2.0 INTRODUCTION

This study was conducted to independently validate the analytical method GRM042.02A (Ref. 1) for the determination of residues of SYN545192 in soil matrix at the LOQ of 0.001 mg/kg, using commercially available instrumentation. The sections of this method necessary to its implementation were translated and referenced at Eurofins|ADME BIOANALYSES under the number AGR/MOA/SYN545192-1.

This study was conducted in accordance with European guidelines SANCO/825/00 Rev. 8.1 and SANCO/3029/99 Rev.4,US EPA guideline OPPTS 860.1340, OPPTS 850.7100 and OECD guidance document ENV/JM/MONO (2007) 17.

Specifically:

a) To establish that the method will produce recovery values which are within an acceptable range (i.e. mean recoveries between 70% and 120%, with a relative standard deviation within a run ≤ 20%), for each fortification level and overall. To establish the 95% confidence intervals.

b) To establish that the limit of quantification (LOQ) of the analytical method is 0.001 mg/kg for SYN545192.

c) To establish that residue levels of SYN545192 in the control samples are not present at levels above 30% of the LOQ.

d) To investigate the relationship between instrument response and analyte concentration for SYN545192 over concentration ranges typical of those for which the method will be used.

e) To assess suppression or enhancement of instrument response to SYN545192 in the presence of soil matrix.

3.0 MATERIALS AND METHODS

3.1 Reference Item

Code Number	SYN545192
Chemical name (IUPAC)	3-difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid (9-dichloromethen-1,2,3,4-tetrahydro-1,4-methano-naphthalen-5-yl)-amide
Molecular formula	$C_{18}H_{15}Cl_2F_2N_3O$
Molecular mass	398.2 g/mol

The test/reference item used for this validation study was the following:

Reference Item	Batch	Purity (%)	Valid until:	Storage Conditions
SYN545192	AMS 1295/2	99.4	31 Oct 2014	20 ± 4 °C

The certificate of analysis has been provided by the sponsor. The remaining test / reference item will be stored at Eurofins|ADME BIOANALYSES as long as its quality can be maintained. The structure is shown in Figure 1.

3.2 Test System

The validation study was carried out using control sample L10-02312-01-005A from study S10-02312 (a soil dissipation study being performed on SYN545192). This control sample (0-30 cm depth) was supplied by the sponsor. Details of the soil characterisation (performed as part of study S10-02312) are given in Table 1.

3.3 Preparation and Stability of Analytical Standard Solutions

A 200 µg/mL stock solution of SYN545192 was prepared in acetonitrile. This solution was stable 6 months (Ref.1) when stored between 0 and 9°C and protected from light.

Fortification solutions of SYN545192 at 1 µg/mL and 0.1 µg/mL were prepared in acetonitrile from the primary stock solution.

Calibration standards for analytical determination by LC-MS/MS were prepared from the fortification solutions over an appropriate range in acetonitrile then were ten-fold diluted in acetonitrile/ultra pure water (50/50, v/v) to achieve the target concentration range.

The fortification and the calibration standards solutions were freshly prepared.

3.4 Fortification Levels

Recovery of SYN545192 through the analytical procedure was assessed by fortifying five aliquots of control soil with SYN545192 at the LOQ (0.001 mg/kg) and five aliquots with SYN545192 at 10 times the LOQ (0.01 mg/kg). In addition, two control samples and one reagent blank were analysed with each sample batch. Fortification levels are summarised in Table 2.

3.5 Analytical Procedures

3.5.1 Limit of Quantification (LOQ)

The LOQ of the method is defined as the lowest analyte concentration in a sample at which the methodology has been successfully validated. A LOQ of 0.001 mg/kg was confirmed for SYN545192 in soil.

3.5.2 Sample Analysis

Samples were analysed according to the procedures described in analytical method GRM042.02A. The method is detailed in Appendix 1.

10 g sub samples of soil were extracted by reflux in 80/20 v/v acetonitrile/ultra pure water**.** After cooling to room temperature, extracts were decanted and centrifuged. Aliquots of the soil extracts were diluted in ultra pure water and purified by a solid phase extraction procedure. Samples at 0.01 mg/kg were 10-fold diluted in acetonitrile/ultra pure water (50/50, v/v). Final determination was by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

The limit of quantification of the method is 0.001 mg/kg (0.001 ppm, 1 ppb) for SYN545192.

The percentage recovery obtained for each sample was calculated and these results were used to assess the relative standard deviation and limit of quantification of the analytical method (see Tables 3 and 4).

3.5.3 Detector Linearity

Standard solutions containing SYN545192 at concentrations ranging from 0.02 to 1.0 ng/mL (equivalent to 1 to 50 pg of SYN545192 injected on to the column, based on a 50 µL injection) were analysed by LC-MS/MS, using the conditions specified in the analytical method. The detector response for LC-MS/MS was plotted against standard concentration injected. The lowest concentration injected was equivalent to 16% of the LOQ of the method. The highest concentration injected was equivalent to 8×LOQ (see Table 5).

3.5.4 Matrix Effects

Each sample set included an appropriate matrix-matched standard, prepared in soil matrix. The response obtained from the matrix-matched standard was compared against the response obtained from the standard in acetonitrile to allow calculation of any matrix effect (either suppression or enhancement of response). The results are presented in Table 6.

TABLE 6: Determination of LC-MS/MS Matrix Effects

The effect of soil matrix on the LC-MS/MS response was assessed by preparing standards in the presence of soil matrix and comparing the peak areas of SYN545192 against non-matrix standards at an equivalent concentration in acetonitrile/ultra pure water (50/50, v/v).

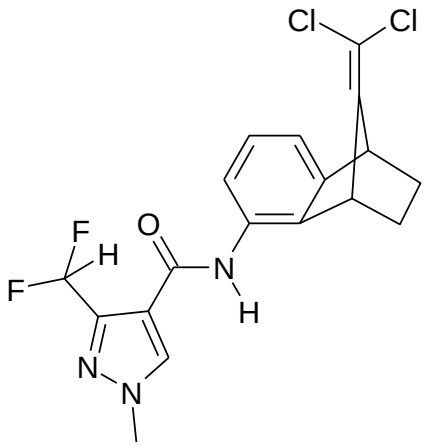
The matrix effect was calculated as follows:

$$Matrix\ Effect\ (\%) \quad \frac{Mean\ Peak\ Area\ of\ Analyte\ in\ Matrix \quad Mean\ Peak\ Area\ of\ Analyte\ in\ Solvent}{Mean\ Peak\ Area\ of\ Analyte\ in\ Solvent} \quad 100$$

No significant matrix effect were observed for SYN545192 in soil tested. Non matrix-matched standards were used for calibration and quantification.

Matrix	**% Matrix Effect for SYN545192**	
	m/z 396/368	*m/z 396/91*
Soil	-3.6	-4.9

FIGURE 1: Structure of SYN545192.



APPENDIX 1 Analytical Method Description

1. PREPARATION AND USE OF THE STANDARD SOLUTIONS

The stock solutions must be stored in a refrigerator when not in use. The stock solutions used for this study were stored between 0 and 9°C and are stable for 6 months protected from light.

1.1. Stock solution

- Between 2 and 50 mg of SYN545192 was accurately weighed into a volumetric flask.
- Adequate volume of acetonitrile for SYN545192 was added in order to obtain stock solutions at 200 µg/mL, taking into account the chemical purity. These solutions were sonicated until total dissolution.

1.2. Fortification solutions

Appropriate serial dilutions of SYN545192 primary stock solutions were performed in acetonitrile to obtain solutions at 1 µg/mL and 0.1 µg/mL.

1.3. Calibration solutions

Appropriate serial dilutions of the fortification solutions were performed in acetonitrile, at the following concentrations.

0.0002 – 0.0005 – 0.001 – 0.002 – 0.005 and 0.01 µg/mL

Calibration standards were prepared by ten-fold dilution of the above standards in acetonitrile/ultra pure water (50/50, v/v). The following calibration solutions were obtained:

0.02 – 0.05 – 0.1 – 0.2 – 0.5 and 1 ng/mL

APPENDIX 1 Analytical Method Description (Continued)

2. ANALYTICAL SUPPLIES AND APPARATUS

According to availability and laboratory equipment, analytical supplies from other suppliers and apparatus of different design may be used.

2.1. Apparatus and material

- LC-MS/MS: API 4000 (Sciex)
- Pump+autosampler LC20AD+SIL20AC (Shimatzu) + HTC Pal (CTC Analytics)
- Column oven CTO-20AC (Shimadzu)
- HPLC column: SB AQ 50 x 4.6 mm - 3.5µm
- Cartridge: Oasis HLB (60 mg/3 mL)
- Centrifuge
- Analytical balances (Mettler, Sartorius)
- Ultrasonic bath
- Reflux
- Vortex
- General glassware
- Various pipettes

2.2. Reagents

- Acetonitrile
- Acetic acid
- Formic acid
- Methanol
- Ultra pure water

APPENDIX 1 Analytical Method Description (Continued)

2.3. Preparation of Reagents

- Acetonitrile / ultra pure water (80/20, v/v)
  Mix 800 mL of acetonitrile with 200 mL of ultra pure water. Stopper flask securely and mix thoroughly by shaking.

- Acetonitrile / ultra pure water (50/50, v/v)
  Mix 500 mL of acetonitrile with 500 mL of ultra pure water. Stopper flask securely and mix thoroughly by shaking.

- Acetonitrile / ultra pure water (10/90, v/v)
  Mix 100 mL of acetonitrile with 900 mL of ultra pure water. Stopper flask securely and mix thoroughly by shaking.

APPENDIX 1 Analytical Method Description (Continued)

3. ANALYTICAL PROCEDURE

a) Weigh a representative amount of soil (10 g) into a round bottom flask (100 mL size). Fortify samples with SYN545192 in acetonitrile as required at this point.

b) Add 80/20 v/v acetonitrile/ultra pure water (50 mL) and record the weight of the flask and contents on a suitable balance. This allows correction for any loss of solvent due to evaporation during reflux.

c) Place the flasks in a suitable electric heating mantle and securely attach a reflux condenser to each flask. Heat at reflux for 1 hour then allow the samples to cool to room temperature with the condensers still attached.

d) Carefully remove the condensers and flasks from the heating mantle and check the weight of the flask and contents. Any losses due to evaporation should be corrected for by addition of acetonitrile (typically 1-5 mL). Swirl the flask and contents to mix thoroughly.

e) Decant the soil and extract into a plastic centrifuge tube (50 mL size) and centrifuge at a speed that visibly separates the supernatant from the soil e.g. 4000 rpm for 5 minutes. The sample concentration is 0.2 g/mL.

f) Transfer 2.5 mL aliquots (equivalent to 0.5 g soil) of extracts into a plastic graduated centrifuge tubes (50 mL size). Dilute to 25 mL with ultra pure water. Cap the tubes securely and shake to mix thoroughly.

g) Take one Waters Oasis HLB SPE cartridge (60 mg, 3 mL size) for each sample to be analysed and place on a suitable vacuum manifold. Add methanol (2 mL) and allow to percolate through 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.

h) Load the soil extracts onto the SPE cartridges 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.

i) Add 90/10 v/v ultra pure water/acetonitrile (2 mL) to the top of the SPE 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. Remove the excess water under vacuum by application of high vacuum for a few seconds.

j) Place suitable centrifuge tubes (15 mL size) under each port, as required, in the manifold rack. Add acetonitrile (2 mL) to the top of each cartridge and allow to percolate through under gravity. Collect the column eluate containing SYN545192. Remove the excess solvent from the cartridges by application of positive pressure or vacuum, collecting the column eluate.

k) Adjust the final volume to 4 mL with ultra pure water so that the solution is approximately 50/50 v/v acetonitrile/ultra pure water. Mix the sample thoroughly by ultrasonicating the contents of centrifuge tube briefly.

l) Transfer the samples into suitable autosampler vials ready for final determination by LC-MS/MS. The final sample concentration is 0.125 g/mL.

APPENDIX 1 Analytical Method Description (Continued)

4. PARAMETERS FOR CHROMATOGRAPHIC ANALYSIS

4.1. Operating conditions

The following parameters were used during the study. They may be adapted if alternative equipment is used.

LC-MS/MS:

- Pump + Autosampler: LC20AD+SIL20AC, Shimadzu or HTC Pal (CTC Analytics)
- Detector: API 4000, Sciex
- Data Acquisition: Analyst 1.5.1, Sciex
- Column HPLC: SB AQ 50 x 4.6 mm - 3.5µm
- Column temperature: 40 °C
- Retention time: approximately 3.05 minutes
- Injection volume: 50 µL
- Autosampler temperature: 4°C
- Flow: 1 mL/minute
- Mobile phase: Solvent A: Acetonitrile
  Solvent B: Ultra pure water + 0.2% acetic acid
- Gradient:

Time (minute)	% A	% B
0	20	80
3.0	90	10
4.0	90	10
4.1	20	80
5.0	20	80

- Ionisation mode: ESI^-
- Scan Type: MRM
- Calibration range: 0.02 to 1 ng/mL

APPENDIX 1 Analytical Method Description (Continued)

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z)*	DP (V)	EP (V)	CXP (V)	CE (V)	Dwell (ms)
SYN545192	396	368 (primary)	-75	-10	-11	-22	500
	396	91 (confirmatory)	-75	-10	-5	-60	500

CAD (collision gas)	8	TEM (°C)	500
CUR (curtain gas)	30	RESOLUTION Q1	Unit
GS1 (ion source gas 1)	50	RESOLUTION Q3	Unit
GS2 (ion source gas 2)	50		
IS (ion spray voltage)	-4200		

4.2. Calibration

Calibration standards were injected before each series of test sample analyses. Confirmatory standards were injected within each series after every four samples. The determination coefficient R^2 was found to be higher than 0.990 in all cases.

4.3. Result calculation

The chromatographic system was calibrated using a calibration curve of SYN545192 external standards. A linear calibration curve was calculated using the method of least squares:

$Y = A \times C + B$

Y = detector response (as peak area) for SYN545192
A = slope of the linear least squares fit of the calibration curve
C = concentration determined from standard curve (ng/mL)
B = Y-intercept of the linear least squares fit of the calibration curve.

The concentration determined from standard curve is: $C = (Y-B)/A$

APPENDIX 1 Analytical Method Description (Continued)

The residue of analyte in each test sample is calculated as follows:

$$\text{Residue } (mg/kg) = \frac{V_1 \quad V_f}{M \quad V_2} \quad extract\ concentration\ (ng/mL) \quad dilution \quad \frac{1}{f}$$

V_1 = Initial extraction volume (50 mL)
V_2 = Volume of aliquot (2.5 mL)
V_f = Final volume (4 mL)
M = Sample weight (10 g)
f = conversion factor from ng/mL to mg/kg (1000)

where the final volume includes dilution steps, if applicable.

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery } (\%) = \frac{A}{S} \times 100$$

where:

A = concentration of SYN545192 found in test sample (mg/kg).
S = concentration of SYN545192 added in test sample (mg/kg).