2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation of the Syngenta Method GRM023.04A, an analytical method for the determination of residues of the metabolite SYN508272 in soil [1].

This study was designed to fulfill the requirements of the EPA's Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods, (d) Independent Laboratory Validation [2] and guideline requirements described in the EPA FIFRA Pesticide Assessment Guidelines for Subdivisions N, E, and K, addenda for Data Reporting Guideline for Environmental Methods [3]. The EPA's Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods, (d) Independent Laboratory Validation (ILV), requires that analytical methods used for a terrestrial field dissipation study be independently validated. This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 [4].

3.0 MATERIALS AND METHODS

3.1 Test Substance

Syngenta Code: SYN508272
IUPAC name: 3-difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid amide
CAS name: 1H-Pyrazole-4-carboxamide, 3-(difluoromethyl)-1-methyl
CAS number: 925689-10-7
Batch identification: DAH-XXXIII-3
Stated purity: 99.1%
Expiration date: September 30, 2009
Storage conditions: Refrigerator

3.2 Test Matrix

The bulk control soil used for the validation was provided by the sponsor and arrived in good frozen condition on dry ice on November 25, 2008. The bulk control soil was stored frozen in a limited-access freezer at the CPS laboratory until needed for analysis.

The control soil sample used in this ILV was collected from Cass County, North Dakota, for Syngenta Study T016831-04 (labeled as "PA.ND.K.BULK CON 0-4" with Sample Numeric ID of RIEN00205-0004) and was supplied by the sponsor. This control soil sample was checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in the validated Syngenta Method GRM023.4A. This control soil sample was characterized by Agvise Laboratories of Northwood, North Dakota, and reported to Syngenta for Syngenta Study T016831-04, and the original raw data for the soil characterization are stored in Syngenta Archive under the Syngenta Study Number T016831-04. The characterization results of the control soil are summarized below.

USDA Texural Class	Percent Sand	Percent Silt	Percent Clay	Percent Organic Matter	pH	Cation Exchange Capacity (meq/100g)	Bulk Density Disturbed (g/cc)	Percent Moisture at 1/3 bar Disturbed
Sandy Loam	71	12	17	6.5	8.1	28.7	1.1	24.3

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Syngenta Method GRM023.04A (Section 2.0, Materials and Apparatus, and Appendices 1 and 2). Identical or equivalent apparatus and materials were used.

3.3.1 Equipment and Apparatus

General Laboratory Glassware (VWR)
Polypropylene Centrifuge Tubes 50 mL (Falcon)
Polypropylene Centrifuge Tubes 15 mL (Falcon)
Polypropylene Bottle 250 mL (VWR)
Analytical Balance (Mettler Toledo)
Top-loading Balance (Mettler Toledo)
Refrigerator/Freezer (Revco)
Freezer -30°C (Revco)
Bench top Temperature Controlled Centrifuge (Beckman Coulter)
Ultrasonic cleaner (Branson)
Electronic Pipettor 120 μL (Biohit)
Electronic Pipettor 1000 μL (Biohit)
Manual Pipettor 10000 μL (VWR)
Nitrogen Evaporator (Organomation)
Eberbach Laboratory Shaker (Eberbach)
Vacuum Manifold (Visiprep)
SPE Cartridges (Waters Oasis HLB 60 mg/3 mL)
Autosampler Vials (Agilent)
Autosampler Vial Red Silicone Snap Caps (Agilent)
API 4000 LC-MS/MS equipped with a TurboIonSpray Source (Applied Biosystems)
Peak Scientific NM20Z Gas Station (Peak Scientific Instruments)
HPLC System (Agilent 1200)
HPLC Column – Phenomenex Synergi, Hydro-RP 80A, 4 μm, 150 × 3 mm (HiChrom)
Column Reservoirs 30 mL fitted with 20 μm polyethylene frits (Varian)

3.3.2 Reagents

Glacial Acetic Acid Reagent grade (J.T.Baker)
Acetonitrile HPLC grade (J.T. Baker)
Methanol HPLC grade (J.T. Baker)

Water HPLC grade (J.T. Baker)

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and an extract of the control soil was shown to be free of interferences at appropriate retention times.

One request for clarification was made by the study director to the study monitor regarding the type of shaker to be used and if it was acceptable to use an N-Evap waterbath evaporator instead of a heating block. It was determined that a horizontal type shaker should be used and that an N-Evap waterbath could be used in place of the heating block. Another request for clarification was made by the study director to the study monitor regarding the type of HPLC column to be used when it was discovered that the designated column was no longer manufactured. It was determined by the developer of the original method that a Phenomenex column with similar properties to the originally designated column could be used.

3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

Sample Validation Sets:
Each analytical set consisted of 13 samples: one reagent blank, two control samples, five control samples fortified at the LOQ of 0.1 ng/g (0.1 ppb), and five control samples fortified at 10× LOQ of 1.0 ng/g (1.0 ppb). Twelve 10-g soil samples were weighed from the bulk control into 250-mL plastic bottles to be used for the validation. Two samples were used as unspiked matrix control samples, five were spiked at the LOQ concentration of 0.1 ng/g (0.1 ppb), and the remaining five samples were spiked at 10× LOQ concentration of 1.0 ng/g (1.0 ppb). All validation samples were assigned a unique identification number during preparation and analysis. A reagent (method) blank was also included in the sample set. The reagent blank sample is a blend of reagents applied through the entire extraction and cleanup procedures according to the method without any soil sample.

Fortification:
The LOQ and 10× LOQ recovery samples were fortified with 0.100 mL of 0.01- and 0.10-µg/mL fortification standard solutions, respectively. The recovery samples were allowed to sit at room temperature for at least 5.00 minutes before proceeding to the extraction step.

Extraction and Workup:
The following extraction steps were followed for each sample:

1. Weigh a representative amount of soil (10 ± 0.1 grams) into separate 250-mL disposable plastic centrifuge bottles.

2. Fortify the recovery samples and allow at least 5 minutes equilibration at room temperature.
3. Add 50 mL of 80:20, v:v, acetonitrile/HPLC-grade water to each 250-mL bottle.
4. Shake on a horizontal shaker at room temperature for approximately 1 hour.
5. Centrifuge the samples at approximately 3000 rpm for 10 minutes.
6. Transfer 10.0-mL aliquots of each supernatant into clean, plastic, graduated 15-mL centrifuge tubes.
7. Reduce sample volume to dryness using a nitrogen evaporator with the water bath set at 50.0°C. This should take approximately 1–1.5 hours.
8. Dilute each sample to 10 mL with HPLC-grade water.
9. Cap and place each sample in an ultrasonic bath for approximately 25 minutes, then shake each sample to mix thoroughly.

The following Solid Phase Extraction (SPE) purification steps were followed for each sample:

1. Connect the SPE cartridges (Waters Oasis HLB, 60 mg/3 mL) to the vacuum manifold.
2. Condition each column by adding 2.0 mL of acetonitrile. Do not allow the cartridges to become dry. Discard the eluate.
3. Repeat Step 2 using HPLC-grade water instead of acetonitrile.
4. Attach a reservoir (70.0-mL capacity) to each of the SPE cartridges.
5. Load the soil extracts onto each of the SPE cartridges and draw through using vacuum at a rate of approximately 1–2 mL per minute. Do not allow cartridges to become dry.
6. After loading, remove the column reservoir and connector. Add 2.0 mL HPLC-grade water to the 15.0 mL centrifuge tube. Cap, shake well, and add this to the top of each of the SPE cartridges, draw through under vacuum, and discard the column eluate.
7. Remove the excess water by drying the column under vacuum for approximately 15 minutes.
8. Place 15.0-mL falcon tubes in the manifold rack to collect each sample. Add 2 mL acetonitrile to the top of each cartridge and collect the column eluate containing the compound of interest.
9. Evaporate the collected eluates to dryness under a stream of nitrogen with heat set at 50.0°C.
10. Adjust the final volume to 1 mL with HPLC-grade water. Sonicate the contents for the falcon tube briefly.
11. Refrigerate samples until day of analysis. Sonicate briefly.
12. Transfer a suitable amount of each sample into an autosampler vial for LC-MS/MS analysis.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described by the Syngenta Method GRM023.04A [1], with a few exceptions. A Phenomenex Synergi, Hydro-RP 80A 4 µm 150 × 3 mm i.d. HPLC column was used instead of the Phenomenex Develosil RP Aqueous (C30) 3 µm, 150 × 3 mm i.d. HPLC column due to the unavailability of the Develosil column. On the first attempt of the validation, 10 mL of sample extract (from Step 6 of the Extraction and Work-up) was reduced to less than 2 mL in an N-Evap set at 50°C (Step 7 of the Extraction and Work-up). During the second attempt, the 10 mL of sample extract was reduced to dryness, instead of to a volume of less than 2 mL. In order to be sure the dried sample was fully reconstituted step 9 of the method was modified from sonication for a few seconds to sonication for 25 minutes. In addition, the multi-point calibration procedure was used to calculate the concentration of SYN508272. The calibration standards included eight separate levels and ranged in concentration from 0.1 to 10 ng/mL.

3.4.4 Fortification and Standard Solution Preparation

A stock solution of the analytical standard, SYN508272 (99.1% purity), prepared in HPLC-grade acetonitrile (approximately 10 mg of standard, with a precision tolerance of 0.1 mg) was added to a 50-mL volumetric flask and brought to volume with acetonitrile. An intermediate stock containing the analyte was prepared by measuring appropriate aliquots of the primary stock into a 100-mL volumetric flask and bringing to volume with acetonitrile. Fortification standards were also prepared at concentrations of 0.1 µg/mL and 0.01 µg/mL by appropriate dilutions of the intermediate stock with acetonitrile.

Standard calibration solutions were prepared at eight concentrations ranging from 0.100 to 10.0 ng/mL in HPLC grade water. All solutions were stored in a refrigerator when not in use.

3.5 LC/MS/MS Instrumentation

Instrumentation
HPLC System (Agilent 1200)
Tandem Mass Spectrometry, MS/MS (Applied Biosystems API 4000)
Software: Applied Bio-Systems, Analyst 1.4.1

3.6 Data Acquisition and Reporting

Peak integration and quantification were performed by Analyst software version 1.4.1. Analyte quantification was achieved by external calibration. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for each analyte. A best-fit, weighted 1/x linear regression equation was derived and used to calculate the concentration of the analyte in each sample. The correlation coefficients for the calibration curves for each analytical set was greater than 0.99. Recovery results were computed for each sample. The equations used for quantification are presented in Appendix 2.

4.4 Critical Steps

During extraction, instead of reducing the sample volume to less than 2 mL by evaporation in a heating block set to 50°C under a steady stream of air, the sample volume was reduced to dryness using a nitrogen evaporator with the water bath set at 50.0°C. It was determined that any residual acetonitrile left in the samples extract, when applied to the SPE cartridge, would adversely affect the recoveries.

4.5 Time Required for Analysis

It took one person approximately 1 day, or 8.0 person-hours, to complete the extraction of one set of 12 samples (two unfortified matrix control samples and 10 fortified samples). Time of analysis was approximately 3 hours. Therefore, to complete one set, including extraction and analysis, took 1.5 days.

4.6 Communication with Study Sponsor

This method passed the independent laboratory validation with initial clarification before method establishment and a second clarification after the first trial. The study director asked study sponsor for clarification regarding the type of shaker to be used and if it was acceptable to use an N-Evap waterbath evaporator instead of a heating block. It was determined that a horizontal type shaker should be used and that an N-Evap waterbath could be used in place of the heating block. This also included discussion regarding the type of HPLC column, as the designated type is no longer manufactured. Upon completion of the first trial, further clarification between the study director and the study sponsor was necessary. The study sponsor indicated that a change to the written method for the extraction and purification steps was necessary to obtain acceptable recoveries. The change was a reduction of the 10-mL extraction solution (from Step 6 of the Extraction and Work-up) to dryness in the "extraction and purification" step. This step was supported by the field-sample analysis as well. This step removes all the acetonitrile from the extracted sample.

Determination of Residues of the Metabolite SYN508272 in soil." Syngenta Method GRM023.04A (see Appendix 5) was demonstrated to be suitable for the determination of the targeted analyte in the soil type studied at an LOQ of 0.1 ng/g (0.1 ppb). The method was performed as written with no major modifications. One minor modification to the method is the type of HPLC column used, since the designated column was no longer manufactured. A Phenomenex Synergi, Hydro-RP 80A 4 µm 150 × 3 mm i.d. HPLC column was used instead of the Phenomenex Develosil RP Aqueous (C30) 3 µm, 150 × 3 mm i.d. HPLC column. A second minor modification is the 10-mL aliquot of extraction solution was reduced to dryness to remove acetonitrile completely, in order to improve recoveries. It took one person approximately 1 day, or 8.0 person-hours, to complete the extraction of one set of 12 samples (two unfortified matrix control samples and 10 fortified samples). Time of analysis was approximately 3 hours. To complete one set, including extraction and analysis, took 1.5 days. This method passed the independent laboratory validation with initial clarification of equipment type during the method establishment between the sponsor and the study director and further clarification after the first trial between the study director and the study sponsor regarding a change to the written method for the extraction and purification steps (change to method described in Section 4.4, Critical Steps).

TABLE 3 HPLC System Operating Parameters

HPLC System:	Agilent Model 1200
Software:	Applied Bio-Systems, Analyst 1.4.1
Analytical Column:	Phenomenex Synergi, Hydro-RP 80A, 4 µm, 150 × 3 mm
Column Temperature:	40°C
Injection Volume:	50 µL
Run Time:	6.0 minutes

Mobile Phase: (A): 0.1% Glacial Acetic Acid in HPLC-Grade Water
(B): Methanol

Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.00	80.0	20.0	500
6.00	80.0	20.0	500

TABLE 4 MS/MS Operating Parameters

Tandem Mass Spectrometry System, Applied Biosystems API 4000
Software: Applied Bio-Systems, Analyst 1.4.1

The following parameters were used for operation of the mass spectrometer:

Parameter	Setting
Ion Source:	TurboIonSpray
Scan Type:	MRM
Polarity:	Positive
Curtain Gas:	15.0
Temperature:	250
Ionspray Voltage:	5500
Collision Gas Setting:	6.00
Gas 1 (GS1):	40.0
Gas 2 (GS2):	40.0
Interface Heater (ihe):	on
Declustering Potential:	51.00 V
Entrance Potential:	10.00 V
Transitions Monitored:	(Q1) 176.00→(Q3) 156.00 m/z quantitative
	(Q1) 176.00→(Q3) 136.00 m/z confirmatory
(Q1) 176.00→(Q3) 156.00 m/z	
Collision Energy:	13.00 V
Collision Cell Exit Potential:	12.00 V
(Q1) 176.00→(Q3) 136.00 m/z	
Collision Energy:	29.00 V
Collision Cell Exit Potential:	8.00 V

APPENDIX 2 Calculations

Peak integration and quantification were performed by Analyst software version 1.4.1. Analyte quantification was achieved by external calibration. The MS detector responses (peak area) for various injected standard concentrations were used to generate external calibration curves for each analyte. A best-fit, weighted 1/x linear regression equation was derived and used to calculate the concentration of each analyte in each sample. The recoveries of either analyte from fortified samples were calculated as follows:

Linear regression formula from calibration curve $y = mx + b$

$$\text{ng/mL analyte} = \frac{y - b}{m}$$

where y = Sample peak area
b = Calibration intercept
m = Slope

$$\text{ng/g (ppb) analyte} = [(\text{ng/mL analyte} \times \text{init. extract vol. (mL)} \times \frac{\text{final vol. (mL)}}{\text{aliquot vol. (mL)}}) \div 10\text{ g}]$$

$$\text{Percent Recovery} = \frac{\text{concentration of analyte (ng/g) - concentration of analyte control (ng/g)}}{\text{analyte fortification level (ng/g)}} \times 100$$