2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation of the method GRM023.02A, a residue method for the determination of SYN524464 as SYN508210 and SYN508211 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:	SYN508210 (trans isomeric form)
IUPAC name:	3-Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid ((1RS,2SR)-2-bicyclopropyl-2-yl-phenyl)-amide
CAS name:	1H-Pyrazole-4-carboxamide, N-[2-(1R,2S)-[1,1'-bicyclopropyl]-2-ylphenyl]-3-(difluoromethyl)-1-methyl-, rel-
CAS number:	599197-38-3
Batch identification:	DAH-XXXIII-37
Stated purity:	98.2%
Expiration date:	August 31, 2010
Storage conditions:	Refrigerator

Product name:	SYN508211 (cis isomeric form)
IUPAC name:	3-Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid ((1RS,2RS)-2-bicyclopropyl-2-yl-phenyl)-amide
CAS name:	1H-Pyrazole-4-carboxamide, N-[2-(1R,2R)-[1,1'-bicyclopropyl]-2-ylphenyl]-3-(difluoromethyl)-1-methyl-, rel-
CAS number:	599194-51-1
Batch identification:	DAH-XXXIII-38
Stated purity:	98.6%
Expiration date:	August 31, 2010
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 GRM023.2A. 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 Method GRM023.02A (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 1000 µL (VWR)
Manual Pipettor 10000 µL (VWR)
Nitrogen Evaporator (Organomation)

Eberbach Laboratory Shaker (Eberbach)
Vacuum Manifold (Supelco)
SPE Cartridges (Varian SAX 2 gm/12 cc)
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 – ACE C18, 5.0 μm, 50 × 3.0 mm (HiChrom)

3.3.2 Reagents

Formic Acid (98%) Reagent grade (EMD)
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.

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 0.1 ng/g (0.1 ppb), and five control samples fortified at 10× LOQ 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 ± 0.3 grams) into separate 250-mL disposable plastic centrifuge tubes.
2. Fortify the recovery samples and allow at least 5 minutes equilibration at room temperature.
3. Add 50 mL of 80:20, v:v, ACN/HPLC 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 5 minutes.
6. Transfer 10.0-mL aliquots of each supernatant into clean plastic graduated 50-mL centrifuge tubes.
7. Dilute each sample to 50 mL with HPLC-grade water.

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 methanol. Do not allow the cartridges to become dry. Discard the eluate.
3. Repeat Step 2 using HPLC water instead of methanol.
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. Wash the loaded SPE cartridges by adding 90:10 HPLC water/ACN (2.0 mL) to the top of each of the SPE cartridges, draw through under vacuum, and discard the column eluate.
7. Place 15.0-mL falcon tubes in the manifold rack to collect each sample. Add 1 mL ACN to the top of each cartridge and collect the column eluate containing the two compounds of interest.
8. Evaporate the collected eluates to 0.5 mL ± 0.1 mL under a stream of nitrogen with heat set at 30°C.
9. Adjust the final volume to 1 mL with HPLC-grade water. Sonicate the contents for the falcon tube briefly.
10. Transfer a suitable amount of each sample into an autosampler vial for LC-MS/MS analysis.

3.4.3 Sample Analysis

The alternative chromatographic conditions described method GRM023.02A, which utilized an ACE C18 5 μm 50 × 3.0 mm i.d. HPLC column, were chosen for analysis of samples. In addition, the multi-point calibration procedure was used to calculate concentrations of SYN508210 and SYN508211. 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 each of the analytical standards, SYN-508210 (98.2% purity) and SYN-508211 (98.6% purity), was 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 both analytes was prepared by measuring appropriate aliquots of each of the primary stocks 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 solutions of acetonitrile/water, 50/50, v/v. 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 each 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. A statistical treatment of the data includes the calculation of averages, standard deviations, RSDs, and the 95% confidence limits. Mean percent recoveries, standard deviations, RSDs, and 95% confidence intervals were calculated using Microsoft® Office Excel 2002. Results were rounded off for reporting purposes but not during calculations.

TABLE 3 HPLC System Operating Parameters

HPLC System:	Agilent Model 1200
Software:	Applied Bio-Systems, Analyst 1.4.1
Analytical Column:	ACE C18 5.0 µm, 50 × 3.0 mm
Column Temperature:	40°C
Injection Volume:	10 µL
Run Time:	7.5 minutes
Mobile Phase:	(A): 0.2% Formic Acid in HPLC-Grade Water
	(B): Methanol

Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.00	50.0	50.0	500
5.00	10.0	90.0	500
6.00	10.0	90.0	500
6.10	50.0	50.0	500
7.50	50.0	50.0	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:	Negative
Curtain Gas:	15.0
Temperature:	450
Ionspray Voltage:	-4500
Collision Gas Setting:	8.00
Gas 1 (GS1):	60.0
Gas 2 (GS2):	60.0
Interface Heater (ihe):	on
Declustering Potential:	-80.0 V
Entrance Potential:	-10.0 V
Transitions Monitored:	(Q1) 330.0→(Q3) 131.0 m/z quantitative (Q1) 330.0→(Q3) 91.0 m/z confirmatory
(Q1) 330.0→(Q3) 131.0 m/z	
Collision Energy:	-30.0 V
Collision Cell Exit Potential:	-7.00 V
(Q1) 330.0→(Q3) 91.0 m/z	
Collision Energy:	-48.0 V
Collision Cell Exit Potential:	-5.00 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

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

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