2.0 INTRODUCTION

The purpose of this study was to conduct a validation of Syngenta Analytical Method GRM061.01A entitled "SYN545974 – Residue Method for the Determination of SYN545974 in Water," as written.

This study was designed to satisfy requirements described in OECD ENV/JM/MONO(2007)17[1], US EPA OCSPP 850.6100[2], 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 validation study to meet all requirements.

The method was successfully validated for SYN545974 at 0.05 µg/L (LOQ) and 0.50 µg/L (10X LOQ) in ground water (drinking water) and surface water using external solvent calibration.

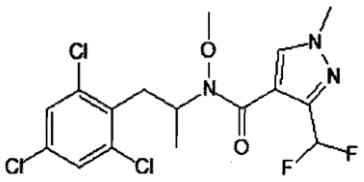
To summarize the method, a 4 mL sample was aliquoted into 20 mL scintillation vials. The sample was fortified, as necessary. Using an auto pipette, 1 mL of 0.2% acetic acid in acetonitrile was added to each sample and the solution was mixed. An aliquot of this sample was transferred to a LC vial. The sample set submitted for LC-MS/MS analysis. The limit of quantitation (LOQ) is 0.05 ppb for SYN545974.

The analytical procedure was performed as written. The clean-up step was not required.

3.0 MATERIALS AND METHODS

3.1 Test Item/Reference Substance

The test substance, SYN545974, was received on 15 December 2014 from Syngenta Crop Protection, Greensboro, North Carolina. The following information was provided:

Compound Structure	
Syngenta Code:	SYN545974
Common Name:	SYN545974
CAS Name:	1H-Pyrazole-4-carboxamide, 3-(difluoromethyl)-N-methoxy-1-methyl-N-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]
CAS Number:	1228284-64-7
Batch Number:	AMS 1432/1
Molecular Weight:	426.7
Structural Formula	$C_{16}H_{16}Cl_3F_2N_3O_2$
Storage Conditions:	< 30 °C
Purity:	99.5 %, Certificate of Analysis (Appendix 2)
Recertification Date:	End of December 2017

Upon receipt, the test substance (AMS 1432/1) was stored at room temperature in the original container. Concentrations were adjusted for the purity of the test substance.

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 (drinking) water. These matrices were chosen because they are representative of the water the method is designed for.

Refrigerator storage temperatures were monitored on a daily basis and were typically at 4.0 ± 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: Mettler Toledo XS205
HPLC:	Water Acquity I Class (UPLC)
Detector:	Sciex 5500QTRAP with Analyst™ ver. 1.5.1 Software
HPLC column:	50 mm × 2.1 mm i.d. Acquity BEH C18, 1.7 µm particle size

3.3.2 Reagents

Acetonitrile:	Optima Grade (Fisher Scientific)
Formic Acid:	Reagent Grade (Fisher Scientific)
Acetic Acid:	Glacial (Fisher Scientific)
Water (H_2O):	Optima 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, 4°C).

3.4.1 Stock Standard Solution

Ten (10) 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 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

Calibration standards 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:	1.0 mL of a 2.0 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
1.0 ng/mL:	1.0 mL of a 10 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.5 ng/mL:	1.0 mL of a 5.0 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.1 ng/mL:	1.0 mL of a 1.0 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.05 ng/mL:	1.0 mL of a 0.5 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.02 ng/mL:	2.0 mL of a 0.1 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).
0.01 ng/mL:	1.0 mL of a 0.1 ng/mL fortification solution diluted to 10 mL with acetonitrile:water (2:8, v/v).

3.5 Analytical Method

Analytical method GRM061.01A was successfully validated in this study. See Appendix 1 for the complete text of the method. The following is a summary of that method:

> A 4 mL aliquot of water sample was transferred to a 20 mL scintillation vial. The sample was fortified, as necessary. A 1 mL portion 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 autosampler vial. The LC vial was capped then submitted for LC-MS/MS analysis. The limit of quantitation (LOQ) is 0.05 ppb for SYN545974.

The analytical procedure was followed exactly as written, with the exception of performed the clean-up step. The clean-up step was omitted due to sufficient sensitivity.

Residue calculations were performed as specified in the analytical method and was conducted using Analyst software to prepare the calibration curve with 1/x weighting. The calculation worksheet can be found in Appendix 3.

3.5.1 Fortifications

Untreated control surface and ground water samples were fortified using microliter amounts of the appropriate fortification standard for 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. After fortification, the sample was mixed thoroughly before vialing.

Untreated control samples were fortified as follows:

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 UPLC- MS/MS detection. Typical conditions were as follows:

Chromatography Conditions

UPLC System	:	Waters Acquity I Class
Detector	:	Sciex 5500QTRAP with Analyst™ Software
Column	:	Waters Acquity BEH C18, 2.1 x 50 mm, 1.7 μm
Column Oven Temperature	:	25°C
Injection volume	:	50 μL
Stop Time	:	6.0 minutes
Injection protocol	:	Analyze calibration standard after 4 to 5 sample Injections
Sample Tray Temperature	:	15°C
Mobile phase	:	Solvent A: 0.1% formic acid in Optima water Solvent B: 0.1% formic acid in Acetonitrile

Mobile Phase Composition

Time (min)	%A	%B	Flow Rate (mL/min)
0.0	70	30	0.35
1.0	70	30	0.35
3.0	10	90	0.35
5.0	10	90	0.35
5.1	70	30	0.35
6.0	70	30	0.35

Mass Spectrometer Conditions

Ion Source Parameters:

Ionization Mode	Positive (+)
Curtain Gas (CUR)	5000
Collision Gas (CAD)	Medium
IonSpray Voltage (V)	5000
Temperature (TEM)	500
Ion Source Gas 1 (GS1)	70
Ion Source Gas 2 (GS2)	50
Declustering Potential (DP)	60
Entrance Potential (EP)	10
Collision Cell Exit Potential	10

Note: The mass spectrometer tuning parameters shown here are for reference only. The analyst should always consult with instrument operation manual to obtain optimum conditions for all the analytes prior to residue analysis.

MRM Operating Parameters:

SYN545974	MS/MS Transition	Time (msec.)	CE (Volts)
Quantification	425.90 → 192.90	25	40
Confirmation	425.90 → 166.10	25	30

3.7 Modifications, Interpretations, and Critical Steps

The analytical method was followed exactly as written with the exception of the clean-up step. The clean-up step was omitted due to sufficient sensitivity of the instrument.

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® and Applied BioSystems/MDS Sciex Analyst software (version 1.5.1), were employed to develop all regression analysis and statistical data.

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM061.01A is suitable for the determination of SYN545974 (Figure 1) in water. The limit of quantitation (LOQ) of the method has been established at a 0.050 µg/L (0.050 ppb).

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, US EPA guidelines EPA OCSPP 850.6100 (2012) and EC Guidance Documents SANCO/3029/99 rev 4 (2000) and SANCO/825/00 rev 8.1 (2010).

1.2 Method Summary

Environmental water samples are analyzed directly upon treatment of acidic acetonitrile using liquid chromatography tandem mass spectrometry (LC-MS/MS) technique when the instrument sensitivity allows. Alternatively, the water samples are concentrated using solid phase extraction (SPE) procedures prior to LC-MS/MS analysis. Thus, upon sample loading, SPE cartridge washing and drying, the samples are eluted with acidic methanol (0.01% formic acid; v/v) from the SPE cartridges and collected. The collected methanol fractions of are evaporated to dryness under a gentle stream of N_2 in a water bath at approximately 35°C and re-constitute with acetonitrile/H_2O (20/80; v/v; HPLC grade) then subjected to LC-MS/MS analysis. The LOQ of the method is 0.050 µg/L (ppb) for the environmental water samples.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare a 100 µg/mL stock solution for SYN545974 by one of the following methods:

Weigh out accurately, using a five figure balance, sufficient SYN545974 analytical standard into an amber "Class A" volumetric flask (100-mL). Dilute to the mark with acetonitrile and mix well to give a 100 µg/mL stock solution of SYN545974.

Alternatively, the appropriate volume of acetonitrile to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V \quad \frac{W \times P}{C} \times 1000$$

P	=	Standard purity in decimal form (P(%)/100)
V	=	Volume of methanol required
W	=	Weight, in mg, of the solid analytical standard
C	=	Desired concentration of the final solution, (µg/mL)
1000	=	Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing SYN545974 should be prepared by serial dilution in acetonitrile from the stock solution. It is recommended that the following solutions are prepared: 1.0 µg/mL, 0.10 µg/mL and 0.01 µg/mL for fortification purposes.

2.3.3 Standards for LC-MS/MS

Calibration standard solutions should be prepared by serial dilution in acetonitrile/H_2O (20/80; v/v; HPLC grade) from the stock solution or fortification solution. For example, transfer 10 mL of the 1.0 µg/mL fortification standard into a 100-mL volumetric flask and diluted with acetonitrile/H_2O (20/80; v/v; HPLC grade) solution to the mark to yield a calibration standard solution at 0.10 µg/mL concentration.

A calibration curve should be generated to quantify SYN545974 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of SYN545974 standard in acetonitrile/H_2O (20/80; v/v; HPLC grade) solution. A

minimum of five levels of calibration standards should be used for calibration plot establishment. In the method validation, following concentration levels of standards were prepared for calibration plots: 0.02 pg/µL, 0.04 pg/µL, 0.10 pg/µL, 0.20 pg/µL, 0.50 pg/µL, 1.0 pg/µL, 2.0 pg/µL, 5.0 pg/µL, 10 pg/µL and 20 pg/µL. Single point calibrations are not recommended for this method.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figure 2; indicating ms/ms transitions from *m/z* (425.9 → 192.9) and *m/z* (425.9 → 166.1).

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of six months for SYN545974 is recommended unless additional data are generated to support a longer expiration date.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S. G. Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent hazards

	Acetonitrile	Methanol	Formic Acid	Acetic Acid
Harmful Vapour	✓	✓	✓	✓
Highly Flammable	✓	✓	✗	✗
Harmful by Skin Absorption	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓
Causes severe burns	✗	✗	✓	✓
Syngenta Hazard Category (SHC)	SHC-C, S	SHC-C, S	SHC-D,S	SHC-C, S
OES Short Term (mg/m^3)	105	310	N/A	37
OES Long Term (mg/m^3)	70	260	N/A	25

N/A not known

At present there are insufficient data available to assign a Syngenta Hazard Classification for SYN545974. It should be treated as a category SHC-D compounds until further information indicates otherwise. The Syngenta Hazard Category scale rates highly toxic chemicals as

category SHC-E and non-toxic chemicals as category SHC-A. An additional hazard category of S indicates the compound is a severe skin and eye irritant.

In all cases avoid breathing vapor. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart as shown in Appendix 5. In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included in each sample set. At least one untreated control and two control samples fortified with a known amount of SYN545974 should be analyzed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired. Note that plastic containers should be avoided without presence of acetonitrile (minimum of 20% v/v is recommended), due to possible strong surface interactions are suspected.

3.1 Sample Preparation

a) If water samples are received frozen they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to subsequent aliquot for further treatment or analysis.

b) Accurately transfer 4.0 mL of the water sample (sample size may be adjusted as necessary to accommodate sensitivity requirement of instrument and injection volume) to be analyzed into a 20-mL glass scintillation vial. Sample fortification is carried out at this time, if required.

c) Accurately add 1.0 mL of acidic acetonitrile (0.2% acetic acid; v/v) to the sample containing vial from step 3.1(b) and mix well. Proceed to Section 3.2 if solid phase extraction (SPE) procedure is required.

d) Vial the acidic acetonitrile treated sample from step 3.1(c) in HPLC vial and subjected to LC-MS/MS analysis for residue determination.

3.2 Solid Phase Extraction Procedure

a) Take one Bond Elut-C18 cartridge (100 mg, 3 mL) for each sample to be analyzed and place on a suitable vacuum manifold. Add 3 mL of methanol and allow solvent to percolate through each cartridge under gravity (or draw through under low vacuum) to the level of the top frit at a rate of approximately 1 mL/min, discarding the column eluate. Do not allow the cartridges to become dry. Add ultra pure water (3 mL) to the top of each cartridge and allow solvent to percolate through under gravity (or draw through under low vacuum) to the level of the top frit at a rate of approximately 1 mL/min, discard the conditioning solution. Do not allow the cartridges to become dry.
Note: If larger volumes of water are analyzed or sample appears too dirty, 200mg, 3 mL Bond Elut-C18 cartridges have been used successfully.

b) Load water samples from Section 3.1 (c) onto the SPE cartridges (a suitable column reservoir may be used if desired) and allow sample to percolate through under gravity (or draw through under slight vacuum) to the level of the top frit at a rate of approximately 1 mL/min), to the level of the top frit. Do not allow cartridges to become dry. SYN545974 is retained on the SPE cartridges.

c) Upon completion of loading, rinse the empty sample vials with MeOH/H_2O (20/80; v/v; HPLC grade) solution (1 mL) and add the rinse solution to the column reservoir. Allow to percolate through under gravity (or draw through under low vacuum) to the level of the top frit at the same rate, again discarding the column eluate. Do not allow the cartridges to become dry. SYN545974 is retained on the SPE cartridges.

d) Repeat step (c). SYN545974 is retained on the SPE cartridges.

e) Remove the column reservoir and column connector from the SPE cartridge if used. Briefly apply a high vacuum for approximately 5 - 10 seconds to remove excess water from the cartridges and discard all the liquids in the SPE box from sample above sample loading and rinsing procedures.

f) Apply high vacuum to dry the SPE cartridges for approximately 20-30 minutes. SYN545974 is retained on the SPE cartridges.

g) Place suitable collection tubes (*e.g.* 15 mL polypropylene tubes) under each port, as required, in the manifold rack. Elute the cartridges with 3 mL (2-mL followed by 1-mL) of 0.01% formic acidic in methanol (v/v; HPLC grade) under gravity (or draw through under low vacuum) at a rate of approximately 1 mL/min to the level of the top frit collecting the column eluate. Apply gentle pressure, using a pipette bulb, on each cartridge to collect the remaining solvent from the SPE cartridges. SYN545974 is eluted in this step.

h) Evaporate the samples to dryness under a gentle stream of clean nitrogen or air in a sample concentrator with the bath temperature set to ≤ 35 ºC. This evaporation procedure should take approximately 45-60 minutes.
Note: Do not allow samples to go dry for extended amount of time at this point.

i) Reconstitute the sample with acetonitrile/H_2O (20/80; v/v; HPLC grade) solution to bring the sample to a volume of 1.0 mL and vortex for about 10 - 20 seconds to ensure the sample is completely dissolved and thoroughly mixed. If residues of greater than 5.0 ppb are expected, samples should diluted further with acetonitrile/H_2O (20/80; v/v; HPLC grade) solution as necessary.

j) Transfer an aliquot to a suitable autosampler vial and subjected to final residue determination by LC-MS/MS.

3.3 Problems and Modifications

a) The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases

however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Bottled Optima grade ultra pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

c) To prevent contamination of the instrument and/or to minimize possible carry-over issues, it is recommended that high level recoveries (≥ 10 ppb) and samples with expected residues greater than 20 ppb should be diluted so that the final analyte concentration does not exceed 10 µg/L concentrations. It may also be useful to include blank injections of HPLC reagent blanks (acetonitrile/H_2O; 20/80; v/v) after high level samples and standards to clear any observed carry-over greater than 10% of the LOQ.

3.4 Time Required for Analysis

The methodology is normally performed with a batch of 20 samples. One skilled analyst can complete the analysis of 20 samples in 1 day (8 hour working period).

3.5 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekends unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

An integrated Thermo Electron TSQ Quantum Ultra mass spectrometer was used to establish the method. The system is controlled and data is processed by Thermo Electron Xcalibur™ Software. Other instruments may also be used, however optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum instrument operation.

Following are the typical instrumental parameters applied for this method during method validation using a Thermo Electron TSQ Quantum Ultra mass spectrometer. The analyst should make necessary adjustments and tuning to these parameters to obtain optimum operational conditions based on the actual instrument used for the specific study. Alternative instrument conditions using AB Sciex API 4000 in method validation study (TK0057870) is included in Appendix 4.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; therefore no further confirmatory conditions are included.

4.1 Instrument Description

HPLC System	:	Surveyor Plus LC System – A quaternary solvent system equip with MS Pump Plus)
Autosampler	:	Surveyor MS Plus
Detector	:	Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ Software
Collision Gas	:	Purified Argon in compressed cylinder
Gas Supply	:	House Nitrogen supply

4.2 Chromatography System Conditions

Column	:	Discovery C8, 2.1 x 50 mm, 5.0 µm (Supelco Cat. no. ~~53848-U~~) 59352-U21 SDH 7/16/13 RE
Column Oven Temperature	:	25°C
Injection volume	:	50 µL
Stop Time	:	6.0 minutes
Injection protocol	:	Analyze calibration standard after 4 to 5 sample injections
Sample Tray Temperature	:	15°C
Mobile phase	:	Solvent A: 0.1% formic acid in Optima water Solvent B: 0.1% formic acid in Acetonitrile

Mobile Phase Composition

Time (min)	%A	%B	Flow Rate (mL/min)
0.0	70	30	0.35
1.0	70	30	0.35
3.0	10	90	0.35
5.0	10	90	0.35
5.1	70	30	0.35
6.0	70	30	0.35

The typical retention times for the analytes are listed in Section 4.3 when using this instrumentation and conditions. The retention time may vary depending upon chromatographic conditions and systems. The chromatographic conditions employed in this method are not designed to resolve the stereoisomers in racemic mixtures.

Note: To help minimizing instrument contamination, a timed event controlled switching valve may be used to divert the LC stream to waste during periods of no data collection.

4.3 Mass Spectrometer Conditions

Ion Source Parameters:

	Positive Mode
Spray Voltage (V)	3200
Vaporization Temperature (°C)	350
Sheath Gas Pressure (psi)	45
Ion Sweep Gas Pressure (psi)	5.0
Aux Gas Pressure (psi)	40
Capillary Temperature (°C)	300
Tube Lens Offset	50 - 110
Skimmer Offset (V)	0
Collision Pressure (mTorr)	1.0

Note: The mass spectrometer tuning parameters shown here are for reference only. The analyst should always consult with instrument operation manual to obtain optimum conditions for all the analytes prior to residue analysis.

MRM (SRM) Operating Parameters:

Analyte	MS/MS Transition*	Scan Width	Dwell (sec.)	CE (Volts)	Q1 PW	Q3 PW	RT (min.)
SYN545974 (with all ^{35}Cl isotope)	Positive mode						
Quantification	425.90 → 192.90	0.01	0.05	38	0.7	0.7	4.17
Confirmation	425.91 → 166.10	0.01	0.05	27	0.7	0.7	4.17
SYN545974** (with one ^{37}Cl isotope)	Positive mode						
Quantification	427.90 → 194.90	0.01	0.05	38	0.7	0.7	4.17
Confirmation	427.91 → 166.10	0.01	0.05	27	0.7	0.7	4.17

Data collection windows: 3.0 - 5.0 minutes

* The specified mass difference of 0.01 amu for precursor ions in quantification and confirmation detections is required for channel separation of signals on the Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ software. The MS/MS transitions listed were the most sensitive and stable transitions for the corresponding analytes based on the optimal tuning parameters obtained prior to method validation with Thermo Electron TSQ Quantum Ultra instrument. Alternative MS/MS transitions may be used if different (or comparable) instrument is applied or if interferences are encountered. Analysts should consult with instrument operation manuals for the specifics and adjustments when using instruments from different manufacturers to obtain optimum results.

** Although the isotopic species with all ^{35}Cl atoms are used for analyte quantification and confirmation, ms/ms transitions form the isotopic species containing one ^{37}Cl isotopic atom are also recorded for further confirmation purposes when needed.

Typical chromatograms with ms/ms transitions of *m/z* (425.9 → 192.9) and (425.9 → 166.1) are shown in the Figures Section. The chromatograms for one ^{37}Cl isotopic species are not used for method validation and therefore not shown.

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

Residues of SYN545974 may be calculated in ppb for each sample as follows:

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 10 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five levels).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to respective ms/ms transition. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where *y* is the instrument response value, *x* is the standard concentration, *m* is the gradient (slope) of the line of best fit ("X-variable 1" in MS Excel) and *c* is the intercept value. An example of this equation generated using the experimental values of *m* and *c* should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Calculate residues of interest in a sample, expressed as µg/L, as follows:

$$Residue\ (\mu g/L\ or\ ppb) = \frac{Analyte\ Found\ (pg)}{Water\ Sample\ Injected\ (mg\ or\ \mu L)}$$

Where on-column *Analyte Found (pg)* is calculated from the standard calibration curve and on-column *Water Sample (matrix) Injected* is calculated as follows:

$$Water\ Sample\ Injected\ (mg\ or\ \mu L) = Sample\ Volume\ (mL) \times \frac{Injection\ Volume\ (\mu L)}{Sample\ Final\ Volume\ (mL)}$$

f) Determine the recovery by first subtracting the residue found in the control sample, if any, from the residue found in the recovery sample. Calculate the recovery as a percentage (%) by the equation:

$$Recovery\ (\%) = \frac{(Residue\ in\ Recovery\ Sample) - (Residue\ in\ Control)}{Amount\ Fortified} \times 100\%$$

g) If residues need to be corrected for average percentage recovery, *e.g.* for storage stability studies, then the equation below should be used.

$$Corrected\ Residue\ (\mu g/L\ or\ ppb) = \frac{Residue\ (\mu g/L\ or\ ppb)}{Average\ Precent\ Recovery}$$

5.2 Single Point Calibration Procedure

Although single point calibration may be used to quantify residues, it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 2).

SYN545974 residues may be calculated in µg/L (ppb) for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard containing SYN545974 at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for SYN545974.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to SYN545974.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the SYN545974 residues in the sample, expressed as µg/L (ppb) using a mean standard response from each of the injections bracketing the sample as follows:

$$Residue\ (\mu g/L\ or\ ppb) = \frac{PK\ area\ (SA)}{PK\ area\ (STD)} \times \frac{Standard\ Conc.}{Sample\ Conc.}$$

PK area (SA) = Peak response for sample

PK area (STD) = Average peak response for bracketing standards

Standard Conc. = Concentration of standard (µg/mL)

Sample Conc. = Sample concentration (L/mL)

e) If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$Corrected\ Residue\ (\mu g/L\ or\ ppb) = \frac{Residue\ (\mu g/L\ or\ ppb)}{Average\ Precent\ Recovery}$$

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analyzed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of analyte), including one at the method LOQ and one at the expected residue level, should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found in the sample. The fortification levels should be appropriate to the residue levels expected in the sample.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 110% and with a relative standard deviation of ≤ 20%.

When the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

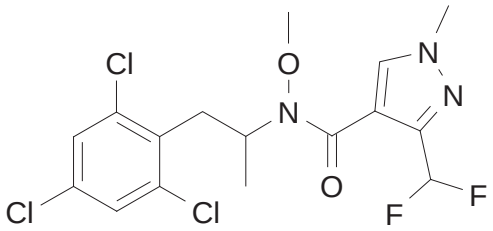
No significant matrix effects were observed in the water types tested during method validation and non-matrix standards should generally be used for quantification.

7.2 Reagent and Solvent Interference

No interference has been observed from using of high purity solvents and reagents.

FIGURE 1 Chemical Structure

Compound Code Number	:	SYN545974
Alternative Compound Code Number	:	CSCD678790
CAS Number	:	1228284-64-7
IUPAC Name	:	3-Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichloro-phenyl)-ethyl]-amide
Molecular Formula	:	$C_{16}H_{16}Cl_3F_2N_3O_2$
Molecular Weight	:	426.7
Molecular Mass	:	425.0



APPENDIX 1 Apparatus

Recommended Suppliers

Equipment	Description	Supplier
General lab glassware	General lab glassware	www.thermoscientific.com
General lab plastic-ware	General lab plastic-ware	www.thermoscientific.com
Sample processing station/Vacuum manifold	Waters Extraction Manifold	www.waters.com
Solid Phase Extraction cartridges	Bond Elut-C18; 100 mg, 3-mL	www.agilent.com
Column connectors	Suitable for various sizes of reservoirs	www.Biotage.com
Column reservoirs	Various sizes	www.Biotage.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
LC-MS/MS system Includes HPLC and autosampler units	Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ Software	www.thermoscientific.com
HPLC column	Discovery C8; 5 µm 2.1 mm i.d × 50 mm	www.sigmaaldrich.com/analytical-chromatography/hplc/columns.html

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	Optima grade	www.thermoscientific.com
Methanol	Optima grade	www.thermoscientific.com
Ultra pure water	HPLC grade	www.thermoscientific.com
Methanol	HPLC grade	www.thermoscientific.com
Glacial Acetic Acid	A.C.S. grade	www.thermoscientific.com
SYN545974 analytical standards	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) "0.2%" acetic acid in acetonitrile; prepared by mixing 2 mL of glacial acetic acid in acetonitrile with 1,000 mL of HPLC grade acetonitrile.

b) Methanol/Water (20/80; v/v; HPLC grade): prepared by mixing 200mL of HPLC grade methanol with 800 mL of HPLC grade water.

c) "0.01%" formic acid in methanol: prepared by mixing 0.10 mL of formic acid with 1,000 mL of Optima LC/MS grade methanol.

d) Acetonitrile/Water (20/80; v/v; HPLC grade): prepared by mixing 200 mL of HPLC grade acetonitrile with 800 mL of HPLC grade water.

e) "0.1%" Formic Acid in H_2O; prepared by mixing 1.0 mL of formic acid with 1,000 mL of Optima LC/MS grade water.

f) "0.1%" Formic Acid in HPLC acetonitrile; prepared by mixing 1.0 mL of formic acid with 1000 mL of HPLC grade acetonitrile.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polytyrosine-1,3,6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for SYN545974

Infuse a standard solutions of SYN545974 (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* 425.9 for SYN545974 in positive ionization mode.

Using the Xcalibur™ Software optimization routine, tune the instrument for SYN545974, ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of a SYN545974 standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position and temperature, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas pressure for maximum sensitivity.

For SYN545974, in positive ionization mode, the protonated molecular ion generated in the ion source (*m/z* 425.9) is selected and subjected to further fragmentation by collision induced fragmentation. The two most sensitive product ions (*m/z* 192.9 and *m/z* 166.1) are then selected and used for quantitative and confirmation analysis.

The fragment *m/z* = 192.9 corresponds to the $[C_7H_4{}^{35}Cl_3]$ fragment and *m/z* = 166.1 represent a stable but un-identified fragment from the product ion.

Alternatively, the isotopic species with one ^{37}Cl atom of SYN545974 can be used as product ion in positive ionization mode. Therefore, the protonated molecular ion generated in the ion source ion source (*m/z* 427.9) is selected and subjected to further fragmentation by collision induced fragmentation. The two most sensitive product ions (*m/z* 194.9 and *m/z* 166.1) are then selected and used for quantitative and confirmation analysis.

The fragment *m/z* = 194.9 corresponds to the $[C_7H_4{}^{35}Cl_2{}^{37}Cl]$ fragment and *m/z* = 166.1 represent a stable but un-identified fragment from the product ion.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

APPENDIX 4 Alternative LC-MS/MS Conditions

Following instrument and conditions were applied in a validation study (TK0057870):

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

APPENDIX 4 Alternative LC-MS/MS Conditions (Continued)

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

APPENDIX 5 Method Flow Chart