3 APPARATUS AND EQUIPMENT

Due to the potential for contamination resulting from low detection limits, disposable materials should be used where possible. If glassware is used, care should be taken to minimize the potential for contamination from insufficient cleaning of the glassware.

Analytical Balances (weighing reference standards):	Analytical balance capable of weighing to ±0.1 mg
Top-loading Balance (weighing samples):	Top-loading balance capable of weighing to ±0.01 g
Centrifuge:	IEC HN-SII centrifuge (International Equipment Co., Needham Heights, MA)
HPLC-MS System:	Shimadzu LC-20AD high pressure liquid chromatograph system/DGU-20A5 vacuum solvent degasser equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, Shimadzu SIL-20AC autosampler and CBM-20A communication bus module (system controller) with Applied BioSystems/MDX Sciex Analyst Software for data collection and system control

HPLC Column:	Phenomenex Luna C18(2)-HST, 100 mm × 2.0 mm, 2.5 µ particle size
Pipets (adjustable):	Finnpipette pipettor: 40-200 µL: VWR Scientific Catalog #53515-052 Eppendorf pipettor: 1-10 mL: Fisher Catalog #05-403-121 100-1000 µL: Fisher Catalog #14-386-74 Pipet tips: 1-10 mL Fisher Catalog #05-403-116 1-200 µL: Fisher Catalog #02-681-140 101-1000 µL: Fisher Catalog #02-681-421
Pipets (glass):	Graduated, serological; various sizes Volumetric; various sizes Pasteur, 9-inch and 5½-inch, disposable
Containers:	Polypropylene centrifuge tubes with screw cap closures for retain storage, 15-mL (VWR Scientific, Bridgeport, NJ). HDPE centrifuge bottles for extraction, 250-mL Standard bottles, glass, amber, various sizes Test (culture) tubes, glass, 13 x 100 mm, 16 x 100 mm Volumetric flasks, glass, various sizes
Graduated cylinders	glass, various sizes mixing, 100- and 250-mL
Microliter syringes	various sizes (Hamilton Co., Reno, NV)
Funnels:	Glass, 100 mm i.d.
Shaker:	Wrist-action, Model 75 (Burrell Corp., Pittsburgh, PA)
Solid Phase Extraction Apparatus:	Visiprep 12 or 24-port SPE vacuum manifold with disposable flow control liners (Supelco, Bellefonte, PA)
Ultrasonic Bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)

4 REAGENTS AND MATERIALS

Reagents are HPLC-grade or higher, except where noted. Unless otherwise noted, alternative sources of reagents and materials may be used.

Acetone:	Fisher Optima® grade (Fisher Scientific, Fair Lawn, NJ)
Acetonitrile:	B&J Brand High Purity solvent, HPLC grade (Burdick and Jackson, Muskegon, MI) Fisher (Fisher Scientific, Fair Lawn, NJ)
Ammonium Formate:	100% GR ACD (Fisher Scientific, Fair Lawn, NJ)
Formic Acid:	98% GR ACS (EMD Chemicals, Gibbstown, NJ)
Glass Wool:	Pyrex®, fiber glass (Fisher Scientific, Fair Lawn, NJ)
HPLC Column:	100 mm × 2.0 mm i.d., Phenomenex Luna C18(2)-HST, 2.5 µ particle size
L-ascorbic Acid:	ACROS Organics, 99% (Fisher Scientific, Fair Lawn, NJ, Catalog #AC105021000)
Methanol:	B&J Brand High Purity solvent, HPLC grade (Burdick and Jackson, Muskegon, MI) Fisher Scientific, Fair Lawn, NJ
Sodium Hydroxide:	Pellets, ACS grade (J.T. Baker, Phillipsburg, NJ)
Sodium Phosphate Tribasic Dodecahydrate:	Fisher, ACS grade, certified crystalline (Fisher Scientific, Fair Lawn, NJ, Catalog #S377-500)
Solid Phase Extraction Cartridges:	Oasis® HLB 3 cc (60 mg) extraction cartridges (Waters Corporation, Milford, MA; Catalog #WAT094226)
Water:	Deionized (DI) water (Polymetrics System, Morse Laboratories, LLC HPLC-grade water (Fisher Scientific, Fair Lawn, NJ)
Pyrifluquinazon:	Analytical grade
Pyrifluquinazon NNI-0101-1H (IV-01):	Analytical grade
Pyrifluquinazon NNI-0101-1H-imino (IV-02):	Analytical grade
Pyrifluquinazon NNI-0101-01-4-oxo (IV-15):	Analytical grade
Pyrifluquinazon NNI-0101-1H-4-OH (IV-27):	Analytical grade
Pyrifluquinazon NNI-0101-1H-imino-4-OH (IV-28):	Analytical grade
Pyrifluquinazon NNI-0101-quinazolinedione (IV-203):	Analytical grade

4.1 Reagents and Materials to be Prepared

Volumes may be adjusted accordingly for different quantities. Reagent solution stability was not determined.

Sodium Ascorbate Buffer (50 mM Ascorbic Acid:25 mM Sodium Phosphate Buffer Adjusted to pH 7, w/w)

To a 1000-mL volumetric flask, add 8.7 g ascorbic acid and 9.5 g sodium phosphate tribasic dodecahydrate, then add ~750 mL of deionized water. Carefully swirl flask to mix contents. Adjust the solution to pH 7 with 1 M sodium hydroxide using a pH meter. Bring to a final volume of 1000-mL with deionized water. Transfer to a properly labeled secondary container. Mix well. Prepare as needed. Store in the refrigerator when not in use. Sufficient for approximately 20 samples.

1 M Sodium Hydroxide (w/v)

Weigh 5.15 g sodium hydroxide pellets into a beaker. Transfer pellets to a 100-mL volumetric flask containing approximately 20 mL deionized water. Bring to a final volume of 100-mL with deionized water. Mix thoroughly. Transfer to a properly labeled container. Mix well. Prepare as needed.

Neutral Extraction Solvent [Acetontrile:Sodium Ascorbate Buffer (4:1, v/v)]

To a 4000-mL bottle, add 3200 mL acetonitrile and 800 mL sodium ascorbate buffer. Mix thoroughly. Prepare as needed. Store in the refrigerator when not in use. Sufficient for approximately 16 samples.

Acetonitrile:Water (5:95, v/v)

In a 100-mL mixing cylinder, add 5.0 mL acetonitrile and 95 mL deionized water. Transfer to a properly labeled secondary container. Mix well. Prepare as needed. Sufficient for approximately 100 samples.

Acetonitrile:Methanol (1:1, v/v)

In a 100-mL mixing cylinder, add 50 mL HPLC acetonitrile and 50 mL HPLC methanol. Transfer to a properly labeled secondary container. Mix well. Prepare as needed.

Acetonitrile:Acetone (1:4, v/v)

In a 100-mL mixing cylinder, add 20 mL HPLC acetonitrile and 80 mL HPLC acetone. Transfer to a properly labeled secondary container. Mix well. Prepare as needed.

HPLC Mobile Phase (0.1% Formic Acid in Water)

To a 1 liter graduated cylinder, add HPLC grade water to the 1000 mL mark. Add 1.0 mL of formic acid using a 2.0 mL graduated pipet. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

5 REFERENCE STANDARDS

Pyrifluquinazon:

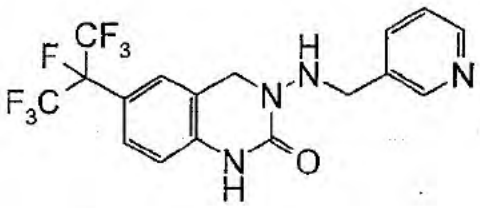
Common Name: Pyrifluquinazon
Experimental Name: NNI-0101
Chemical Names: 1-acetyl-3,4-dihydro-3-[(3-pyridinylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-2(1*H*)-quinazolinone (CAS)

1-acetyl-1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one (IUPAC)
CAS No.: 337458-27-2
Structural Formula:

Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ0017P
Purity: 99.9%
Expiration Date: April 03, 2013

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

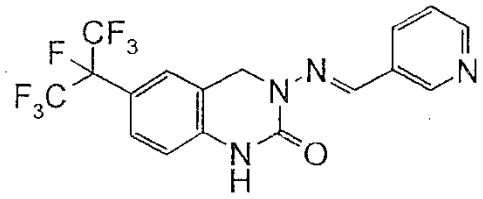
Common Name: NNI-0101-1H, pyrifluquinazon metabolite
Experimental Name: NNI-0101-1H
Chemical Name: 1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one (IUPAC)
CAS No.: Not available
Structural Formula:



Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ6404P
Purity: 98.7%
Expiration Date: August 19, 2017

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

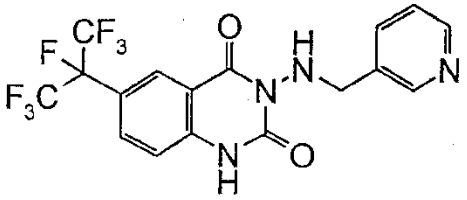
Common Name: NNI-0101-1H-imino, pyrifluquinazon metabolite
Experimental Name: NNI-0101-1H-imino
Chemical Name: 1,2,3,4-tetrahydro-3-[(3-pyridylmethylene)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one (IUPAC)
CAS No.: Not available
Structural Formula:



Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ6304P
Purity: 99.3%
Expiration Date: October 13, 2020

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

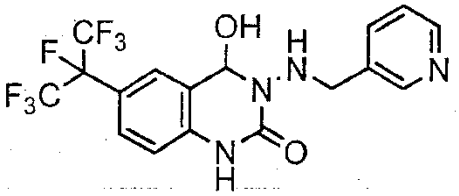
Common Name: NNI-0101-1H-4-oxo, pyrifluquinazon metabolite
Experimental Name: NNI-0101-1H-4-oxo
Chemical Names: 1,2,3,4-tetrahydro-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2,4-dione (IUPAC)
CAS No.: Not available
Structural Formula:



Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ0301S
Purity: 99.5%
Expiration Date: October 09, 2020

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

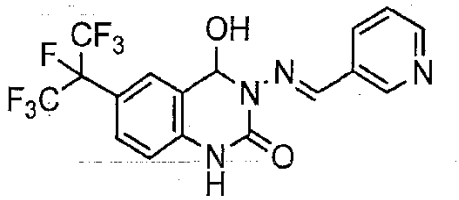
Common Name: NNI-0101-1H-4-OH, pyrifluquinazon metabolite
Experimental Name: NNI-0101-1H-4-OH
Chemical Name: 1,2,3,4-tetrahydro-4-hydroxy-3-[(3-pyridylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2-one (IUPAC)
CAS No.: Not available
Structural Formula:



Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ0901S
Purity: 91.5%
Expiration Date: October 22, 2020

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

Common Name: NNI-0101-1H-imino-4-OH, pyrifluquinazon metabolite
Experimental 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 (IUPAC)
CAS No.: Not available
Structural Formula:

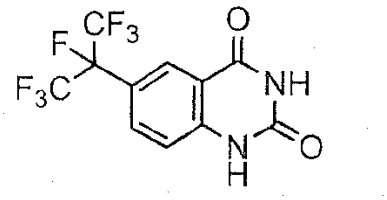


Source: Nihon Nohyaku Co., Ltd.
Lot Number: 5FZ1301S
Purity: 96.9%
Expiration Date: November 03, 2013

NNI-0101-quinazolinedione (IV-203):

Common Name: NNI-0101-quinazolinedione, pyrifluquinazon metabolite
Experimental Name: NNI-0101-quinazolinedione
Chemical Name: 1,2,3,4-tetrahydro-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl]quinazolin-2,4-dione (IUPAC)

CAS No.: Not available
Structural Formula:



Source: Nihon Nohyaku Co., Ltd.
Lot Number: 4FZ0601S
Purity: 97.2%
Expiration Date: September 24, 2012

6 STANDARD PREPARATION

Prepare all standard solutions in HPLC-grade solvents using appropriate analytical techniques. Alternative or additional standard concentrations and volumes may be prepared as needed. All standard solutions prepared in this section are stored in amber glass bottles in the refrigerator when not in use. Typically, the following standard solutions are prepared:

6.1 Stock Standard Solutions

Pyrifluquinazon

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

NNI-0101-1H (IV-01)

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

NNI-0101-imino (IV-02)

Approximately 10.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 50-mL volumetric flask and brought to volume with acetonitrile: acetone (1:4, v/v). The contents were sonicated with gentle heat (~30-35 °C) until completely dissolved. Final concentration of the stock standard solution is 200 µg/mL. This solution is to be stored refrigerated when not in use.

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

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

NNI-01010-1H-4-OH (IV-27)

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

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

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile: methanol (1:1, v/v) to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

NNI-0101-1H-quinazolinedione (IV-203)

Approximately 25.0 mg (corrected for purity) of the analytical standard is accurately weighed, quantitatively transferred to a 25-mL volumetric flask and brought to volume with acetonitrile to make a stock standard solution having a concentration of 1000 µg/mL. This solution is to be stored refrigerated when not in use.

6.2 Fortification Standard Solutions

Typically, the following concentrations of fortification standard solutions are prepared from the stock standard solutions. These solutions are prepared as a mixture containing all targeted analytes. The concentration listed represents the concentration of each analyte.

Sonicate the IV-203 stock standard solution for 10-minutes prior to preparing any dilutions.

5.0 µg/mL: Transfer 250 µL of each 1000-µg/mL stock standard solutions (pyrifluquinazon, IV-01, IV-02, IV-15, IV-27, IV-28, IV-203) and 1.25 mL of the 200-µg/mL stock standard solution (IV-02) to a 50-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

1.0 µg/mL: Transfer 5.0 mL of the 5.0-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

6.3 HPLC Calibration Standard Solutions

Typically, the calibration standard solutions are prepared from the fortification standard solutions. These solutions are prepared as mixtures containing all targeted analytes. The concentration listed represents the concentration of each analyte. Prepare these standards every two weeks.

Calibration Standard Solutions:

10.0 ng/mL: Transfer 500 µL of 1.0-µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile: methanol (1:1, v/v). Mix well.

5.0 ng/mL: Transfer 25 mL of 10.0-ng/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile: methanol (1:1, v/v). Mix well.

2.0 ng/mL: Transfer 20 mL of 5.0-ng/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile: methanol (1:1, v/v). Mix well.

0.4 ng/mL: Transfer 10 mL of 2.0-ng/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile: methanol (1:1, v/v). Mix well.

0.2 ng/mL: Transfer 25 mL of 0.4-ng/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile: methanol (1:1, v/v). Mix well.

7 SAMPLE FORTIFICATION

All samples must be kept in the frozen state until the addition of the extraction solvent. Upon removal of samples from the freezer, immediately place them into a suitable storage container containing dry-ice. At time of weigh-out, use a rubber mallet to break apart the frozen sample into small pieces and thoroughly mix the sample. If at any time there is a delay in weighing, return sample to the container containing dry-ice.

1. Weigh 10.0 g of frozen homogenized sample into a 250-mL HDPE centrifuge bottle. Cap and keep the sample frozen in a suitable storage container containing dry-ice until fortified.

2. In the frozen state, fortify the sample with the appropriate amount of standard solution. Disperse solution over as much of the sample as possible. Use a volume ≤1.0 mL.

3. Allow standard solution to soak into the sample for a moment before proceeding with Step 8.2.

8 SAMPLE EXTRACTION

All samples must be kept in frozen state until the addition of the extraction solvent. See Section 7 introductory paragraph.

1. Weigh 10.0 g of frozen homogenized sample into a 250-mL HDPE centrifuge bottle. Cap and keep the sample frozen in a suitable storage container containing dry-ice, fortify appropriate samples at this time.

2. To the frozen sample, add 120 mL of neutral extraction solvent and cap the bottle. Shake on a Wrist-Action shaker (at full speed) for 15 minutes.

3. Centrifuge mixture for ~30 minutes at ~2500 rpm. Decant supernatant through a glass funnel containing a loosely-packed plug of glass wool into a 250-mL mixing cylinder.

4. Add an additional 120 mL of neutral extraction solvent. Shake on a Wrist-Action shaker (at full speed) for 15 minutes. Centrifuge for ~30 minutes at ~2500 rpm. Decant supernatant through the same glass wool into the mixing cylinder, combining the extracts. Bring to a final volume of 250 mL with neutral extraction solvent.

5. Transfer 2.0 mL of the extract to a 15-mL polypropylene centrifuge tube. Add 8.0 mL deionized water. Mix well.

6. Proceed to Section 9.

9 OASIS® HLB SPE CARTRIDGE CLEANUP

NOTE: Due to the potential for lot to lot variation for SPE columns it is recommend that the elution profile for each lot be confirmed prior to use. See Appendix II for quality control procedure for the SPE Cartridges.

1. Set up Visiprep system and support apparatus and proceed with Oasis® HLB SPE cleanup. In general, set vacuum to produce a flow rate of approximately 2 mL/minute (not continuous flow).

2. Take one Oasis® HLB cartridge (size 60 mg, 3 mL) for each sample to be analyzed and place on the vacuum manifold. Condition the cartridge with 2 mL acetonitrile followed by 3 × 2 mL deionized water, drawing each addition under vacuum to the level of the top of the frit at a rate of ~2 mL/minute. Do not allow the cartridge to become dry in between any of the conditioning steps or between conditioning and sample introduction. Discard all eluates.

3. Transfer the extracts from Step 8.6 onto the cartridges and draw through under vacuum at a rate of approximately 2 mL/minute, discarding the column eluates. Residues of pyrifluquinazon and its metabolites are retained on the cartridge.

4. Add deionized water (2 mL) to the tubes that contained the extracts. Rinse the tubes and transfer to the SPE cartridges. Draw through under vacuum to the level of the top frit, discarding the column eluate.

5. Further wash the sample laden cartridge with 1 mL of acetonitrile:water (5:95, v/v) and dry the column for one minute using vacuum.

6. Place suitable collection tubes (e.g. 13 × 100 mm test tubes calibrated at 1.0 and 2.0 mL) under each port, as required, in the manifold rack.

7. Elute the analytes with 1.0 mL HPLC-grade acetonitrile and allow column to go dry using vacuum.

8. Bring to final volume of 2.0 mL with HPLC-grade methanol. Mix well. Submit to LC-MS/MS analysis. Final sample concentration: 1 mL = 0.04 g sample.

10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

The column and conditions stated below have been satisfactory for the matrix being analyzed. The specific column packing, mobile phase, column temperature, and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted with each chromatographic run and will not otherwise be documented.

10.1 Operating Conditions

Typical HPLC conditions used for this analysis were as follows:

Instrument:	Shimadzu LC-20AD high pressure liquid chromatograph system/DGU-20A5 vacuum solvent degasser equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, Shimadzu SIL-20AC autosampler and CBM-20A communication bus module (system controller) with Applied BioSystems/MDS Sciex Analyst Software for data collection and system control.		
HPLC Column:	Phenomenex Luna C18(2)-HST, 100 mm × 2.0 mm, 2.5 µ particle size		
Mobile Phase:	Component A: 0.1% formic acid in HPLC grade water		
	Component B: 100% Acetonitrile		
Injection Volume:	10 µL		
Column Temperature:	40 °C		
Gradient:	Time (min.)	% A	% B
	0.00-0.50	90	10
	1.00	75	25
	3.50	55	45
	9.00	40	60
	9.51-11.50	0	100
	11.51-14.51	90	10

Divert Valve:	Programmed to divert LC flow from column to waste (bypassing detector) from 0.00 to 4.50 minutes and again from 8.75 to 14.51 minutes. LC flow is directed to detector during the 4.50 to 8.75 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.
Flow Rate:	250 µL/min. (or 0.250 mL/min.)
Interface:	TIS (turbo ion spray)

10.1.1 Pyrifluquinazon, IV-01, IV-02, IV-15, IV-27, and IV-28

Ionization Mode:	Positive (+); duration 0 to ~7.5 minutes				
Acquisition Mode:	MRM				
Resolution:	Q1 – unit, Q3 – unit (Note: Unit equivalent to medium)				
Source Temperature:	650 °C				
Curtain Gas:	Nitrogen @ setting of "20"				
Collision Gas:	Nitrogen @ setting of "7"				
Transitions Monitored:		Ion, *m/z*			
	Analyte	Q1	Q3	Time, *ms*	CE,*v*
	Pyrifluquinazon:	465.2	423.2	50	31 (quantitation)
		465.2	92.3	50	60 (confirmation)
	IV-01:	423.2	106.9	50	39 (quantitation)
		423.2	92.3	50	57 (confirmation)
	IV-02:	421.0	104.9	50	59 (quantitation)
		421.0	107.2	50	35 (confirmation)
	IV-15:	437.0	93.1	50	77 (quantitation)
		437.0	107.1	50	63 (confirmation)
	IV-27:	438.6	421.0	50	29 (quantitation)
		438.6	107.0	50	49 (confirmation)
	IV-28:	437.0	104.5	50	57 (quantitation)
		437.0	92.2	50	59 (confirmation)
Injection Volume:	10 µL				
Column Temperature:	40 °C				
Retention Times:	Pyrifluquinazon:	~7.0 minutes			
	IV-01:	~6.0 minutes			
	IV-02:	~7.2 minutes			
	IV-15:	~5.7 minutes			
	IV-27:	~5.3 minutes			
	IV-28:	~6.8 minutes			

10.1.2 IV-203

Ionization Mode:	Negative (-); duration 7.5 to ~12.5 minutes
Acquisition Mode:	MRM
Resolution:	Q1 – unit, Q3 – unit (Note: Unit equivalent to medium)
Source Temperature:	650 °C

Curtain Gas:	Nitrogen @ setting of "20"				
Collision Gas:	Nitrogen @ setting of "10"				
Transitions Monitored:	Analyte	Ion, *m/z* Q1	Q3	Time, *ms*	CE,*v*
	IV-203:	329.0	309.0	150	-32 (quantitation)
		329.0	240.0	150	-44 (confirmation)
		329.0	289.0	150	-34 (confirmation)
		329.0	268.9	150	-40 (confirmation)
Injection Volume:	10 µL				
Column Temperature:	40 °C				
Retention Time:	IV-203:	~8.0 minutes			

10.2 Sample Analysis

Prepare a standard curve by injecting constant volumes of standard solutions (at least 5 concentrations). Use constant volume injections for sample extracts as well. Sample responses found greater than those produced by the highest concentration of standard in the standard curve require dilution and reinjection. Calibration standards should be injected intermixed with test samples before and after every 1-4 samples in each analytical set.

A set of 12 samples, including QC, requires approximately 8 hours to extract/purify and prepare for LC-MS/MS analysis, followed by approximately 5 hours of instrumental analysis time.

11 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application (e.g., Applied BioSystems/MDS Sciex Analyst, version 1.6.2) to create a standard curve based on linear regression. The regression functions are used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak area response) and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. For each analytical set, calibration standards are injected over the linear range of the instrument (typically 0.20 to 10.0 ng/mL). All standards injected and their corresponding peak responses are entered into the program to create the standard curve. Weighting (1/x) is used. With no weighting, the slope of the line (curve) tends to be dominated by the highest point. When weighting of 1/concentration (1/x) is used, the slope more closely approximates the majority of the points used to construct it.

The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = detector response (peak area) for each analyte

X = analyte concentration in the sample in ng/mL

$$X = \frac{Y - \text{intercept}}{\text{Slope}} = \text{ng/mL}$$

The standard (calibration) curve generated for each analytical set is used for the quantitation of Pyrifluquinazon or metabolites (IV-01, IV-02, IV-15, IV-27, IV-28, and IV-203) in the samples for a given set. Correlation coefficient (r) for each calibration curve was greater than 0.995 (r^2 equal to or greater than 0.99).

For the determination of Pyrifluquinazon or metabolites (IV-01, IV-02, IV-15, IV-27, IV-28, and IV-203) in soil (in terms of ppm), the following equation is used:

$$\text{ppm} = \text{ng/mL} \times \frac{\text{HPLC FV (mL)}}{\text{sample wt (g)}} \times \frac{\text{ext. solv. (mL)}}{\text{aliq. (mL)}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} \times \text{HPLC dil. fact.}$$

where:

ng/mL found	=	ng/mL of analyte found as determined by the analysis
sample wt (g)	=	amount of sample taken through the extraction process (typically 10.0 g)
HPLC FV (mL)	=	volume of final extract submitted to instrumentation (typically 2.0 mL)
ext. solv. (mL)	=	extraction solvent added (typically 250 mL)
aliq. (mL)	=	volume of extract taken through the procedure (typically 2.0 mL)
1/1000	=	conversion factor from ng to µg
HPLC dil. fact.	=	dilution of sample extract required to produce an analyte response bracketed by standards

The percent recovery for fortified control samples is calculated as follows:

$$\%\ Recovery = \frac{ppm\ found\ in\ fortified\ control - ppm\ found\ in\ control}{ppm\ added} \times 100$$

APPENDIX I Analysis Flowchart