2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation on Syngenta Analytical Method No. GRM061.01A entitled "SYN545974 – Residue Method for the Determination of SYN545974 in Water," 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[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 in water. Surface and ground water were selected for evaluation in this study as they represent a diverse types of natural waters.

To summarize the method, a 4 mL sample was aliquoted into 20 mL scintillation vials. The sample was fortified, as necessary. A portion (1 mL) of 0.2% acetic acid in acetonitrile was added to each sample and the solution was mixed. 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 0.05 ppb for SYN545974.

The analytical method was run exactly as written. The clean-up step was not required. The analyst substituted acetic acid in the chromatography solvents, instead of using formic acid as described in the method. No negative impact on the study was detected.

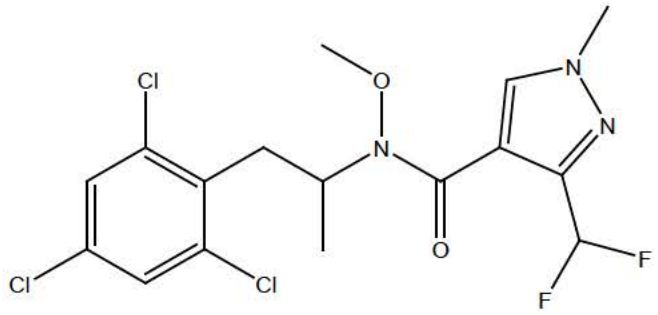
3.0 MATERIALS AND METHODS

3.1 Test Item/Reference Substance

The analytical (reference) substance used for this study was:

SYN545974

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.:	1228284-64-7
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

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

3.2 Test Systems

The test systems evaluated in this study were surface water and ground (well) water. These matrices were chosen because they are representative of the water the method is designed for. The control samples used in this study were provided by the Sponsor.

Control samples were received cool and in good condition at PTRL West on November 16, 2012. Upon receipt, they were transferred to a limited-access refrigerator (R-23) for storage where they remained until they were removed to aliquot for analysis. Both samples were logged in according to PTRL West SOPs using the original sample numbers assigned to them and assigning unique PTRL West sample numbers. Additional designations such as "control" and "fortified control," as appropriate, were assigned by the laboratory during the method validation experiments.

Refrigerator storage temperatures were monitored on a daily basis and were typically at 2.0 ± 1 °C. Except for the periods during which the samples were removed for analysis, the samples were stored refrigerated.

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:	Analytical balance: Model OHaus Analytical Plus
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:	50 mm × 2.1 mm i.d. ACE Excel C18-AR, 2 µm particle size

3.3.2 Reagents

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

3.4 Preparation of Standard Solutions

The preparation of SYN545974 standard solutions used for this study is described below. The solutions were stored as recommended in the method when not in use (refrigerated, 0.8 to 6.9 °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 acetonitrile. An additional 3.58 mL of acetonitrile was added to make a stock standard solution of SYN545974 having a concentration of 100 µg/mL.

3.4.2 Intermediate and Fortification Standard Solutions

Fortification Standard Solutions

1.0-µg/mL:	0.1 mL of a 100-µg/mL SYN545974 stock standard solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.
0.1-µg/mL:	1.0 mL of a 1.0-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.
0.01-µg/mL:	0.1 mL of a 1.0-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile 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.

20 ng/mL:	0.2 mL of a 1.0-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
10 ng/mL:	0.1 mL of a 1.0-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
5 ng/mL:	0.05 mL of a 1.0-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
2.0 ng/mL:	0.02 mL of a 1.0-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
1.0 ng/mL:	0.01 mL of a 1.0-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.5 ng/mL:	0.05 mL of a 0.1-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.1 ng/mL:	0.01 mL of a 0.1-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.04 ng/mL:	0.04 mL of a 0.01-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).

0.02 ng/mL: 0.02 mL of a 0.01-µg/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).

3.5 Analytical Method

The analytical method independently validated in this study Syngenta Analytical Method No. GRM061.01A entitled "SYN545974 – Residue Method for the Determination of SYN545974 in Water," See Appendix 2 for the complete text of the method. The following is a summary of that method:

> A 4 mL sample was aliquoted into 20 mL scintillation vials. The sample was fortified, as necessary. A portion (1 mL) of 0.2% acetic acid in acetonitrile was added to each sample and the solution was mixed. 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 0.05 ppb for SYN545974.

The analytical method was run exactly as written. The clean-up step was not required. The analyst substituted acetic acid in the chromatography solvents, instead of using formic acid as described in the method. No negative impact on the study was detected.

Residue calculations were performed as specified in the analytical method and 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 surface and ground water 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)	Final Volume (mL)	Final Conc. (µg/L)	Replicates
Surface	20	0.01	4	LOQ	5
Ground	20	0.10	4	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 water phase.

Untreated control samples were fortified according to the following scheme:

Matrix	Sample Type	Fortifying Compound	Fortification Level (ppb)	# of Samples
Surface Water	Control	None	0.0	2
	Fortified control	SYN545974	0.05 (LOQ)	5
	Fortified control	SYN545974	0.5 (10 × LOQ)	5
Ground Water	Control	None	0.0	2
	Fortified control	SYN545974	0.05 (LOQ)	5
	Fortified control	SYN545974	0.5 (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:	50 mm × 2.1 mm i.d. ACE Excel C18-AR, 2 µm particle size
Column Oven Temp:	25°C
Injection Volume:	50µL
Run Time:	6 minutes
Retention Time:	4.5 minutes
Flow rate:	0.35 mL/minute
Mobile phase:	Component A: Burdick and Jackson High Purity Water 0.1% (v/v) acetic acid Component B: Burdick and Jackson High Purity Acetonitrile 0.1% (v/v) acetic acid

Gradient:

Time (min.)	% A	% B
0.0	70	30
1.0	70	30
3.0	10	90
5.0	10	90
5.1	70	30
6.0	70	30

Sample Temperature: 15°C

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): 35.00

Ionspray voltage: 5500.00

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

Gas 1 (GS1): 70.00

Gas 2 (GS2): 20.00

Interface heater (ihe): ON

Transitions monitored:

	Ion, *m/z*		Time, *ms*
	Q1	Q3	
SYN545974	426.4	193.2	200
	426.4	166.4	200

Acquisition mode: MRM

Declustering potential (DP): 70.00

Entrance potential (EP): 10.00

Collision energy (CE): 44 (quantitation), 39 (confirmation)

Collision cell exit potential (CXP): 4.20 (quantitation), 8.50 (confirmation

Calibration/Sample Analysis

An eight-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. The clean-up step was not required. The analyst substituted acetic acid in the chromatography solvents, instead of using formic acid as described in the method. No negative impact on the study was detected.

3.8 Statistics

Statistical methods used were limited to calculations of the mean, range, standard deviation, 1/x weighting of linear regression and relative standard deviation. Software programs, Microsoft Excel® 2007 and Applied BioSystems/MDS Sciex Analyst software (version 1.5.1), were employed to develop all regression analysis and statistical data.

4.4 Time Requirements

A single analyst completed a sample set consisting of 12 samples plus 1 reagent blank in approximately 2 hours with LC-MS/MS analysis performed overnight.

4.5 Protocol/SOP/Method Deviations

One method deviation was generated in this study. Due to analyst error, the LC-MS/MS analysis was conducted with addition of acetic acid instead of formic acid.

No SOP deviations were generated for this study.

The deviation described above is considered to have no negative impact on the integrity of this study.

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. GRM061.01A entitled "SYN545974 – Independent Laboratory Validation of Residue Method (GRM061.01A) for the Determination of SYN545974 in Water." The final extracts were found to be stable when stored refrigerated for 7 days. See Table 4. No significant matrix suppression or enhancement was observed.

The method was demonstrated to be suitable for the determination of SYN545974 in water. An LOQ of 0.05 ppb (µg/L) was demonstrated.

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) curve generated for the analytical set was used for the quantitation of SYN545974 in the samples. For this study, the correlation coefficient (r^2) for the calibration curves (quantitation and confirmation) was equal to or greater than 0.999.

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

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

$$\text{ppb} = \frac{\text{analyte conc. found (ng/mL) x Final Volume (mL) x Dil. Factor}}{\text{Sample Volume (mL)}}$$

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. 2386W-002 (Surface water, control sample). (Figure 1.1):

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

$$ppb = 0$$

2. PTRL West Sample No. 2386W-002 (Surface water, fortified control sample, replicate F1A), quantitation ion:

Fortified Control @ 0.05 ppb, (Figure 1.2):

$$\text{ng/mL} = 0.04$$

$$\text{ppb} = [0.04 \text{ ng/mL} \times 5 \text{ mL}] \div [4 \text{ mL}]$$

$$\text{ppb} = 0.05$$

$$\% \text{ Recovery} = \frac{0.05 \text{ ppb} - 0.000 \text{ ppm}}{0.05 \text{ ppb}} \times 100$$

$$= 100\%$$