

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM042.01A is suitable for the determination of SYN545192 (Figure 1) in water. The limit of quantification (LOQ) of the method has been established at 0.05 µg/L (or 0.05 ppb).

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, US EPA guidelines EPA OPPTS 860.1340 and EPA OPPTS 850.7100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 7.

1.2 Method Summary

Environmental water samples are concentrated using solid phase extraction (SPE). After elution with acetonitrile, samples are diluted with ultra pure water and analysed by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS). The limit of quantification of the method is 0.05 µg/L (0.05 ppb).

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 2. 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 3.

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 200 µg/mL stock solution for SYN545192 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient SYN545192 analytical standard into an amber “Class A” volumetric flask (50 mL). Dilute to the mark with acetonitrile to give a 200 µg/mL stock solution of SYN545192.

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.

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

- P = Standard purity in decimal form (P%/100)
V = Volume of acetonitrile 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 SYN545192 should be prepared by serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant matrix effects, suppression or enhancement of the instrument response for SYN545192 has been observed in the water types tested using the procedures described in Section 3 during method development and non-matrix calibration standards should normally be used for quantification.

To prepare e.g., a 0.5 ng/mL standard, transfer acetonitrile/ultra pure water 50/50 v/v (8 mL) into a 10 mL volumetric flask and add 50 µL of a 0.1 µg/mL SYN545192 standard in acetonitrile. Adjust to the 10 mL mark with acetonitrile/ultra pure water 50/50 v/v. Stopper the flask securely and shake to mix thoroughly. Transfer an aliquot of the standard into a suitable autosampler vial ready for analysis by LC-MS/MS.

A calibration curve may also be generated to quantify SYN545192 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of SYN545192 standard in acetonitrile.

Any matrix effects observed may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with acetonitrile/ultra pure water 50/50 v/v should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer 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 SYN545192 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	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-C, S
OES Short Term (mg/m ³)	105	310	37
OES Long Term (mg/m ³)	70	260	25

N/A not known

At present there are insufficient data available to assign a Syngenta Hazard Classification to SYN545192. It should be treated as category a SHC-D compound 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 vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

- If water samples are received deep frozen they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to analysis.

- b) Transfer 10 mL of the water sample to be analysed into a polypropylene centrifuge tube (15 mL size). Sample fortification, if required, is to be carried out at this time.

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-measured control water sample, add the appropriate amount of standard solution containing SYN545192 in acetonitrile. At least one untreated control and two control samples fortified with a known amount of SYN545192 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

- c) Add acetonitrile (2 mL). Cap the tubes securely and shake gently to mix. .

3.2 Solid Phase Extraction Procedure.

- a) Take one Waters Oasis™ HLB cartridge (60 mg, 3 mL) for each sample to be analysed and place on a suitable vacuum manifold (e.g. IST Vacmaster). Add methanol (2 mL) and allow to percolate through each cartridge under gravity or draw through under 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 (2 mL) to the top of each cartridge and allow to percolate through under gravity or draw through under 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.
- b) Load water samples from Section 3.1 (c) onto the SPE cartridges (a suitable column reservoir may be used if desired) and allow to percolate through under gravity or under low vacuum, at a rate of approximately 1 - 2 mL/min, to the level of the top frit. Do not allow cartridges to become dry. SYN545192 is retained on the SPE cartridges.
- c) On completion of loading, wash the empty sample tubes with ultra pure water (2 mL) and add the rinse to the column reservoir. Allow to percolate through under gravity or draw through under 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.
- d) 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 but do not dry for extended periods.
- e) Place suitable collection tubes (e.g. graduated, plastic disposable centrifuge tubes) under each port, as required, in the manifold rack. Elute the cartridges with acetonitrile (2 mL), under gravity or draw through under low vacuum at a rate of approximately 1 - 2 mL/min to the level of the top frit collecting the column

eluate. Apply high vacuum for approximately 5 seconds to collect the excess solvent from the SPE cartridges. SYN545192 is eluted in this step.

- f) Dilute the samples to 4 mL with ultra pure water and transfer and aliquot to a suitable autosampler vial for analysis by LC-MS/MS. The final sample concentration is 2.5 mL/L (or 0.0025 L/mL)
- g) Alternatively, where there is insufficient instrument sensitivity to achieve the desired LOQ, evaporate the samples to dryness under a stream of clean, dry air in a sample concentrator with the temperature set to 45 °C. This should take approximately 20 minutes. Although there is no information to suggest that losses occur if samples are left at dryness for extended periods it is recommended that samples are removed soon after the solvent has evaporated.
- h) Add acetonitrile (0.5 mL) and ultrasonicate thoroughly. Add ultra pure water (0.5 mL) and again ultrasonicate thoroughly to ensure the sample is completely dissolved and thoroughly mixed.
- i) Transfer an aliquot to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 10 mL/mL (or 0.01 L/mL).

3.3 Experimental Precautions

- 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 HPLC 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 to minimise possible carry-over issues, it is recommended that high level recoveries (>0.1 mg/kg) and samples with expected residues greater than 0.1 mg/kg should be diluted so that the final analyte concentration does not exceed 0.005 µg/mL. It may also be useful to include blank injections of acetonitrile/ultra pure water (50/50 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.
- d) SYN545192 has been observed to adsorb to glass surfaces over a period of time in solutions with < 50% organic solvent. Storing samples containing SYN545192 in 100% aqueous solutions must be avoided.

3.4 Time Required for Analysis

The methodology is normally performed with a batch of 20 samples. One person 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 weekend breaks 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 conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation 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 use. The method has been developed for use on an Applied Biosystems API4000.

4.1 Instrument Description

HPLC system	: Perkin Elmer 200 series pumps, degasser, column oven and autosampler.
Detector	: Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.1
Gas Supply	: Peak Scientific NM20ZA gas station

4.2 Chromatography Conditions

Column	: ZORBAX SB-AQ column 50 mm x 4.6 mm i.d., 3.5 µm particle size
Column Oven Temperature	: 40°C
Injection volume	: 25 µL
Stop Time	: 5.5 minutes
Injection protocol	: Analyse calibration standard after 3 to 4 sample injections
Mobile phase	: Solvent 1 = acetonitrile Solvent 2 = 0.2% acetic acid in ultra pure water

Mobile Phase Composition

Time (min)	% Solvent 1	% Solvent 2	Flow, mL/min
0.00	20	80	1.0
3.00	90	10	1.0
5.00	90	10	1.0
5.1	20	80	1.0
5.5	20	80	1.0

Under these conditions the retention SYN545192 is 2.7 minutes.

Notes : The column eluate is diverted to waste for the first 1 minute to prevent ionic material from the sample contaminating the mass spectrometer front plate. A secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste has been found to be unnecessary. Under these conditions the retention time of the analytes is tabulated below

4.3 Mass Spectrometer Conditions

Interface : TurboIonSpray
Polarity : Negative
Curtain gas (CUR) : Nitrogen set at 40 (arbitrary units)
Temperature (TEM) : 450 °C
Ionspray voltage : -3500 V
Collision gas setting (CAD) : Nitrogen set at 8 (arbitrary units)
Gas 1 (GS1) : Air set at 50 (arbitrary units)
Gas 2 (GS2) : Air set at 50 (arbitrary units)
Interface heater (ihe) : On
Scan type : MRM

MRM Conditions		SYN545192 primary transition	SYN545192 confirmatory transition
Q1 <i>m/z</i>	:	396	396
Q3 <i>m/z</i>	:	368	91
Dwell time	:	150 ms	150 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	-75 V	-75 V
Entrance potential (EP)	:	-10 V	-10 V
Collision energy (CE)	:	-22 V	-60 V
Collision cell exit potential (CXP)	:	-11 V	-5 V

Typical chromatograms are shown in the Figures Section.

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

SYN545192 residues may be calculated in µg/L 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).
- b) Make an injection of each sample solution and measure the areas of the peaks corresponding to SYN545192. 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 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 SYN545192 residues in the sample, expressed as $\mu\text{g/L}$, as follows

$$\text{Residue } (\mu\text{g/L}) = \frac{\text{Analyte found } (\mu\text{g/mL})}{\text{Sample conc. (L/mL)}}$$

Where analyte found ($\mu\text{g/mL}$) is calculated from the standard calibration curve and sample conc. is the final sample concentration in L/mL.

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

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} (\mu\text{g/L})$$

5.2 Single Point Calibration Procedure

SYN545192 residues may be calculated in $\mu\text{g/L}$ 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 SYN545192 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 SYN545192.
- b) Make an injection of each sample solution and measure the areas of the peaks corresponding to SYN545192.
- c) Re-inject the standard solution after a maximum of four injections of sample solutions.
- d) Calculate the SYN545192 residues in the sample, expressed as $\mu\text{g/L}$ using a mean standard response from each of the injections bracketing the sample as follows.

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

Peak response for sample

Average peak response for bracketing standards

Concentration of standard ($\mu\text{g/mL}$)

Sample concentration (L/mL)

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

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} (\mu\text{g/L})$$

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).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analysed 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 analysed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of SYN545192 in acetonitrile) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

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

Where 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

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

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.

8.0 METHOD VALIDATION

8.1 Recovery Data and Repeatability

Method validation has been carried out on the procedures described in Sections 3 and 4. The method validation data are reported in Syngenta report number TK0002507 (Reference 3) and a summary is included in Tables 2 and 3.

8.2 Limit of Quantification (LOQ)

The limit of quantification of the method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and a mean recovery of 70-110% with a relative standard deviation of $\leq 20\%$ has been obtained. Generally, for accurate quantification, the response for an analyte peak should be no lower than four times the mean amplitude of the background noise in an untreated sample at the corresponding retention time.

The limit of quantification has been set at 0.05 $\mu\text{g/L}$ (0.05 ppb).

8.3 Limit of Detection (LOD)

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as three times background noise. Note that the LOD may vary between runs and from instrument to instrument.

8.4 Matrix Effects

No significant matrix effects were observed in the water types tested during method validation and non-matrix standards should generally be used for quantification. A summary of the matrix effects is included in Table 4.

8.5 Detector Linearity

For accurate quantification of residue concentrations, analyses should be carried out within the linear range of the detector. For multi point calibration, detector range and linearity will be demonstrated within each sample set.

The linearity of the LC-MS/MS detector response for SYN545192 was tested in the range from 1.25 ng to 50 ng injected on column (equivalent to 0.05 ng/mL to 2 ng/mL standards when using a 25 µL injection volume) and was found to be linear.

If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantification.

Standards of 6 different concentration levels were injected and the response plotted against the standard concentration, using Microsoft Excel for both primary and confirmatory transitions for SYN545192.

Detector linearity graphs are presented in Figures 24 - 25.

8.6 Extract Stability

Final water samples in acetonitrile/ultra pure water 50/50 v/v retained in vials and stored at a temperature of approximately 4°C were suitable for SYN545192 residue analysis, for storage periods of up to 7 days. A summary of the stability data is presented in Table 5.

9.0 LIMITATIONS

The method has been tested on representative water types. It can reasonably be assumed that the method can be applied to other water matrices not tested in this method provided successful recovery tests at the relevant levels validate the suitability of the method.

10.0 CONCLUSIONS

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of SYN545192 residues in water. Only commercially available laboratory equipment and reagents are required. The analysis of 20 water samples for SYN545192 can be completed by one person in 1 day (8 working hour period). Untreated and fortified samples should be analysed with each set of samples to demonstrate absence of any interference and adequate recovery, if possible. The limit of quantification of the method is 0.05 µg/L (0.05 ppb).

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, US EPA guidelines EPA OPPTS 860.1340 and EPA OPPTS 850.7100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 7.

11.0 REFERENCES

1. Luxon S G (1992): Hazards in the Chemical Laboratory 5th Edition. The Royal Society of Chemistry. Thomas Graham House, The Science Park, Cambridge CB4 4WF, UK. ISBN 0-85186-229-2.
2. Cardone M J, Palermo P J and Sybrand L B: Potential error in single point ratio calculations based on linear calibration curves with a significant intercept. Anal Chem., 52 pp 1187-1191, 1980.
3. Mayer L (2010): Validation of SYN545192 - Residue Method GRM042.01A for the Determination of SYN545192 in Water. Syngenta report number TK0002507.

Determination of LC-MS/MS Matrix Effects

The effect of different water types on the LC-MS/MS signal was assessed by preparing standards in the presence of water matrix and comparing the peak areas of SYN545192 against non-matrix standards at an equivalent concentration.

Table 4 : LC-MS/MS Matrix Effects

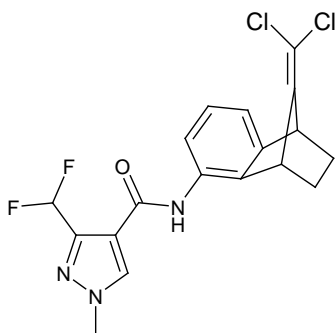
Analyte	Matrix Effect		
	Drinking water	Surface Water	Groundwater
SYN545192 $m/z = 396 \rightarrow 368$	-16% suppression	-4% suppression	1% enhancement
SYN545192 $m/z = 396 \rightarrow 91$	-19% suppression	-3% suppression	-2% suppression

The magnitude of the matrix effects were considered not to be significant for the water types tested during method validation and non-matrix standards were used for calibration. Matrix matched standards may be used to compensate for any significant effects, at the discretion of the study director.

CHEMICAL STRUCTURES

Figure 1

Compound Code Number	: SYN545192
Alternative compound code number	: CSCD064398
CAS Number	: Not in registry
IUPAC Name	: <i>N</i> -[(1 <i>R</i> S,4 <i>S</i> R)-9-(Dichloromethylidene)-1,2,3,4-tetrahydro-1,4-methanonaphthalen-5-yl]-3-(difluoromethyl)-1-methyl-1 <i>H</i> -pyrazole-4-carboxamide
Molecular Formula	: C ₁₈ H ₁₅ Cl ₂ F ₂ N ₃ O
Molecular Weight	: 398



APPENDIX 1 APPARATUS

Recommended Suppliers

Equipment	Description	Supplier
General glassware	General glassware	www.thermofisher.com/global/en/home.asp
Plastic centrifuge tubes	15 mL HDPE	www.thermofisher.com/global/en/home.asp
Sample processing station/Vacuum manifold	Isolute [®] Vacmaster-20 [®] sample processing station	www.Biotage.com
Solid Phase Extraction cartridges	Oasis HLB, 60 mg, 3 mL size	www.waters.com
Column connectors	Suitable for 30 mL reservoirs	www.Biotage.com
Column reservoirs	30 mL size	www.Biotage.com
Autosampler vials	Crimp cap, 2 mL size	www.Agilent.com
LC-MS/MS system	API 4000 equipped with a TurboIonSpray source	www.AppliedBiosystems.com
HPLC system	Perkin 200 series HPLC system equipped with binary pump, vacuum degasser, column compartment with column switching valve and autosampler	www.Agilent.co.uk
HPLC column	Zorbax SB-AQ 50 x 4.6 mm, 3.5 µm	www.Agilent.co.uk
Nitrogen generator	Peak Scientific NM20ZA gas station	www.peakscientific.com

APPENDIX 2 REAGENTS

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	HPLC grade	www.thermofisher.com/global/en/home.asp
Acetonitrile	HPLC grade	www.thermofisher.com/global/en/home.asp
Methanol	HPLC grade	www.thermofisher.com/global/en/home.asp
Acetic acid	Analytical grade	www.sigmaaldrich.com
SYN545192 analytical standards	GLP certified	GLP Testing Facility, Syngenta, CH-4333, Munchweilen, Switzerland or Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

- a) Acetonitrile/ultra pure water 50/50 v/v:
Measure 500 mL acetonitrile into a 1 L volumetric flask. Add 500 mL ultra pure water. Stopper flask securely and shake to mix thoroughly.
- b) Acetic acid in ultra pure water 0.2% v/v.
Add concentrated acetic acid (2 mL) to ultra pure water in a 1L volumetric flask. Adjust volume to the 1 L mark with ultra pure water. Stopper flask securely and shake to mix thoroughly.

APPENDIX 3 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 SYN545192

Note: The analyte for this method has 2 Chlorine atoms. Chlorine has two isotopic forms ^{35}Cl and ^{37}Cl and therefore the mass spectrum shows ions at M ($^{35}\text{Cl} \times 2$), $M+2$ ($^{35}\text{Cl} + ^{37}\text{Cl}$) and $M+4$ ($^{37}\text{Cl} \times 2$). For SYN545192 on the instruments tested, the most abundant molecular ion and daughter ions were for M (i.e. $^{35}\text{Cl} \times 2$).

Infuse a standard solutions of SYN545192 (0.1 to 1.0 $\mu\text{g/mL}$) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 $\mu\text{L/min}$. Roughly adjust interface parameters (sprayer position, 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 for SYN545192 in negative ionisation mode.

Using the Analyst software quantitative optimisation routine, tune the instrument for SYN545192, 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 SYN545192 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.

For SYN545192, in negative ionisation mode, the deprotonated molecular ion generated in the ion source (m/z 396) is selected and subjected to further fragmentation by collisional activation. The two most sensitive daughter ions (m/z 368 and m/z 91) are then selected and used for quantitative analysis.

The fragment $m/z = 368$ corresponds to loss of $\text{H}_2\text{C}=\text{CH}_2$ from the deprotonated molecular ion and $m/z = 91$ corresponds to the deprotonated 1,3 dimethyl pyrazole fragment.

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

APPENDIX 4 METHOD FLOW CHART

