC/MS/MS system. The system parameters are shown in the tables below. Peak area was used for quantitation.

Typical HPLC Conditions (positive mode):

System:	Applied Biosystems/Sciex API 5000 LC-MS/MS, a Waters Acquity Column Manager, Waters Acquity Sample Manager, a Waters Acquity Binary Solvent Manager, and a Waters Acquity Sample Organizer. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software.			
Column:	50 mm × 2 mm, Phenomenex Aqua C18 analytical column with 5 µm particle size			
Column Temperature:	30 °C			
Injection Volume:	10 µL			
Autosampler Temperature:	10 °C			
Flow Rate:	No split			
Mobile Phase:	A: Ammonium formate in water:Methanol:Formic acid (90:10:0.1)			
	B: Methanol:Formic acid (100:0.1)			
Mobile Phase Conditions:	Time	%A	%B	Flow (mL/min)
	0.00	100	0	0.3
	2.00	0	100	0.3
	5.00	0	100	0.3
	5.50	100	0	0.3
	8.00	100	0	0.3

Retention Times:	Pyrifluquinazon	~2.6 minutes
	IV-01	~2.5 minutes
	IV-02	~2.6 minutes
	IV-15	~2.5 minutes
	IV-27	~2.5 minutes
	IV-28	~2.5 minutes
Total Run Time:	~8 minutes	

Typical HPLC Conditions (negative mode):

System:	Applied Biosystems/Sciex API 5000 LC-MS/MS, a Waters Acquity Column Manager, Waters Acquity Sample Manager, a Waters Acquity Binary Solvent Manager, and a Waters Acquity Sample Organizer. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software.
Column:	50 mm × 2.1 mm, Acquity BEH C18 analytical column with 1.7 µm particle size
Column Temperature:	45 °C
Injection Volume:	10 µL
Autosampler Temperature:	10 °C
Flow Rate:	No split
Mobile Phase:	A: Water:Acetonitrile:Acetic acid (90:10:0.1) B: 0.1% Acetic acid in acetonitrile

Mobile Phase Conditions:

Time	%A	%B	Flow (mL/min)
0.00	100	0	0.5
0.20	100	0	0.5
2.00	5	95	0.5
2.50	5	95	0.5
3.00	100	0	0.5
4.00	100	0	0.5

Retention Times:	IV-203	~1.6 minutes
Total Run Time:	~4 minutes	

The detection method utilized was LC-MS/MS employing turbo ion spray (TIS) interface in the positive 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:

MS Conditions:

System: Applied BioSystems/MDS Sciex API 5000 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	465.1 → 107[a]	135	50	0.05	10	18	2-3
	465.1 → 92[b]	135	50	0.05			
IV-01	423 → 107[a]	145	38	0.05	10	18	2-3
	423 → 92[b]	145	45	0.05			
IV-02	421 → 105[a]	160	45	0.05	10	18	2-3
	421 → 107[b]	160	35	0.05			
IV-15	437 → 92[b]	160	40	0.05	10	18	2-3
	437 → 107[a]	160	50	0.05			
IV-27	453 → 107[a]	140	45	0.05	10	18	2-3
	453 → 105[b]	140	55	0.05			
IV-28	437 → 105[a]	160	50	0.05	10	18	2-3
	437 → 148[b]	160	35	0.05			
IV-203	329 → 309[a]	-140	-30	0.2	-10	-20	1-2
	329 → 289[b]		-35	0.2			

[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.:	500 °C	Source Temp.:	500 °C
Nebulizer (GS1):	80	Nebulizer (GS1):	80
Auxiliary Gas (GS2):	40	Auxiliary Gas (GS2):	40
Curtain Gas:	40	Curtain Gas:	40
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 minimum 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:

$$y = mx + b$$

where:

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

Equations

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

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

$$\text{ppb Found} = \text{ng/mL found} \times \frac{\text{mL FV} \times \text{HPLC dil.factor}}{\text{mL samp. vol}}$$

where:

ng/mL found	=	ng/mL of analyte found as determined by the analysis
mL FV	=	volume of final extract submitted to instrumentation (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
mL samp. vol.	=	amount of sample taken through the extraction process (200 mL)

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

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