2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation on Syngenta Analytical Method No. GRM061.02A entitled "SYN545974 – Analytical Method for the Determination of SYN545974 and SYN545547 in Soil Commodities," as written.

This study was designed to satisfy guideline requirements described in OECD ENV/JM/MONO(2007)17[1] and US EPA OCSPP 850.6100(2012)[2]. This study complied with the EC SANCO/3029/99 rev. 4 (2000)[3] and EC SANCO/825/00 rev. 8.1 (2010)[4] guidelines. This study was conducted in compliance with US EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160.

The residue analytical method is suitable for the determination of SYN545974 and SYN545547 in soil. Silt loam soil was selected for evaluation in this study as they represent a diverse type of soil.

To summarize the method, a 10 gram sample was aliquoted into 125 mL Nalgene plastic centrifuge bottle. The sample was fortified, as necessary. A portion (40 mL) of acetonitrile:0.1 M ammonium acetate (80:20, v/v) was added to each sample and the solution was shaken on a wrist action shaker for 60 minutes. The mixture was centrifuged at ~3,500 rpm for approximately 5 minutes. The supernatant was aliquoted to a clean bottle. A portion (30 mL) of acetonitrile:0.1% acetic acid was added to the soil and the centrifuge bottle was capped. The mixture was placed on a wrist action shaker for 60 minutes. The sample mixture was centrifuges at ~3500 rpm for ~5 minutes. The supernatant was combined with the first supernatant. The acetonitrile:0.1% acetic acid extraction was repeated with a second portion (30 mL) and the supernatants were combined and mixed well. The combined extracts were filtered an a 10 mL portion was put through an SPE cartridge cleanup. A slight change to the elution method was made to yield a final extract, which was concentrated prior to analysis. After adjusting the final sample volume, an aliquot of this sample was transferred to an analysis vial. The analysis vial was capped then submitted for LC-MS/MS analysis. The limit of quantitation (LOQ) is 0.5 ppb for SYN545974 and SYN545547.

The method was used as written with a minor change to the SPE elution profile.

3.0 MATERIALS AND METHODS

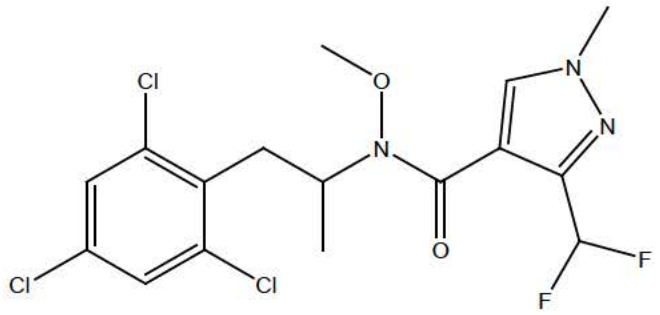
3.1 Test Item/Reference Substance

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

<u>SYN545974</u>

Common Name: SYN545974
Other Code Name: AMS 1432/1

IUPAC Name:	3-Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichloro-phenyl)-ethyl]-amide
CAS Name:	1H-pyrazole-4-carboxamide, 3-(difluormethyl-N-methoxy-1-methyl-N-[1-methyl-2(2,4,6-trichlorphenyl)ethyl]-
Structural Formula:	$C_{16}H_{16}Cl_3F_2N_3O_2$
Molecular Weight:	426.7 grams/mole

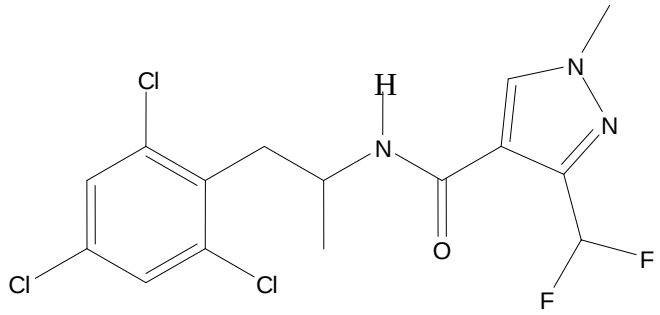


CAS No.:	122/284-64-7
Source:	Syngenta Crop Protection Inc.
Lot no.:	659114 (or AMS 1432/1)
Purity:	99.5%
Date received:	November 16, 2012
Expiration date:	End of May 2014
Storage conditions:	<30°C

SYN545547

Common Name:	SYN545547
Other Code Name:	CSCD550897
CAS Name:	1H-pyrazole-4-carboxamide, 3-(difluoromethyl)-1-methyl-N-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-

Structural Formula: $C_{15}H_{14}Cl_3F_2N_3O$
Molecular Weight: 396.65 grams/mole



CAS No.:	960053-63-8
Source:	Syngenta Crop Protection Inc.
Lot no.:	DAH-XXXV-19
Purity:	97.0%
Date received:	November 29, 2012
Expiration date:	May 31, 2014
Storage conditions:	<30°C

Characterization data for the test/reference standard are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificates of Analysis are included in Appendix 3.

The test/reference substances (analytical standards) used in this study were procured from the Sponsor and stored as directed on "Analytical Standards Chain of Custody" documents. All solutions made from the reference substances (analytical standards) were stored according to the method.

3.2 Test Systems

The test system evaluated in this study was soil. This matrix was chosen because it is representative of the soil the method is designed for. The control samples used in this study were provided by the Sponsor. Characterization data is provided in Table I.

The control soil sample was received in good condition at PTRL West on November 16, 2012. Upon receipt, the sample was transferred to a limited-access room temperature storage unit (RT4) for storage where the sample remained until it was removed to aliquot for analysis. The sample was logged in according to PTRL West SOPs using the original sample number assigned by the Sponsor and assigning a unique PTRL West sample number. Additional designations such as "control" and "fortified control," as appropriate, were assigned by the laboratory during the method validation experiments.

Room temperature storage was monitored on a daily basis and was typically at 14.0 ± 6 °C.

3.3 Equipment and Reagents/Supplies

The equipment and reagents/supplies used for the method validation were as outlined in the method. Identical or equivalent equipment and materials were used, as permitted by the method. The equivalent equipment and reagents used were as follows:

3.3.1 Equipment

Balance:	Top-loading: Model Analytical balance: Model
HPLC/MS/MS System:	Dionex Ultimate3000 high pressure liquid chromatography system/vacuum solvent degasser SRD 3600, Pump DGP3600SD, autosampler WPS3000 TSL and Column compartment TCC 3000SD equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, with Applied BioSystems/MDS Sciex Analyst Software for data collection and system control.
HPLC column:	100 mm × 2.1 mm i.d. Kinetex Phenyl-Hexyl 2.6 µm particle size
SPE Cartridge Wrist action shaker Centrifuge	Bond Elut C18 (100 mg, 3 mL)

3.3.2 Reagents

Acetonitrile:	High Purity (Burdick and Jackson) Optima Grade (Fisher Scientific)
Acetic acid:	Glacial (Fisher Scientific)
Ammonium Acetate:	Fisher Chemical
Methanol:	Optima Grade (Fisher Scientific)
Water (H_2O):	HPLC grade (Fisher Scientific)

3.4 Preparation of Standard Solutions

The preparation of SYN545974 and SYN545547 standard solutions used for this study is described below. The solutions were stored as recommended in the method when not in use (refrigerated, 1.5 to 6.3 °C).

3.4.1 Stock Standard Solution

Ten (10.36) milligrams (corrected for purity) of SYN545974 reference substance were accurately weighed and quantitatively transferred to a 100-mL volumetric flask. The contents were brought to volume with methanol. An additional 3.58 mL of methanol was added to make a stock standard solution of SYN545974 having a concentration of 100 µg/mL. 5 (5.219) milligrams (corrected for purity) of SYN545547 reference substance were accurately weighted and quantitatively transferred to a 50-mL volumetric flask. The contents were brought to volume with methanol. An additional 2.186 mL of methanol was added to make a stock standard solution of SYN545547 having a concentration of 100 µg/mL.

3.4.2 Intermediate and Fortification Standard Solutions

Fortification Standard Solutions

0.1-µg/mL:	0.025 mL of each a 100-µg/mL stock solution was transferred to a 25-mL volumetric flask. The contents were brought to volume with methanol and mixed well.
0.01-µg/mL:	2.5 mL of a 1.0-µg/mL fortification solution was transferred to a 25-mL volumetric flask. The contents were brought to volume with methanol and mixed well.

3.4.3 HPLC (Calibration) Standard Solutions

Calibrant solutions were prepared from the fortification solutions and were stored refrigerated when not in use.

1.0 ng/mL:	5.0 mL of a 0.01-µg/mL fortification solution diluted to 50 mL with 10 mL methanol and diluting to the mark with 0.1% acetic acid in HPLC grade water.
0.5 ng/mL:	5.0 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)
0.2 ng/mL:	2.0 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)
0.1 ng/mL:	1.0 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)
0.05 ng/mL:	0.5 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)

0.02 ng/mL:	0.2 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)
0.01 ng/mL:	0.1 mL of a 1.0-ng/mL calibrant solution diluted to 10 mL with methanol:0.1% actic acid (30:70, v/v)

3.5 Analytical Method

The analytical method independently validated in this study Syngenta Analytical Method No. GRM061.02A entitled "SYN545974 – Analytical Method (GRM061.02A) for Determination of SYN545974 and SYN545547 in Soil Commodities," See Appendix 2 for the complete text of the method. The following is a summary of that method:

> To summarize the method, a 10 gram sample was aliquoted into 125 mL Nalgene plastic centrifuge bottle. The sample was fortified, as necessary. A portion (40 mL) of acetonitrile:0.1 M ammonium acetate (80:20, v/v) was added to each sample and the sample was shaken on a wrist action shaker for 60 minutes. The mixture was centrifuged at ~3,500 rpm for approximately 5 minutes. The supernatant was aliquoted to a clean bottle. A portion (30 mL) of acetonitrile:0.1% acetic acid was added to the soil and the centrifuge bottle was capped. The mixture was placed on a wrist action shaker for 60 minutes. The sample mixture was centrifuged at ~3500 rpm for ~5 minutes. The supernatant was combined with the first supernatant. The acetonitrile:0.1% acetic acid extraction was repeated with a second portion (30 mL) and the supernatants were combined and mixed well. The combined extracts were filtered (Whatman 2V) and a 10 mL portion was transferred to a ppolypropylene centrifuge tube and evaporated to <0.5 mL under a gentle stream of nitrogen at ~40°C. A Bond Elut-C18 (100 mg/ 3 mL) was mounted on a suitable vacuum maniford and pre-rinsed with 3 mL of methanol, then 3 mL of ultra pure water, without allowing the SPE cartridge to go dry. The concentrated sample was loaded onto the SPE cartridge and allowed to percolate through the SPE cartridge under gravity to the level of the top frit at a rate of approximately 1 mL/minute and the column eluate was discarded. The sample concentration centrifuge tube was rinsed with 2.5 mL of methanol:0.1% acetic acid in ultra pure water (60:40, v/v) and the rinse solution was added to the SPE cartridge. The volume was allowed to percolate through under gravity to the level of the top frit and the eluate was discarded. A clean polypropylene centrifuge tube (15 mL) was placed under the outlet port of the SPE cartridge and the analytes were eluted with 2 mL of methanol:0.1% acetic acid in ultra pure water (60:40, v/v) plus 3 mL of methanol under gravity at a rate of approximately 1mL/minute. The combined eluates were concentrated under a gentle stream of nitrogen at ~40°C to remove the organic solvent. A portion of methanol was added to yield 1.5 mL final volume. The sample was further diluted to 5 mL with 0.1% acetic acid in ultra pure water to yield the final sample. The sample was vortexed well to mix. An aliquot of this sample was transferred to an analysis vial and the vial was capped and submitted for LC-MS/MS analysis. The limit of quantitation (LOQ) is 0.5 ppb for SYN545974 and SYN545547.

The method was used as written, with a minor modification to the SPE cleanup step. The collection of the 1 mL of methanol:0.1% acetic acid in ultra pure water (60:40, v/v) was suggested by the Sponsor via email on January 30, 2013. PTRL West modified this to collect the last 2.0 mL of the 4.5 ml of the 60/40 (v/v) MeOH/0.1% acetic acid in ultra pure water and combining this portion with the 3 ml MeOH fraction to achieve acceptable recovery. Dilution of the final 10X LOQ samples was not necessary to achieve acceptable recoveries.

Residue calculations were conducted using Analyst software to prepare the calibration curve with 1/x weighting. Equations used for calculation of residues and example calculations can be found in Appendix 4. The calculation spreadsheets can be found in Appendix 5.

3.5.1 Fortifications

Untreated control soil samples were fortified using microliter amounts of the appropriate fortification standard to LOQ and 10X concentrations as per method. Fortifications used in this method validation are as follows:

Matrix	Fortification Volume (µL)	Fortification Conc. (µg/mL)	Sample Weight (g)	Final Conc. (ppb)	Replicates
Soil	500	0.01	10	LOQ	5
Soil	500	0.1	10	10X LOQ	5

Aliquots of untreated control sample were fortified with microliter amounts of the fortification standard solution. During fortification, the standard solution was mixed into the soil prior to addition of solvent.

Untreated control samples were fortified according to the following scheme:

Matrix	**Sample Type**	**Fortifying Compounds**	**Fortification Level (ppb)**	**# of Samples**
Soil	Control	None	0.0	2
	Fortified control	SYN545974 + SYN545547	0.5 (LOQ)	5
	Fortified control	SYN545974 + SYN545547	5.0 (10 × LOQ)	5

3.6 Instrumentation Conditions

All samples were analyzed by HPLC employing tandem mass spectrometric (MS/MS) detection. Typical conditions were as follows:

LC Operating Conditions

Instrument: Dionex Ultimate 3000 high pressure liquid chromatography system/ vacuum solvent degasser SRD 3600, Pump DGP3600SD, autosampler WPS3000 TSL and Column compartment TCC 3000SD equipped with an Applied BioSystems API 4000 mass spectrometer (MS/MS) detector, with Applied BioSystems/MDS Sciex Analyst Software for data collection and system control.

Analytical column: 100 mm × 2.1 mm i.d. Kinetex Phenyl-Hexyl
2.6 µm particle size

Column Oven Temp: 40°C

Injection Volume: 10µL

Run Time: 10.5 minutes

Retention Time: 5.4 minutes for SYN545974, 5.0 minutes for SYN545547

Flow rate: 0.5 mL/minute

Mobile phase: Component A: Burdick and Jackson High Purity Water
Component B: Burdick and Jackson High Purity Methanol

Gradient:

Time (min.)	% A	% B
0.0	70	30
5.0	0	100
7.5	0	100
7.6	70	30
10.5	70	30

Mass Spectrometer Conditions

Interface: TIS (Turbo Ion Spray)

Ionization mode: Positive (+)

Resolution: Q1-Unit, Q3-Unit (Note: Unit is equivalent to medium)

Curtain gas (CUR): 25.00

Ionspray voltage: 5500.00

Collision gas setting (CAD) : Nitrogen @ setting of "6"

Gas 1 (GS1): 50.00

Gas 2 (GS2): 60.00

Interface heater (ihe): ON

Transitions monitored:

	Ion, *m/z*		Time, *ms*
	Q1	Q3	
SYN545974	426.4	193.2	200
	426.4	166.4	200
SYN545547	396.0	376.3	100
	396.0	136.3	100

Acquisition mode: MRM

Declustering potential (DP): 70.00 for SYN545974, 56.00 for SYN545547

Entrance potential (EP): 10.00

Collision energy (CE): SYN545974: 44 (quantitation), 39 (confirmation)
SYN545547: 22 (quantitation), 43 (confirmation)

Collision cell exit potential (CXP):
SYN545974: 4.20 (quantitation), 8.50 (confirmation
SYN545547: 4.50 (quantitation), 10.5 (confirmation)

Calibration/Sample Analysis

A six- or seven-point standard curve was prepared by injecting constant volumes of calibration standards at specified concentrations. Constant volume injections were used for sample extracts as well. A calibration standard was injected every 3 to 5 sample injections.

3.7 Modifications, Interpretations, and Critical Steps

The analytical method was run exactly as written, with a minor change to the SPE cartridge cleanup procedure. This minor modification is described in Section 3.5.

4.5 Protocol/SOP/Method Deviations

One method modification was generated in this study. Due to slight differences in SPE cartridge lots, the volume collected was modified slightly to include the final 2 mL of fraction 3.5.e (methanol:0.1% acetic acid (60:40, v/v) plus fraction 3.5.f (methanol)

No SOP deviations were generated for this study.

The modification to the method described above was considered to have no negative impact on the integrity of this study. The extract stability test was conducted after 21 days of refrigerator storage.

5.0 CIRCUMSTANCES AFFECTING DATA

No circumstances occurred during this validation that affected quality or integrity of the data.

6.0 CONCLUSION

PTRL West successfully independently validated Syngenta Analytical Method No. GRM061.02A entitled "SYN545974 – Independent Laboratory Validation of Residue Method (GRM061.02A) for the Determination of SYN545974 and SYN545547 in Soil." No significant matrix suppression or enhancement was observed.

The method was demonstrated to be suitable for the determination of SYN545974 and SYN545547 in soil. An LOQ of 0.5 ppb was demonstrated. The final extracts were found to be stable over a period of 21 days of refrigerator storage. No significant matrix effects on the LC-MS/MS analysis were detected.

APPENDIX 4 Example Calculations

Equations

Calculations for instrumental analysis were conducted using a software application to create a standard curve based on linear regression. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. In all cases, a 1/x weighting was applied to the curve.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

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

The standard (calibration) curves generated for the analytical set was used for the quantitation of SYN545974 and SYN545547 in the samples. For this study, the correlation coefficient (r^2) for the calibration curves (quantitation and confirmation) was equal to or greater than 0.998.

The calculations for ppm found and percent recovery (for fortified samples) for SYN545974 and SYN545547 are:

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

$$\text{ppb} = \frac{\text{analyte conc. found (ng/mL) x Final Volume (mL) x Adjusted Extract Vol. (mL) x Dil. Factor}}{\text{Sample Wt. (g) x Aliquot Vol (mL)}}$$

2. The percent recovery in fortified control samples is calculated as follows:

$$\%\ \text{Recovery} = \frac{\text{ppb found in fortified control - ppm found in control}}{\text{fortification level (ppb) added}} \times 100$$

Example Calculations

1. PTRL Sample No. 2387W-001 (soil, control sample). (Figure 1.1):

$$0 \text{ peak response} \rightarrow 0.00 \text{ ng/mL}$$

$$ppb = 0$$

2. PTRL West Sample No. 2387W-001 (soil, fortified control sample, replicate F1A), quantitation ion:

Fortified Control @ 0.5 ppb, (Figure 1.2):

$$ng/mL = 0.0804$$

$$ppb = [0.0804 \text{ ng/mL} \times 5 \text{ mL} \times 100.2 \div [10 \text{ mL} \times 10 \text{ g}]$$

$$ppb = 0.40$$

$$\% \text{ Recovery} = \frac{0.40 \text{ ppb} - 0.000 \text{ ppm}}{0.5 \text{ ppb}} \times 100$$

$$= 80\%$$