4. DESCRIPTION OF THE ANALYTICAL METHOD

4.1. Reference Substances, Standard Solutions, and Fortification Solutions

The reference substances (analytical reference standards) used in this validation were IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 (mixture of diastereomers), and pyrazole carboxamide. The details are described below:

Compound Name:	**IR9792/F9990**
Reference Number (Lot #):	PL13-0195
Percent purity:	98.6%
CAS Number:	Not registered
FMC ID:	510142
Storage Conditions:	Ambient
Expiration date:	July 01, 2018
Received date:	August 20, 2014

Compound Name:	**3-hydroxy-IR9792/F9990**
Reference Number (Lot #):	30440/43
Percent purity:	97.2%
CAS Number:	Not registered
FMC ID:	510152
Storage Conditions:	Freezer
Expiration date:	April 30, 2017
Received date:	April 27, 2015

Compound Name:	**1-carboxy-IR9792/F9990 (mixture of diastereomers)**
Reference Number (Lot #):	55261-4-8
Percent purity:	97.8%
Diastereomeric ratio	1.72:1
CAS Number:	Not registered
FMC ID:	510169
Storage Conditions:	Freezer
Expiration date:	March 31, 2017
Received date:	April 27, 2015

Compound Name:	**Pyrazole carboxamide**
Reference Number (Lot #):	R14151
Percent purity:	99.5%
CAS Number:	925689-10-7
FMC ID:	510151
Storage Conditions:	Freezer
Expiration date:	June 30, 2017
Received date:	June 24, 2015

4.1.1. IR9792/F9990 Stock Standard Solution

A stock solution containing IR9792/F9990 was prepared by weighing approximately 0.010 g of IR9792/F9990 reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of IR9792/F9990.

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.1. 3-hydroxy-IR9792/F9990 Stock Standard Solution

A stock solution containing 3-hydroxy-IR9792/F9990 was prepared by weighing approximately 0.010 g of 3-hydroxy-IR9792/F9990 reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of 3-hydroxy-IR9792/F9990.

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.2. 1-carboxy-IR9792/F9990 Stock Standard Solution

A stock solution containing the 1-carboxy-IR9792/F9990 diastereomers was prepared by weighing approximately 0.010 g of 1-carboxy-IR9792/F9990 reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of total 1-carboxy-IR9792/F9990. Based on the diasteremeric ratio, the stock solution contained approximately 632 µg/mL and 368 µg/mL for diastereomers 1 and 2, respectively.

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.3. Pyrazole carboxamide Stock Standard Solution

A stock solution containing pyrazole carboxamide was prepared by weighing approximately 0.010 g of pyrazole carboxamide reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of pyrazole carboxamide.

For each compound, the following intermediate standards and fortification solutions were made individually. This was done so all four compounds could be used to spike the soil samples individually for a storage stability study, once the method was validated.

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.4. 10 µg/mL Intermediate Standard

An intermediate standard containing 10 µg/mL of compound was prepared by pipetting approximately 100 µL of the compound stock standard solution into a 10 mL class A volumetric flask and bringing it to volume with methanol.

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.5. 1 µg/mL Intermediate Standard / 10X LOQ Fortification Solution

An intermediate standard containing 1 µg/mL of compound was prepared by pipetting approximately 5 mL of the compound 10 µg/mL intermediate standard solution into a 50 mL class A volumetric flask and bringing it to volume with methanol.

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.6. 100 ng/mL Intermediate Standard / LOQ Fortification Solution

An intermediate standard containing 100 ng/mL of compound was prepared by pipetting approximately 1 mL of the 1 µg/mL intermediate standard solution into a 10 mL class A volumetric flask and bringing it to volume with methanol.

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

4.1.7. Working Standards (Calibration Standards)

Sets of working standards were prepared in volumetric flasks, and brought up to volume using methanol:water (8:2) (v:v).

The working standards were stored chilled at approximately 1-6 °C when not being used in the laboratory.

Example Preparation:

Target Concentration (ng/mL)	Volume of Intermediate Standard (mL)	Concentration of Intermediate Standard	Final Volume (mL)
0.1	1.0	1 ng/mL Working Std	10.0
1.0	1.0	10 ng/mL Working Std	10.0
2.5	0.25	100 ng/mL Int Std	10.0
5.0	0.5	100 ng/mL Int Std	10.0
10.0	1.0	100 ng/mL Int Std	10.0

4.2. Determination of IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 (diastereomer 1), 1-carboxy-IR9792/F9990 (diastereomer 2), and pyrazole carboxamide in Soil by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

4.2.1. Reagents and Solutions

Formic acid
Ammonium Acetate
Methanol, Pesticide Grade
Methanol, HPLC Grade
Acetone, pesticide grade

Hydrochloric acid
MilliQ Deionized (DI) Water, 18.2 Ω

Extraction solution 1: Acetone:water (9:1) (v:v). Using a graduated cylinder, add 900 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 100 mL of deionized water to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation.

Extraction solution 2: Acetone:water (1:1) (v:v). Using a graduated cylinder, add 500 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 500 mL of deionized water to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation.

Extraction solution 3: Acetone:0.5N HCl (1:1) (v:v). Using a graduated cylinder, add 500 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 500 mL of 0.5 N HCl to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation. Note: 0.5N HCl was prepared by adding approximately 20.83 mL of concentrated HCl to 500 mL of deionized water.

Mobile Phase A: 10mM ammonium acetate and 0.2% formic acid in water. Using a graduated cylinder, 1000 mL of DI water was measured and transferred to a mobile phase bottle. 2.0 mL of formic acid and 0.7708g of ammonium acetate was added to the bottle, and mixed well. The solution was stored at room temperature for up to 30 days after preparation.

Mobile phase B: 0.2% formic acid in methanol. Using a graduated cylinder, 1000 mL of methanol was measured and transferred to a mobile phase bottle. 2.0 mL formic acid was added to the bottle and mixed well. The solution was stored at room temperature for up to 30 days after preparation.

4.2.2. Equipment and Instruments

Freezer(s), capable of maintaining -20 °C ± 10 °C
Refrigerator(s), capable of maintaining 1-6 °C
Balance, Analytical, capable of weighing to the nearest 0.01 mg
Balance, Analytical, capable of weighing to the nearest 0.1 mg
Balance, Top Loading, capable of weighing to the nearest 0.1g
Sonicator
Vortex
Adjustable pipettes, 200 µL, 1000 µL, 5000 µL
Graduated cylinders
Wrist action shaker
Centrifuge capable of 4000 rpm
Autosampler vials and caps, 2-mL, screw cap
UHPLC column,
UHPLC system, Dionex Ultimate 3000
Mass Spectrometer, ABSciex Qtrap 4000
UHPLC system, Shimadzu Nexera XR
Mass Spectrometer, Sciex 6500+ QQQ
UPLC software, Dionex Chromeleon, bundled with Analyst version 1.6.2

MS/MS software, ABSciex Analyst, version 1.6.2
Data analysis software, ABSciex MultiQuant, version 2.0.2
Microsoft Office Excel 2007

4.2.3. Sample Extraction

- Weigh 5.0 ± 0.1 g of each sample into a 50 mL centrifuge tube.
- Using untreated sample, make fortifications at the LOQ and 10X LOQ.
- Add 250 µL of the LOQ fortification and 250 µL of the 10 X LOQ fortification solutions to their respective samples.
- Add 25 mL of acetone:water (9:1) to each centrifuge tube and place on a wrist action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant into a 100 mL volumetric flask. (A funnel with filter paper may be used).
- Repeat extraction by adding 25 mL of acetone:water (1:1) to the sample.
- Sonicate the sample at approximately 40 °C for 60 minutes.
- Shake on wrist-action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant and combine with first supernatant in the 100 mL volumetric flask.
- Repeat extraction by adding 25 mL of acetone:0.5N HCl (1:1) to the sample.
- Sonicate the sample at approximately 40 °C for 60 minutes.
- Shake on wrist-action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant and combine with the other two supernatants in the 100 mL volumetric flask.
- Bring up to the 100 mL volume mark with acetone. Shake well to mix.
- Pour the mixed extract into a 50 mL centrifuge tube and fill to the top. The rest of the sample can be discarded.
- Centrifuge the sample at 4000 rpm for approximately 10 minutes.
- Using a transfer pipette, transfer a portion of the supernatant to a 15 mL centrifuge tube, up to the 5 mL graduation mark.
- Place the sample under nitrogen evaporation in order to evaporate off the acetone. Once this is done, approximately 1 to 2 mL of water should be left.
- Remove from nitrogen evaporation, and bring back up to the 5 mL graduation mark with methanol.
- Shake well to mix.
- Transfer a portion to an HPLC vial for LC-MS/MS analysis.

4.2.4. Instrument Analysis

UPLC-MS/MS was used to quantify residues of IR9792/F9990 its metabolites, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 diastereomers, and pyrazole carboxamide in soil. Two different sets of instruments were utilized over the course of the study. At first, a Dionex HPLC with a Sciex QTrap 4000 mass spectrometer, and later a Shimadzu HPLC with a Sciex 6500+ mass spectrometer. Both sets of parameters are below:

UHPLC system:	Dionex Ultimate 3000
MS Detector:	Sciex Triple Quadrapole API 4000

UHPLC column:	Phenomenex Kinetex 2.6µ C18 100A, 50 x 4.6 mm
Guard column:	SecurityGuard Ultra Cartridge UHPLC C18 for 4.6 mm
Column temp:	20°C
Injection volume:	10 µL
Run Time:	24.0 minutes
Mobile phase A:	10mM ammonium acetate and 0.2% formic acid in water
Mobile phase B:	0.2% formic acid in methanol
Flow rate:	0.600 mL/minute
Approx. Retention Times:	pyrazole carboxamide: 2.7 min (positive polarity) 1-carboxy-IR9792/F9990 Diastereomer 1: 11.80 min (positive polarity) 1-carboxy-IR9792/F9990 Diastereomer 2: 12.63 min (positive polarity) 3-hydroxy-IR9792/F9990: 17.63 min (negative polarity) IR9792/F9990: 18.78 min (positive polarity)

Gradient program:

Time (min)	%A	% B
0.0	90	10
3.5	90	10
6.0	60	40
22.0	20	80
23.0	20	80
23.1	90	10
24.0	90	10

Curtain Gas:	10.00
Collision Gas:	High
IonSpray Voltage:	5500, -4500
Temperature:	450 °C
Ion Source Gas 1:	60
Ion Source Gas 2:	60

Compound	Parent Ion (da)	Daughter Ion (da)	Dwell time (msec)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Polarity
Pyrazole carboxamide	176.059	136	500	41	10	23	8	Positive
Pyrazole carboxamide*	176.059	156	500	41	10	13	10	Positive
1-carboxy-IR9792/F9990 (diastereomer 1)	382.1	336.200	500	76	10	21	10	Positive
1-carboxy-IR9792/F9990 (diastereomer 1)*	382.1	296.100	500	76	10	35	20	Positive
1-carboxy-IR9792/F9990 (diastereomer 2)	382.1	336.201**	500	76	10	21	10	Positive
1-carboxy-IR9792/F9990 (diastereomer 2)*	382.1	296.101**	500	76	10	35	20	Positive
3-hydroxy-IR9792/F9990	366.235	174.8	1000	-110	-10	-30	-11	Negative
3-hydroxy-IR9792/F9990*	366.235	130.7	1000	-110	-10	-40	-7	Negative
IR9792/F9990	352.185	332.1	1000	106	10	23	8	Positive
IR9792/F9990*	352.185	256.1	1000	106	10	41	16	Positive

*Confirmatory ion transition.
**For the diastereomers, AB Sciex Analyst program required that the daughter ions not have the exact same molecular weight.

UHPLC system:	Shimadzu Nexera XR HPLC
MS Detector:	Sciex Triple Quadrapole 6500+
UHPLC column:	Phenomenex Kinetex 2.6µ C18 100A, 50 x 4.6 mm
Guard column:	SecurityGuard Ultra Cartridge UHPLC C18 for 4.6 mm
Column temp:	20°C
Injection volume:	10 µL
Run Time:	24.0 minutes

Mobile phase A:	10mM ammonium acetate and 0.2% formic acid in water
Mobile phase B:	0.2% formic acid in methanol
Flow rate:	0.600 mL/minute
Approx. Retention Times:	pyrazole carboxamide: 2.8 min (positive polarity) 1-carboxy-IR9792/F9990 Diastereomer 1: 11.8 min (positive polarity) 1-carboxy-IR9792/F9990 Diastereomer 2: 12.6 min (positive polarity) 3-hydroxy-IR9792/F9990: 17.8 min (negative polarity) IR9792/F9990: 18.9 min (positive polarity)

Gradient program:

Time (min)	%A	% B
0.0	90	10
3.5	90	10
6.0	60	40
22.0	20	80
23.0	20	80
23.1	90	10
24.0	90	10

Curtain Gas:	20.00
Collision Gas:	7.0
IonSpray Voltage:	5500, -4500
Temperature:	450 °C
Ion Source Gas 1:	75.0
Ion Source Gas 2:	75.0

Compound	Parent Ion (da)	Daughter Ion (da)	Dwell time (msec)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Polarity
Pyrazole carboxamide	176.0	136.0	350	26	10	21	16	Positive
Pyrazole carboxamide*	176.0	156.0	350	26	10	13	18	Positive
1-carboxy-IR9792/F9990 (diastereomer 1)	382.1	336.1	350	101	10	19	20	Positive
1-carboxy-IR9792/F9990 (diastereomer 1)*	382.1	296.1	350	101	10	33	20	Positive
1-carboxy-IR9792/F9990 (diastereomer 2)	382.1	336.1	350	101	10	19	20	Positive
1-carboxy-IR9792/F9990 (diastereomer 2)*	382.1	296.1	350	101	10	33	20	Positive
3-hydroxy-IR9792/F9990	366.1	175.0	1000	-130	-10	-28	-11	Negative
3-hydroxy-IR9792/F9990 *	366.1	131.0	1000	-130	-10	-36	-13	Negative
IR9792/F9990	352.1	256.1	1000	86	10	39	16	Positive
IR9792/F9990 *	352.1	312.1	1000	86	10	25	22	Positive

*Confirmatory ion transition.

4.5. Calculations

4.5.1. Linear Regression (IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 diastereomers, and pyrazole carboxamide).

The following formulas were used to calculate IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 diastereomers, and pyrazole carboxamide fortification recoveries. The amount found (ppb) of IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 diastereomers, and pyrazole carboxamide residue was calculated from the linear regression equation with 1/x weighting.

The linear calibration equation is:

$$y = mx + b$$

Where:

y: area count from LC-MS/MS chromatogram
m: slope of the linear regression line
b: y-intercept of the linear regression line
x: sample concentration in ng/mL

4.5.2. Amount Found (ppb)

Below is an example of how the amount of residue found is determined for IR9792/F9990. This same equation is used for all compounds in this validation. The amount found (ppb) IR9792/F9990 was calculated by solving the linear equation for x, concentration, using the detector response (area) substituted in for y, and multiplying the result by the dilution factor.

$$x = \frac{y\text{-}b}{m} * \text{dilution factor}$$

Regression line: y = 2553330 (x) + 18546.41437

Example:	IR9792/F9990
Lab Sample ID:	T1-SC-4-SPA-6-R1
Sample Peak Area:	27940977
Dilution factor:	20

$$x = \frac{27940977 - (-18546.41437)}{2553330} * 20 = 219.004\, ppb$$

Analyst MultiQuant software (ABSciex, Version 2.0.2) was used to calculate linear, 1/x weighted regression calibration curves and amount found (ppb).

4.5.3. Dilution Factor

The dilution factor for IR9792/F9990, 3-hydroxy-IR9792/F9990, 1-carboxy-IR9792/F9990 (diastereomer 1), 1-carboxy-IR9792/F9990 (diastereomer 2), and pyrazole carboxamide samples are calculated with the equation below:

$$dilution\ factor = \frac{total\ dilution\ volume\ (mL)}{sample\ weight\ (g)}$$

Example:	IR9792/F9990
Lab Sample ID:	LOQ
Sample Weight:	5.0 g
Total Dilution Vol:	100 mL

$$dilution\ factor = \frac{100\ mL}{5.0\ g} = 20$$

4.5.4. Fortification Level IR9792/F9990 (ppb added)

Below are examples of the fortifications used for the validation.

$$Fortification\ level\ (ppm) = \frac{Vol.Spiking\ Soln.\ (mL)X\ Spiking\ Soln.\ Conc.\left(\frac{\mu g}{mL}\right)}{Sample\ weight\ (g)}$$

Example:	IR9792/F9990
Lab Sample ID:	Interval 4 LOQ 1
Volume Spiking Soln:	0.250 mL
Identity of Spiking Soln:	LOQ Fortification Solution
Spiking Soln. Conc.:	0.107474 µg/mL
Sample Weight:	5.0 g

Fortification level = 0.250 mL * 0.107474µg/mL / 5.0 g = 0.0053737 ppm (5.3737 ppb)

Example:	IR9792/F9990
Lab Sample ID:	Interval 4 10X LOQ 1
Volume Spiking Soln:	0.250 mL
Identity of Spiking Soln:	10X LOQ Fortification Solution
Spiking Soln. Conc.:	1.07474 µg/mL
Sample Weight:	5.0 g

Fortification level = 0.250 mL * 1.07474 µg/mL / 5.0 g = 0.053737 ppm (53.737ppb)

4.5.5. % Method Recovery

For all samples, the % recovered in fortification samples was corrected for the amount of residue found in the control sample analyzed in the same run, if residue was found in the control sample above the LOD.

$$\%\ Method\ Recovery = \frac{ppm\ found\ in\ fortification\ sample - ppm\ found\ in\ untreated}{Fortification\ level} * 100\%$$

Example:	IR9792/F9990
Lab Sample ID:	Interval 4 10X LOQ Spike 1
ppm Found in spike:	0.05040587 ppm
ppm Found in untreated:	0.00000000 ppm
Fortification level:	0.05373700 ppm

% Method Recovery = (0.05040587 – 0.00000000) ppm / 0.05373700 ppm * 100% = 93.80%

Summary Title

Method Validation of F9990, 3-hydroxy-F9990, F9990-1-carboxylate and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide in soil

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INTRODUCTION

The following is a summary of the steps taken to validate an analytical method for F9990 and its soil degradates, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide in soil. Two types of soil were used, a sandy loam from Nebraska, USA, and a silt loam from New York, USA. This validation is to be used as part of the larger ongoing studies being performed by SGS for FMC. A description of the analytical methods, along with instrumental parameters and raw data from the datasets are found below.

DESCRIPTION OF THE ANALYTICAL METHOD

Reference Substances, Standard Solutions, and Fortification Solutions

The reference substances (analytical reference standards) used in this validation were F9990, 3-hydroxy-F9990, F9990-1-carboxylate, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide. The details are described below:

Compound Name:	**F9990**
Reference Number (Lot #):	PL13-0195
Percent purity:	98.6%
CAS Number:	Not registered
Storage Conditions:	Ambient
Expiration date:	July 01, 2018
Received date:	August 20, 2014

Compound Name:	**3-hydroxy-F9990**
Reference Number (Lot #):	30440/43
Percent purity:	97.2%
CAS Number:	Not registered
Storage Conditions:	Freezer
Expiration date:	April 30, 2017
Received date:	April 27, 2015

Compound Name:	**F9990-1-carboxylate (mixture of diastereomers)**
Reference Number (Lot #):	55261-4-8
Percent purity:	97.8%
Diastereomeric ratio	1.72:1
CAS Number:	Not registered
Storage Conditions:	Freezer
Expiration date:	March 31, 2017
Received date:	April 27, 2015

Compound Name:	3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide
Reference Number (Lot #):	R14151
Percent purity:	99.5%
CAS Number:	925689-10-7
Storage Conditions:	Freezer
Expiration date:	June 30, 2017
Received date:	June 24, 2015

F9990 Stock Standard Solution

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

A stock solution containing F9990 was prepared by weighing approximately 0.010 g of F9990 reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of F9990.

3-hydroxy-F9990 Stock Standard Solution

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

A stock solution containing 3-hydroxy-F9990 was prepared by weighing approximately 0.010 g of 3-hydroxy-F9990 reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of 3-hydroxy-F9990.

F9990-1-carboxylate Stock Standard Solution

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

A stock solution containing the F9990-1-carboxylate diastereomers was prepared by weighing approximately 0.010 g of F9990-1-carboxylate reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of F9990-1-carboxylate.

3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide Stock Standard Solution

The stock standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

A stock solution containing 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide was prepared by weighing approximately 0.010 g of 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide reference substance into a 10 mL volumetric flask. The flask was diluted to volume with methanol, capped, and mixed thoroughly. The stock solution contained approximately 1000 µg/mL of 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide.

For each compound, the following intermediate standards and fortification solutions were made individually. This was done so all four compounds could be used to spike the soil samples individually for a storage stability study, once the method was validated.

10 µg/mL Intermediate Standard

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

An intermediate standard containing 10 µg/mL of compound was prepared by pipetting approximately 100 µL of the compound stock standard solution into a 10 mL class A volumetric flask and bringing it to volume with methanol.

1 µg/mL Intermediate Standard / 10X LOQ Fortification Solution

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

An intermediate standard containing 1 µg/mL of compound was prepared by pipetting approximately 5 mL of the compound 10 µg/mL intermediate standard solution into a 50 mL class A volumetric flask and bringing it to volume with methanol.

100 ng/mL Intermediate Standard / LOQ Fortification Solution

The intermediate standard was stored chilled at approximately 1-6 °C when not being used in the laboratory.

An intermediate standard containing 100 ng/mL of compound was prepared by pipetting approximately 1 mL of the 1 µg/mL intermediate standard solution into a 10 mL class A volumetric flask and bringing it to volume with methanol.

Working Standards (Calibration Standards)

The working standards were stored chilled at approximately 1-6 °C when not being used in the laboratory.

Sets of working standards were prepared in volumetric flasks, and brought up to volume using methanol:water (8:2) (v:v).

Example Preparation:

Target Concentration (ng/mL)	Volume of Intermediate Standard (mL)	Concentration of Intermediate Standard	Final Volume (mL)
0.1	1.0	1 ng/mL Working Std	10.0
1.0	1.0	10 ng/mL Working Std	10.0
2.5	0.25	100 ng/mL Int Std	10.0
5.0	0.5	100 ng/mL Int Std	10.0
10.0	1.0	100 ng/mL Int Std	10.0

Determination of F9990, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide in Soil by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

Reagents and Solutions

Formic acid
Ammonium Acetate
Methanol, Pesticide Grade
Methanol, HPLC Grade
Acetone, pesticide grade
Hydrochloric acid
MilliQ Deionized (DI) Water, 18.2 Ω

Extraction solution 1: Acetone:water (9:1) (v:v). Using a graduated cylinder, add 900 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 100 mL of deionized water to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation.

Extraction solution 2: Acetone:water (5:5) (v:v). Using a graduated cylinder, add 500 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 500 mL of deionized water to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation.

Extraction solution 3: Acetone:0.5N HCl (5:5) (v:v). Using a graduated cylinder, add 500 mL of pesticide grade acetone to a 1L solution bottle. Next, using a graduated cylinder, add 500 mL of 0.5 N HCl to the same 1L solution bottle. Shake well to mix. The solution was stored at room temperature for up to 30 days after preparation. Note: 0.5N HCl was prepared by adding approximately 20.83 mL of concentrated HCl to 500 mL of deionized water.

Mobile Phase A: 10mM ammonium acetate and 0.2% formic acid in water. Using a graduated cylinder, 1000 mL of DI water was measured and transferred to a mobile phase bottle. 2.0 mL of formic acid and 0.7708g of ammonium acetate was added to the bottle, and mixed well. The solution was stored at room temperature for up to 30 days after preparation.

Mobile phase B: 0.2% formic acid in methanol. Using a graduated cylinder, 1000 mL of methanol was measured and transferred to a mobile phase bottle. 2.0 mL formic acid was added to the bottle and mixed well. The solution was stored at room temperature for up to 30 days after preparation.

Equipment and Instruments

Freezer(s), capable of maintaining -20 °C ± 10 °C
Refrigerator(s), capable of maintaining 1-6 °C
Balance, Analytical, capable of weighing to the nearest 0.01 mg
Balance, Analytical, capable of weighing to the nearest 0.1 mg
Balance, Top Loading, capable of weighing to the nearest 0.1g
Sonicator
Vortex
Adjustable pipettes, 200 µL, 1000 µL, 5000 µL

Graduated cylinders
Wrist action shaker
Centrifuge capable of 4000 rpm
Autosampler vials and caps, 2-mL, screw cap
UHPLC column,
UHPLC system, Dionex Ultimate 3000
Mass Spectrometer, ABSciex Qtrap 4000
UPLC software, Dionex Chromeleon, bundled with Analyst version 1.5.2
MS/MS software, ABSciex Analyst, version 1.5.2
Data analysis software, ABSciex MultiQuant, version 2.0.2
Microsoft Office Excel 2007

Sample Extraction of F9990, 3-hydroxy-F9990, F9990-1-carboxylate, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide

- Weigh 5.0 ± 0.1 g of each sample into a 50 mL centrifuge tube.
- Using untreated samples, make fortifications at the LOQ and 10X LOQ.
- Add 250 µL of the LOQ fortification and 250 µL of the 10 X LOQ fortification solutions to their respective samples.
- Add 25 mL of acetone:water (9:1) to each centrifuge tube and place on a wrist action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant into a 100 mL volumetric flask. (A funnel with filter paper may be used).
- Repeat extraction by adding 25 mL of acetone:water (5:5) to the sample.
- Sonicate the sample at approximately 40 °C for 60 minutes.
- Shake on wrist-action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant and combine with first supernatant in the 100 mL volumetric flask.
- Repeat extraction by adding 25 mL of acetone:0.5N HCl (5:5) to the sample.
- Sonicate the sample at approximately 40 °C for 60 minutes.
- Shake on wrist-action shaker for approximately 60 minutes.
- Centrifuge samples at 4000 rpm for approximately 10 minutes.
- Decant supernatant and combine with the other two supernatants in the 100 mL volumetric flask.
- Bring up to the 100 mL volume mark with acetone. Shake well to mix.
- Pour the mixed extract into a 50 mL centrifuge tube and fill to the top. The rest of the sample can be discarded.
- Centrifuge the sample at 4000 rpm for approximately 10 minutes.
- Using a transfer pipette, transfer a portion of the supernatant to a 15 mL centrifuge tube, up to the 5 mL graduation mark.
- Place the sample under nitrogen evaporation in order to evaporate off the acetone. Once this is done, approximately 1.5 mL of water should be left.
- Remove from nitrogen evaporation, and bring back up to the 5 mL graduation mark with methanol.
- Shake well to mix
- Transfer a portion to an HPLC vial for LC-MS/MS analysis.

Instrument Analysis

UPLC-MS/MS was used to quantify residues of F9990 its metabolites, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-(difluoromethyl)-1H-pyrazole-4-carboxamide in soil.

UHPLC system:	Dionex Ultimate 3000
MS Detector:	Triple Quadrapole API 4000
UHPLC column:	Phenomenex Kinetex 2.6µ C18 100A 5µm, 50 x 4.6 mm
Guard column:	SecurityGuard Ultra Cartridge UHPLC C18 for 4.6 mm
Column temp:	20°C
Injection volume:	10 µL
Run Time:	24.0 minutes
Mobile phase A:	10mM ammonium acetate and 0.2% formic acid in water
Mobile phase B:	0.2% formic acid in methanol
Flow rate:	0.600 mL/minute
Approx. Retention Times:	3-(difluoromethyl): 2.7 min (positive polarity) F9990-1-carboxylate Diastereomer 1: 11.80 min (positive polarity) F9990-1-carboxylate Diastereomer 2: 12.63 min (positive polarity) 3-hydroxy-F9990: 17.63 min (negative polarity) F9990: 18.78 min (positive polarity)

Gradient program:

Time (min)	%A	% B
0.0	90	10
3.5	90	10
6.0	60	40
22.0	20	80
23.0	20	80
23.1	90	10
24.0	90	10

Curtain Gas:	10.00
Collision Gas:	High
IonSpray Voltage:	5500, -4500
Temperature:	450 °C
Ion Source Gas 1:	60
Ion Source Gas 2:	60

Compound	Parent Ion (da)	Daughter Ion (da)	Dwell time (msec)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Polarity
3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide	176.059	136	500	41	10	23	8	Positive
3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide*	176.059	156	500	41	10	13	10	Positive
F9990-1-carboxylate (diastereomer 1)	382.1	336.200	500	76	10	21	10	Positive
F9990-1-carboxylate (diastereomer 1)*	382.1	296.100	500	76	10	35	20	Positive
F9990-1-carboxylate (diastereomer 2)	382.1	336.201**	500	76	10	21	10	Positive
F9990-1-carboxylate (diastereomer 2)*	382.1	296.101**	500	76	10	35	20	Positive
3-hydroxy-F9990	366.235	174.8	1000	-110	-10	-30	-11	Negative
3-hydroxy-F9990*	366.235	130.7	1000	-110	-10	-40	-7	Negative
F9990	352.185	332.1	1000	106	10	23	8	Positive
F9990*	352.185	256.1	1000	106	10	41	16	Positive

*Confirmatory ion transition.

**For the diastereomers, AB Sciex Analyst program required that the daughter ions not have the exact same molecular weight.

Calculations

Linear Regression (F9990, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide)

The following formulas were used to calculate F9990, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide fortification recoveries.

The amount found (ppb) of F9990, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide residue was calculated from the linear regression equation with 1/x weighting.

The linear calibration equation is:

$$y = mx + b$$

The slope (m) and intercept (b) are calculated as:

$$m = (\sum w \sum wxy - \sum wx \sum wy) / D_x$$

$$b = (\sum wx^2 \sum wy - \sum wx \sum wxy) / D_x$$

Where:

$$D_x = \sum w \sum wx^2 - (\sum wx)^2$$

$$D_y = \sum w \sum wy^2 - (\sum wy)^2$$

Amount Found (ppb)

Below is an example of how the amount of residue found is determined for F9990. This same equation is used for all compounds in this validation. The amount found (ppb) F9990 was calculated by solving the linear equation for x, concentration, using the detector response (area) substituted in for y, and multiplying the result by the dilution factor.

$$x = \frac{y\text{-}b}{m} * \text{dilution factor}$$

Example: F9990
Lab Sample ID: Nebraska LOQ 1
Sample Peak Area: 8240.95
Dilution factor: 20

$$x = \frac{8240.95 — (1018.48685)}{32234.90} * 20 = 4.4811\ ppb$$

Analyst MultiQuant software (ABSciex, Version 2.0.2) was used to calculate linear, 1/x weighted regression calibration curves and amount found (ppb).

Dilution Factor

The dilution factor for F9990, 3-hydroxy-F9990, F9990-1-carboxylate diastereomers, and 3-difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide samples are calculated with the equation below:

$$dilution\ factor = \frac{total\ dilution\ volume\ (mL)}{sample\ weight\ (g)}$$

Example:	F9990
Lab Sample ID:	Nebraska LOQ
Sample Weight:	5.0 g
Total Dilution Vol:	100 mL

$$dilution\ factor = \frac{100\ mL}{5.0\ g} = 20$$

Fortification Level F9990 (ppb added)

Below are examples of the fortifications used for the validation.

$$Fortification\ level\ (ppm) = \frac{Vol.Spiking\ Soln.(mL)X\ Spiking\ Soln.Conc.\left(\frac{\mu g}{mL}\right)}{Sample\ weight\ (g)}$$

Example:	F9990
Lab Sample ID:	Nebraska LOQ 1
Volume Spiking Soln:	0.250 mL
Identity of Spiking Soln:	LOQ Fortification Solution
Spiking Soln. Conc.:	0.105009 µg/mL
Sample Weight:	5.0 g

Fortification level = 0.250 mL * 0.105009 µg/mL / 5.0 g = 0.00525045 ppm (5.25045 ppb)

Example:	F9990
Lab Sample ID:	Nebraska 10X LOQ 1
Volume Spiking Soln:	0.250 mL
Identity of Spiking Soln:	10X LOQ Fortification Solution
Spiking Soln. Conc.:	1.05009 µg/mL
Sample Weight:	5.0 g

Fortification level = 0.250 mL * 1.05009 µg/mL / 5.0 g = 0.0525045 ppm (52.5045 ppb)

% Method Recovery

For all samples, the % recovered in fortification samples was corrected for the amount of residue found in the control sample analyzed in the same run, if residue was found in the control sample above the LOD.

$$\%\ Method\ Recovery = \frac{ppm\ found\ in\ fortification\ sample - ppm\ found\ in\ untreated}{Fortification\ level} * 100\%$$

Example:	F9990
Lab Sample ID:	Nebraska LOQ Spike 1
ppm Found in spike:	0.0448110 ppm
ppm Found in untreated:	0.0000000 ppm
Fortification level:	0.0525045 ppm

% Method Recovery = (0.0448110 – 0.0000000) ppm / 0.0525045 ppm * 100% = 85.35%