1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM061.02A is suitable for determination of SYN545974 and SYN545547 (Figure 1) in soil. The limit of quantitation (LOQ) of the method has been established at 0.5 ppb (μg/kg) for SYN545974 and SYN545547.

This method satisfies 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

A soil subsample (10 g) is extracted with 40 mL of 80/20 (v/v) acetonitrile/0.1M ammonia acetate aqueous solution follow by two additional extractions each with 30 mL of 80/20 (v/v) acetonitrile/0.1% acetic acid in deionized water. The extracts are combined and filtered through filter papers. An aliquot (10 mL) is evaporated to aqueous for removal of acetonitrile and mixed with 1 mL of 0.1% acetic acid in ultra pure water. The sample is then loaded onto a preconditioned Bond Elute-C18 SPE cartridge (100 mg, 3 mL) and rinsed with 2.5 mL of 60/40 (v/v) MeOH/0.1% acetic acid in ultra pure water. The analytes are finally eluted from the SPE cartridge with 2 mL of 60/40 (v/v) MeOH/0.1% acetic acid in ultra pure water followed by 3 mL of MeOH and evaporated again to remove MeOH. The sample is mixed with 1.5 mL of MeOH and diluted to 5 mL with 0.1% acetic acid for LC-MS/MS analysis. The LOQ of the method is 0.5 μg/kg (ppb) for each of the analytes in soil.

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 individual 100 µg/mL stock solutions for SYN545974 and SYN545547 in MeOH by one of the following methods:

Weigh out accurately, using a five figure balance, sufficient SYN545974 and SYN545547 analytical standards into two separate amber "Class A" volumetric flasks (100 mL), respectively. Dilute to the mark with MeOH and mix well to give 100 µg/mL stock solutions of SYN545974 and SYN545547.

Alternatively, the appropriate volume of methanol 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.

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

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

Combined fortification solutions of SYN545974 and SYN545547 should be prepared by mixing appropriate amounts of the two individual stock solutions and diluting with MeOH. It is recommended that the following combined solutions are prepared: 0.10 µg/mL and 0.01 µg/mL for fortification purposes.

2.3.3 Calibration Standards for LC-MS/MS

Combined calibration standard solutions should be prepared by serial dilutions of the 0.01 µg/mL fortification standard from Section 2.3.2. For example, transfer 5 mL of the 0.01µg/mL fortification standard into a volumetric flask (50 mL) and add 10 mL of MeOH and dilute to the mark with 0.1% acetic acid in ultra pure water to yield a calibration standard solution at 1 ng/mL for SYN545974 and SYN545547. Serial dilutions of 1 ng/mL standard solution with 30/70 (v/v) MeOH/0.1% acetic acid in ultra pure water will yield calibration standard solutions of lower concentrations: 0.01 ng/mL, 0.02 ng/mL, 0.05 ng/mL, 0.10 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL. In general, single point calibrations are not recommended for this method (Reference 1).

Calibration curves should be generated and used for quantitation of SYN545974 and SYN545547 residues. Standards over an appropriate concentration range should be prepared as described above and a minimum of five levels of calibration standards should be used for generation of calibration curves.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figure Section.

Note: If significant matrix effects are observed for any particular matrix, matrix match standards should be used for analysis. Matrix match standards are prepared by mixing 100 µL of appropriate levels of non-matrix match standard with 900 µL of control final extracts. For example, mix 100 µL of the 1 ng/mL non-matrix standard with 900 µL of a matrix control final extract to yield a 0.1 ng/mL matrix match standard for that particular matrix.

2.3.4 Standard Solution Storage and Expiration

Stock solution, fortification standards and calibration standards 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 and SYN545547 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 2).

Solvent and Reagent hazards

	Acetonitrile	Methanol	Formic Acid	Acetic Acid
Harmful Vapor	✓	✓	✓	✓
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 and SYN545547. 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

3.1 Precautions

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

b) To prevent contamination of the instrument and to minimize possible carry-over issues, it is recommended that high level recoveries (> 5 ppb) and samples with expected residues greater than 5 ppb (µg/kg) should be diluted so that the final analyte concentration does not exceed 1 ng/mL. It may also be useful to include blank injections of 30/70 (v/v) MeOH/0.1% acetic acid in ultra pure water after injections of high level samples to clear any observed carry-over greater than 10% of the LOQ;

c) All glassware must be rinsed with ultra pure water followed by HPLC grade MeOH, acetone or acetonitrile prior to use. Polypropylene centrifuge bottles, tubes and beakers are highly recommended to be used for this method.

3.2 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

3.3 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), recovery samples should be prepared and included with each sample set. At least one untreated (control) and two recovery samples should be analyzed concurrently with each sample set. To each pre-weighed control soil sample, add the appropriate amount of mix fortification standard solution containing SYN545974 and SYN545547 in MeOH (Section 2.3.2). For example, add 0.5mL of 0.01 µg/mL fortification standard to 10 g of control soil sample to yield a 0.5 ppb recovery sample. Let the sample stand for at least five minutes after fortification to allow the spiking solution to soak into the matrix before proceeding with the extraction procedure. The volume of fortification standard added to samples should not exceed 1 mL.

3.4 Extraction

Note: A summary of the method is briefly described in a flow-chart form in Appendix 3. The procedures are normally performed with a batch of 20 samples.

a) Weigh a representative amount of soil sample (10 g) into a Nalgene plastic centrifuge bottle (125 mL). Fortify control samples, if needed, with known amounts of SYN545974 AND SYN545547 in MeOH as described in Section 3.3;

b) Add 40 mL of 80/20 (v/v) acetonitrile/0.1 M ammonium acetate aqueous solution to the soil (or 40 mL minus the water content of the sample);

Note: Estimate the percentage water content in each matrix type and hence the total volume of water in the 10 g sub-sample. E.g. for a 10 g sub-sample with 20% natural water content add 40 mL – (10 x 20/100) mL = 38 mL of extraction solvent. It is sufficient to round the natural water content to the nearest ten percent value. Any volume contraction due to mixing organic solvents with water and evaporation loss during extraction is considered to be negligible.

Alternatively, where information is not available, it may be necessary to determine the moisture content experimentally, following a suitable moisture content determination procedure.

c) Cap the plastic centrifuge bottle and shake on a mechanical shaker for 60 minutes. Centrifuge at 3500 rpm for ~5 minutes to separate supernatant. Decant the supernatant into a clean Nalgene centrifuge bottle (125 mL);

d) Add 30 mL of 80/20 (v/v) acetonitrile/0.1% acetic acid in ultra pure water to the soil sample. Cap and shake on a mechanical shaker for 60 minutes. Centrifuge at 3500 rpm for ~5 minutes to separate supernatant. Combine the second extraction solvent with the first extraction solvent collected in the clean Nalgene centrifuge bottle (125 mL);

e) Add additional 30 mL of 80/20 (v/v) acetonitrile/0.1% acetic acid in ultra pure water to the soil sample. Cap and shake on a mechanical shaker for 60 minutes. Add the first and second extracts collected in the clean Nalgene centrifuge bottle (125 mL) back to the centrifuge bottle (125 mL) and mix well;

f) Filter the sample mixture through piggy backed filter papers (Whatman 2V inside Reeve Angel) into a clean Nalgene centrifuge bottle (125 mL) to yield the sample extract and discard the solid soil particles. Store the sample extract (~100 mL) refrigerated if the next step cannot be performed immediately. The sample concentration is 0.1 g/mL.

3.5 Sample SPE Purification Procedures

a) Transfer an aliquot (10 mL) to a polypropylene centrifuge tube and evaporate to <0.5 mL for removal of organic solvent under a gentle stream of clean nitrogen or air in a sample concentrator with the bath temperature set to ~40 ºC. This evaporation procedure should take approximately 30-40 minutes. Add 1 mL of 0.1% acetic acid in ultra pure water to the sample;

b) Place a Bond Elut-C18 cartridge (100 mg, 3 mL) on a suitable vacuum manifold and allow 3 mL of MeOH to percolate through the 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;

c) Add ultra pure water (3 mL) to the SPE cartridge top and allow 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;

d) Allow the sample from Section 3.5 (a) to percolate through the SPE cartridge under gravity (or draw through under slight vacuum) to the level of the top frit at a rate of approximately 1 mL/min and discard the column eluate. Do not allow cartridge to become dry. SYN545974 and SYN545547 are retained on the SPE cartridge;

e) Rinse the empty polypropylene centrifuge tube with 2.5mL of 60/40 (v/v) MeOH/0.1% acetic acid in ultra pure water and add the rinse solution to the SPE. 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 cartridge to become dry. SYN545974 and SYN545547 are retained on the SPE cartridge;

f) Place a clean polypropylene centrifuge tube (15 mL) under the outlet port, as required, in the manifold rack. Elute the cartridge with 2 mL 60/40 (v/v) MeOH/0.1% acetic acid in ultra pure water followed by 3 mL of MeOH under gravity (or draw through under low vacuum) at a rate of approximately 1 mL/min. Collect the SPE column eluates and let the SPE go dry. SYN545974 and SYN545547 are eluted from the SPE cartridge in this step;

3.6 Final Fraction

a) Evaporate the sample from Section 3.5(f) to about ~0.5 mL under a gentle stream of clean nitrogen or air in a sample concentrator with the bath temperature set to 40 ºC. This evaporation procedure should take approximately 10-15 minutes. Add 1.5 mL of MeOH to the sample;

b) Dilute the sample to 5 mL with 0.1% acetic acid in ultra pure water to yield sample final fraction. Vortex for about 10 - 20 seconds to ensure the sample is completely dissolved and thoroughly mixed. If residues of greater than 5 ppb are expected, samples should be diluted further with 30/70 (v/v) MeOH/0.1% acetic acid in ultra pure water as necessary.

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

3.7 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).

3.8 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

The following instrumentation and liquid chromatographic conditions are suitable for analysis of SYN545974 and SYN545547. Other instrumentation can also be used, though optimization may be required to achieve the desired separation and mass spectrometer sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

HPLC System	:	Waters Acquity UPLC® system (H Class) with Sample Manager and Column Manager
Detector	:	Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer with Analyst TM software version 1.4.2

4.2 Chromatographic Conditions for SYN545974 and SYN545547

Column	:	Agilent Varian Pursuit XRs 3 Diphenyl 100 x 4.6 mm i.d., 3.0 µm particle size or Kinetex 100 mm x 2.1 mm i.d. Phenyl-Hexyl 2.6 µm particle size
Column Oven Temperature	:	40°C
Injection volume	:	10 -20 µL
Stop Time	:	10.5 minutes
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1: 0.1% acetic acid in ultra pure water Solvent 2: 0.1% acetic acid in MeOH

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	70	30	0.5
5.0	0	100	0.5
7.5	0	100	0.5
7.6	70	30	0.5
10.5	70	30	0.5

Note: Under these conditions the retention times are 5.0 minutes for SYN545547 and 5.4 minutes for SYN545974.

4.3 API 4000 Mass Spectrometer Conditions for SYN545974 and SYN545547

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 25 (arbitrary units)
Temperature (TEM)	:	250°C
Ionspray voltage	:	5000
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)
Gas 1 (GS1)	:	Air set at 50 (arbitrary units)
Gas 2 (GS2)	:	Air set at 60 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		SYN545974 primary transition	SYN545974 confirmatory transition	SYN545547 primary transition	SYN545547 confirmatory transition
Q1 *m/z*	:	426.0	426.0	396.1	396.1
Q3 *m/z*	:	193.2	166.2	376.1	136.3
Dwell time	:	100 ms	100 ms	100 ms	100 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	81 V	81 V	90 V	90 V
Entrance potential (EP)	:	10 V	10 V	10 V	10 V
Collision energy (CE)	:	43 V	40 V	24 V	40 V
Collision cell exit potential (CXP)	:	15 V	12 V	20 V	11 V

Note: The MS/MS parameters can be directly input into an API 4000 instrument to create a MS/MS method for quantitation or if necessary optimization of an instrument can be conducted by infusing standard solutions of SYN545974 and SYN545547 (0.1 µg/mL) in mobile phase directly into the mass spectrometer interface at a rate at of approximately 10-20 µL/min (Appendix 4).

Typical LC-MS/MS chromatograms from analysis of soil samples are shown in Figure Section.

4.4 Confirmatory Procedures for SYN545974 and SYN545547

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

5.0 CALCULATION OF RESULTS

5.1 Multi-Point Calibration Procedure

The weight/weight concentration of SYN545974 and SYN545547 residues in an unknown sample may be calculated in ppb (µg/kg) as follows:

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

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to SYN545974 and SYN545547. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions;

c) Generate calibration curve and 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 injected on column (pg), m is the gradient 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 the weight/weight concentration in the sample, expressed as ppb (µg/kg), as follows:

$$\text{Analyte (ppb)} = \frac{\text{Analyte Injected on Column (pg)}}{\text{Matrix Injected on Column (mg)}}$$

Where analyte injected on column (pg) is calculated from the standard calibration curve and sample matrix injected on column is calculated as follows:

$$\text{Matrix Injected (mg)} = \left(\frac{\text{Sample Wt (g)}}{\text{Extract Vol} * \text{(mL)}} \right) \times \left(\frac{\text{Aliquot Vol (mL) x Injection Vol (µµL}}{\text{Final Volume (mL)}} \right)$$

$$\text{Extract Volume*} = \text{Extraction Solvent Volume (mL)} + \text{Sample Weight (g)} \times \text{Moisture(\%)}$$

Note: The moisture (%) can be determined experimentally from water content determination of the matrix.

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

$$\text{Recovery Factor} = \frac{\text{ppb Found in Recovery Sample} - \text{ppb Found in Control}}{\text{ppb Fortified}}$$

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

$$\text{Corrected Analyte (ppb)} = \frac{\text{Analyte (ppb)} \times 100}{\text{Average Percentage Recovery}}$$

5.2 Single Point Calibration Procedure

SYN545974 and SYN545547 may be calculated in ppb (µg/kg) 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 and SYN545547 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 and SYN545547.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to SYN545974 and SYN545547.

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

d) Calculate SYN545974 and SYN545547 in the sample, expressed as ppb (µg/kg) using a mean standard response from each of the injections bracketing the sample as follows.

$$\text{Residue (ppb)} = \frac{\text{Peak area (SA)}}{\text{Peak area (STD)}} \times \frac{\text{Standard amount (pg) injected on clumn}}{\text{Matrix injected (mg) on column}}$$

Peak area (SA) = Peak area response for unknown sample
Peak area (STD) = Average peak response for bracketing standards

Note: Although single point calibration may be used for quantitation it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 1).

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 $\leq$ 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.

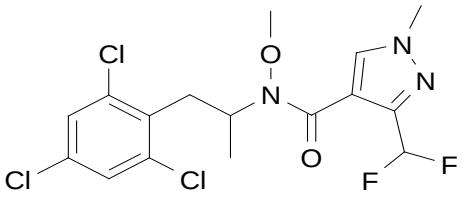
7.2 Reagent and Solvent Interference

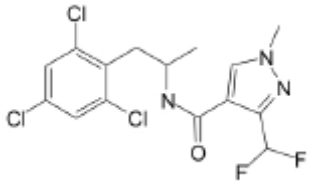
No interference has been observed from using of high purity solvents and reagents.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

FIGURE 1 Chemical Structure

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

Compound	
Common Name:	None
Code Name:	SYN545547
IUPAC Name:	N1H-pyrazole-4-carboxamide, 3-(difluoromethyl)-1-methyl-N-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-
CAS Number:	NA
Molecular Formula:	$C_{15}H_{14}Cl_3F_2N_3O$
Molecular Weight:	396.7

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

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
Formic 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) 80/20 (v/v) acetonitrile/0.1M ammonium acetate aqueous solution, prepared by mixing 800 mL of acetonitrile with 200 mL of 0.1M ammonium acetate aqueous solution;

b) 80/20 (v/v) acetonitrile/0.1% acetic acid in ultra pure water, prepared by mixing 800 mL of acetonitrile with 200 mL of 0.1% acetic acid in ultra pure water;

c) 30/70 (v/v) MeOH/0.1% acetic acid in ultra pure water, prepared by mixing 800 mL of acetonitrile with 200 mL of 0.1% acetic acid in ultra pure water;

d) 0.01% formic acid in methanol, prepared by mixing 0.1 mL of formic acid with 1000 mL of MeOH;

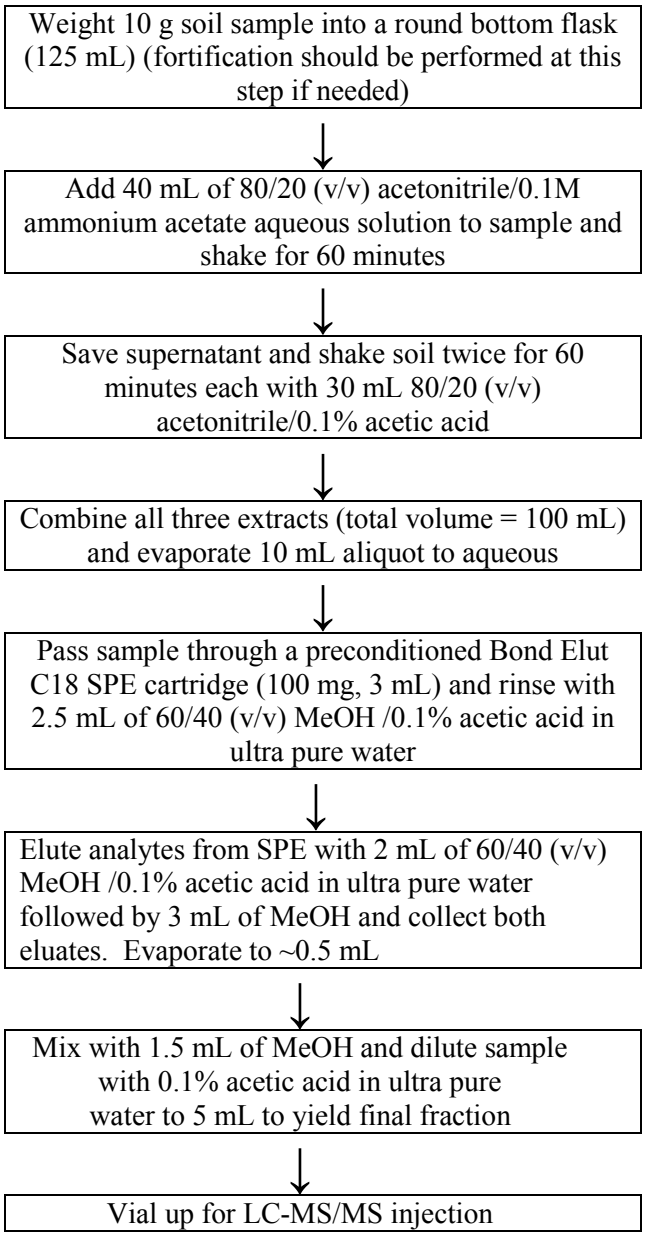
e) 20/80 (v/v) MeOH/ultra pure water, prepared by mixing 200 mL of MeOH with 800 mL of ultra pure water;

f) 0.1% acetic acid in ultra pure water, prepared by mixing 1.0 mL of acetic acid with 999 mL of ultra pure water;

g) 0.1% acetic acid in MeOH, prepared by mixing 1.0 mL of acetic acid with 999 mL of MeOH;

h) 0.1M ammonium acetate aqueous solution, prepared by dissolving 7.7 grams of ammonium acetate in 1000 mL of ultra pure water.

APPENDIX 3 METHOD FLOW-CHART



APPENDIX 4 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polypropylene glycol (PPG) 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 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* 426.0 for SYN545974 in positive ionization mode.

Using the Analyst software quantitative optimization routine, tune the instrument for SYN545974, ensuring that the correct ions are selected (*m/z* 426.0 → *m/z* 193.2 and *m/z* 426.0 → *m/z* 166.2). 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, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.

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

Tuning Instrument for SYN545547

Infuse a standard solutions of SYN545547 (0.1 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* 396.1 for SYN545974 in positive ionization mode.

Using the Analyst software quantitative optimization routine, tune the instrument for SYN545547, ensuring that the correct ions are selected (*m/z* 396.1 → *m/z* 376.1 and *m/z* 396.1 → *m/z* 136.3). 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 SYN545547 standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.