1.0 INTRODUCTION

1.1 Scope

Analytical method GRM023.06A is suitable for the determination of residues of SYN524464 analysed as the isomers SYN508210 (Figure 1) and SYN508211 (Figure 2) and the metabolites CSCC210616 (Figure 3), CSCD465008 (Figure 4) and CSAA798670 (Figure 5). The limit of quantification has been set at 0.05 μg/L.

This method complies with OECD guidance document ENV/JM/MONO(2007)17, US EPA guidelines EPA OPPTS 860.1340 and OPPTS 850.7100 and EU guidelines SANCO/3029/99 rev. 4, SANCO/825/00 rev. 7.

1.2 Method summary

Residues of SYN508210 and, SYN508211 in water may be diluted with methanol and quantified by direct injection by LC-MS/MS, where instrument sensitivity is sufficient. Residues of CSCC210616 in water may be quantified by LC-MS/MS directly without any sample manipulation, where instrument sensitivity is sufficient.

Alternatively, for analysis of SYN508210, SYN508211 and CSCC210616 the water samples are taken through a solid-phase extraction (SPE) procedure using Oasis ™ HLB cartridges. The SPE cartridges are washed with water and the anlytes are eluted with acetonitrile. The final volume is adjusted to 5 mL with acetonitrile. Aliquots of the eluate are diluted with ultra pure water, as required.

For the analysis of CSCD465008 and CSAA798670, water samples are acidified then taken through a solid-phase extraction (SPE) procedure using Oasis ™ HLB cartridges. The SPE cartridges are washed with water and CSCD465008 and CSAA798670 are eluted with acetonitrile. The column eluates are evaporated to remove the acetonitrile and then redissolved in ultra pure water.

For all analytes, final determination is 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 for SYN508210, SYN508211, CSCD465008, CSAA798670 and CSCC210616.

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 provided that 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

Weigh out accurately, using a five-figure balance, sufficient SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 analytical standards and carefully transfer into a “Class A” volumetric flask. Dilute to the mark with acetonitrile to give 200 µg/mL stock solutions.

Alternatively, the appropriate volume of solvent 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 a volumetric flask.

2.3.2 Fortification Solutions

It is recommended that, as a minimum, 10 µg/mL, 1.0 µg/mL, 0.1 µg/mL and 0.01 µg/mL solutions are prepared by serial dilution in acetonitrile for SYN508210 and SYN508211

and in ultra pure water for CSCC210616, CSCD465008 and CSAA798670. The preparation of LC-MS/MS calibration standards is discussed in Section 3.9.

2.3.3 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 before use.

An expiry date of six months is recommended unless additional data are generated to support a longer expiry 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	Formic Acid	Hydrochloric acid
Harmful Vapour	✓	✓	✓	✓	✓
Highly Flammable	✓	✓	✗	✗	✗
Harmful by Skin Absorption	✓	✓	✓	✓	✓
Causes burns	✗	✗	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓	✓
Syngenta Hazard Category	SHC-C, S	SHC-C, S	SHC-C, S	SHC-C, S	SHC-C, S
OES Short Term (mg/m^3)	105	310	37	N/A	7
OES Long Term (mg/m^3)	70	260	25	9	N/A

N/A - not known

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

There are currently insufficient data to assign a Syngenta Hazard Category (SHC) to SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670. The compounds are therefore assumed to be SHC-D until further information becomes available. Suitable precautions must be taken when handling the solid compound and solutions. The toxicity classification scale rates highly toxic chemicals as SHC-E and non-toxic chemicals as SHC-A. An additional hazard category of S indicates the compound is a severe skin and eye irritant.

3.0 ANALYTICAL PROCEDURE

3.1 Modifications and Potential Problems

a) Bottled HPLC-grade 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.

b) To prevent contamination of the instrument and to minimise possible carry-over issues, higher level recoveries (>5.0 µg/L) and samples with expected residues greater than 5.0 µg/L should be diluted so that the final analyte concentration does not exceed 0.5 µg/L. It may also be useful to include blank injections of methanol/ultra pure water (50/50 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

c) Losses of SYN508210 and SYN508211 in 100% aqueous solutions stored in glass vessels may be observed, due to adsorption to the glass surfaces. Storage of samples containing SYN508210 and SYN508211 in 100% aqueous solutions in glass vessels should therefore be avoided.

3.2 Sample Preparation

a) If water samples are received frozen, they should be allowed to defrost thoroughly at room temperature before analysis. Once completely thawed the bulk water samples should be shaken thoroughly prior to analysis.

3.3 Direct Analysis of Water Samples for SYN508210 and SYN508211

a) Transfer 20 mL of the water sample to be analysed into a polypropylene centrifuge tube (50 mL size). Sample fortification, if required, is to be carried out at this point. At least one untreated control and two control samples fortified with a known amount of each compound should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

b) Dilute the sample with an equal volume of methanol (20 mL). Cap the centrifuge tube securely and shake to mix thoroughly.

c) Transfer an aliquot into a suitable autosampler vial for analysis by LC-MS/MS, using an ACE C18 HPLC column (See Sections 4.2 – 4.3)

3.4 Direct Analysis of Water Samples for CSCC201616

a) Transfer 50 mL of the water sample to be analysed into a polypropylene centrifuge tube (50 mL size). Sample fortification, if required, is to be carried out at this point. At least one untreated control and two control samples fortified with a known amount of each compound should be analysed alongside each batch of

samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

b) Transfer an aliquot into a suitable autosampler vial for analysis by LC-MS/MS, using a Develosil RPAqueous-3 HPLC column (See Sections 4.4 – 4.5).

3.5 Solid Phase Extraction Procedure for SYN508210, SYN508211 and CSCC210616 when Direct Injection Analysis is not Feasible

a) Transfer 50 mL of the water sample to be analysed into a polypropylene centrifuge tube (50 mL size). Sample fortification, if required, is to be carried out at this point. At least one untreated control and two control samples fortified with a known amount of SYN508210, SYN508211 and CSCC210616 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

b) Take one Waters Oasis™ HLB SPE cartridge (size 60 mg, 3 mL) for each sample to be analysed and place on a suitable vacuum manifold. Add methanol (2 mL) to the cartridges and draw through under vacuum to the level of the top frit at a rate of approximately 2 mL/min, discarding the column eluate. Do not allow the cartridges to become dry. Add water (2 mL) and draw through under vacuum to the level of the top frit at a rate of approximately 2 mL/min, discarding the column eluate. Do not allow cartridges to become dry.

c) Load water samples onto the SPE cartridges via a suitable column reservoir 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. Residues of SYN508210, SYN508211 and CSCC210616 are retained on the cartridge.

Note: It is recommended that water with visible particulate matter is filtered through a polypropylene frit placed in the column reservoir before loading on to the SPE cartridge, to prevent blockage of the SPE frit. Alternatively, samples may be centrifuged to separate out any particulate material.

d) On completion of loading, remove the column reservoir and connector. Add ultra pure water (2 mL) to the top of the SPE cartridge frit 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.

e) Remove any remaining water droplets adhering to the inside of the cartridges with absorbent tissue and dry under vacuum for approximately 10 minutes.

f) Place suitable, graduated collection tubes (e.g. 15 mL graduated, plastic centrifuge tubes) under each port, as required, in the manifold rack.

g) Add acetonitrile (5 mL) to the SPE cartridge and allow to percolate through under gravity or draw through under vacuum at a rate of approximately 1 - 2 mL/min

collecting the column eluates. Apply positive pressure or a high vacuum for approximately 5 seconds to draw off any remaining droplets of acetonitrile. Residues of SYN508210, SYN508211 and CSCC210616 are eluted in this fraction.

h) Adjust the column eluate volume to 5 mL with acetonitrile. Mix the sample thoroughly by shaking. The sample concentration is 10 mL/mL.

i) Dilute aliquots from 3.5 (h) as required with 50/50 v/v methanol/ultra pure water for SYN508210 and SYN508211 analysis and with ultra pure water for CSCC210616 analysis to achieve the desired instrument sensitivity to accurately quantify residues at the LOQ.

j) Transfer aliquots of the final samples from 3.5 (i) into suitable autosampler vials for final determination of SYN508210, SYN508211 and CSCC210616 by LC-MS/MS, using the chromatography conditions described in Sections 4.2 – 4.3 and 4.4 – 4.5.

3.6 Solid Phase Extraction Procedure for CSAA465008 and CSAA798670

a) Transfer 50 mL of the water sample to be analysed into a polypropylene centrifuge tube (50 mL size). Sample fortification, if required, is to be carried out at this point. At least one untreated control and two control samples fortified with a known amount of CSCD465008 and CSAA798670 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

b) Add concentrated hydrochloric acid (200 µL) to each sample. Cap the centrifuge tubes securely and shake gently to mix. Using suitable indicator paper, check that the pH is no higher than pH 1. Add further hydrochloric acid as required to ensure the correct pH is achieved. The low pH is required to ensure that CSCD465008 and CSAA798670 are fully protonated so that they are retained on the SPE cartridge.

c) 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.

d) Load water samples from Section 3.6 (b) onto the SPE cartridges via a suitable column reservoir 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.

Note: It is recommended that water with visible particulate matter is filtered through a polypropylene frit placed in the column reservoir before loading on to the

SPE cartridge, to prevent blockage of the SPE frit. Alternatively, samples may be centrifuged to separate out any particulate material.

e) On completion of loading, remove the column reservoir and connector. Add ultra pure water (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.

f) Place suitable, disposable, plastic, graduated centrifuge tubes (10 mL size) under each port, as required, in the manifold rack. Add 50/50 v/v acetonitrile/ultra pure water (2 mL) to the top of each cartridge and allow to percolate through under gravity. Collect the column eluate containing CSCD465008 and CSAA798670. Remove the excess solvent from the cartridges by application of positive pressure or vacuum, collecting the column eluate.

g) Evaporate the collected eluates to 0.5 mL under a stream of air or nitrogen in a sample concentrator with the heating block set at 30 ^{o}C so that the acetonitrile is eliminated from the sample. The presence of acetonitrile in the sample will have an adverse effect on the chromatography of CSCD465008 and CSAA798670, with poor retention and peak shape.

h) Adjust the final volume to 1 mL with ultra pure water and mix sample thoroughly by brief ultrasonication of the contents of centrifuge tube.

i) Transfer the samples into suitable autosampler vials ready for final determination by LC-MS/MS, using the chromatography conditions described in Section 4.6 – 4.7. The final sample concentration is 50 mL/mL.

3.7 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).

3.8 Method Stopping Points

It is recommended that the analytical procedure be completed in one day. 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.

3.9 Preparation of Calibration Standards for LC-MS/MS

No significant suppression or enhancement of the instrument response for these compounds has been observed in the water types tested using the above procedure in this laboratory.

LC-MS/MS calibration standards for SYN508210 and SYN508211 should be prepared in acetonitrile/ultra pure water 50/50 (v/v) with each analysis batch. For example, to prepare

a 0.025 µg/L calibration standard, transfer 250 µL of a 0.01 µg/mL standard in acetonitrile in a volumetric flask (10 mL). Add acetonitrile/ultra pure water 50/50 (v/v) to the 10 mL mark. Stopper the flask securely and shake to mix thoroughly, then dilute the solution 10-fold further with acetonitrile/ultra pure water 50/50 (v/v).

Transfer aliquots into suitable autosampler vials for analysis by LC-MS/MS.

LC-MS/MS calibration standards for CSCD465008, CSAA798670 and CSCC210616 are prepared in ultra pure water with each analysis batch. For example, to prepare a 2.5 µg/L calibration standard, transfer 250 µL of a 0.1µg/mL standard in ultra pure water in a volumetric flask (10 mL). Add ultra pure water to the 10 mL mark. Stopper the flask securely and shake to mix thoroughly.

Transfer aliquots into suitable autosampler vials for analysis by LC-MS/MS.

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 the Applied Biosystems API 4000 LC-MS/MS.

4.1 Instrument Description

HPLC system	:	Series 200 (Perkin Elmer) and Shimadzu LC20AD
Detector	:	Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.2
Gas Supply	:	Peak Scientific NM20ZA gas station

4.2 Chromatography Conditions for SYN508210 and SYN508211

Column	:	ACE C18, 5µm, 100 mm × 3.0 mm i.d.
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	13 minutes
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 = Methanol Solvent 2 = 0.1% v/v formic acid in ultra pure water

Mobile Phase Gradient

Time (min)	% Solvent 1	% Solvent 2	Flow (mL/min)
0.0	50	50	0.6
6.0	90	10	0.6
9.0	90	10	0.6
9.1	50	50	0.6
13.0	50	50	0.6

Under these conditions the retention times of SYN508210 and SYN508211 are approximately 6.7 and 7.2 minutes respectively.

4.3 Mass Spectrometer Conditions for SYN508210 and SYN508211

Interface : TurboIonSpray

Polarity : Negative

Curtain gas (CUR) : Nitrogen set at 25 (arbitrary units)

Temperature (TEM) : 550°C

Ionspray voltage : -4500V

Collision gas setting (CAD) : Nitrogen set at 4 (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 : Multiple reaction monitoring (MRM)

MRM Conditions		SYN508210 and SYN508211 primary transition	SYN508210 and SYN508211 confirmatory transition
Q1 *m/z*	:	330	330
Q3 *m/z*	:	131	91
Dwell time	:	600 ms	600 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	-90 V	-90 V
Entrance potential (EP)	:	-10 V	-10 V
Collision energy (CE)	:	-30 V	-46 V
Collision cell exit potential (CXP)	:	-10 V	-6 V

Typical chromatograms for SYN508210 and SYN508211 in drinking water are presented in the Figures 6 - 25. Chromatograms for other water types are similar.

4.4 Chromatography Conditions for CSCC210616

Column	:	Develosil RP Aqueous-3 150 mm × 3 mm
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	6 minutes
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 = Methanol Solvent 2 = 0.1% v/v formic acid in ultra pure water

Mobile Phase Gradient

Time (min)	% Solvent 1	% Solvent 2	Flow (mL/min)
0.0	20	80	0.5
6.0	20	80	0.5

Under these conditions the retention times of CSCC210616 is approximately 3.5 minutes.

4.5 Mass Spectrometer Conditions for CSCC210616

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 25 (arbitrary units)
Temperature (TEM)	:	500°C
Ionspray voltage	:	5500V
Collision gas setting (CAD)	:	Nitrogen set at 4 (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		**CSCCC210616 Primary Transition**	**CSCCC210616 Confirmatory Transition**
Q1 *m/z*	:	176	176
Q3 *m/z*	:	136	156
Dwell time	:	300 ms	300 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	50 V	50 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	23 V	14 V
Collision cell exit potential (CXP)	:	13 V	14 V

Typical chromatograms for CSCC210616 in drinking water are presented in the Figures 26 - 35. Chromatograms for other water types are similar.

4.6 Chromatography Conditions for CSCD465008 and CSAA798670

Column	:	Develosil RP Aqueous 3µm 150 mm × 3 mm
Column Oven Temperature	:	40°C
Injection volume	:	10 µL
Stop Time	:	6 minutes
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 = Acetonitrile Solvent 2 = 0.2% v/v acetic acid in ultra pure water

Mobile Phase Gradient

Time (min)	% Solvent 1	% Solvent 2	Flow (mL/min)
0.0	20	80	0.5
6.0	20	80	0.5

Under these conditions the retention times of CSCD465008 is approximately 2.4 minutes and CSAA798670 is 3.4 minutes.

4.7 Mass Spectrometer Conditions for CSCD465008 and CSAA798670

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 25 (arbitrary units)
Temperature (TEM)	:	550°C
Ionspray voltage	:	-4500V
Collision gas setting (CAD)	:	Nitrogen set at 4 (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		CSCD465008 Primary Transition	CSCD465008 Confirmatory Transition	CSAA798670 Primary Transition	CSAA798670 Confirmatory Transition
Q1 *m/z*	:	161	161	175	175
Q3 *m/z*	:	141	66	91	111
Dwell time	:	100 ms	100 ms	300 ms	300 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	-45 V	-45 V	-50 V	-50 V
Entrance potential (EP)	:	-10 V	-10 V	-10 V	-10 V
Collision energy (CE)	:	-13 V	-30 V	-29 V	-23 V
Collision cell exit potential (CXP)	:	-11 V	-10 V	-13 V	-8 V

Typical chromatograms for CSCD465008 in drinking water are presented in the Figures 36 – 45 and for CSAA798670 in Figures 46 - 55. Chromatograms for other water types are similar.

Note: The mobile phase conditions for CSCD465008, CSAA798670 and CSCC210616 are different. It is recommended that at least 10 primer injections are included in between the analyses to allow sufficient time for the Develosil RP Aqueous HPLC column to fully equilibrate.

5.0 CALCULATION OF RESULTS

5.1 Single Point Calibration Procedure

Residues may be calculated in µg/L for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of each standard containing SYN508210 and SYN508211, CSCC210616, CSCD465008 and CSAA798670 as required 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 the analytes.

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

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

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

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

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)

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

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

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).

5.2 Multi Point Calibration Procedure

Residues may be calculated in μg/L for each sample as follows.

a) Make repeated injections of a standard containing SYN508210 and SYN508211, CSCC210616, CSCD465008 and CSAA798670 as required over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 20 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least four).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to each analyte. 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) Alternatively (depending on the regression analysis software available) a quadratic equation may be used to fit the data. In this case the following general equation should be re-arranged and used to calculate residues:

$$y = a + bx + cx^2$$

Where *y* is the instrument response value, *x* is the standard concentration and *a*, *b*, *c* are constants.

f) Calculate the residues of each compound in the sample, expressed as µg/L, as follows

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

Where analyte found (µg/L) 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.

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

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 SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670) 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%.

7.0 SPECIFICITY

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

7.1 Matrix Interference

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 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

A method validation study has been carried out on the procedures described in Sections 3 and 4. The method validation data are reported in Eurofins|ADME Bioanalyses report TK0009674-REG (Reference 3) and a summary is included in Tables 10 – 25.

8.2 Matrix Effects

Matrix effects, enhancement or suppression, of the instrument response were considered not to be significant and non-matrix calibration standards should be used for calibration. Details are given in Table 39.

8.3 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 ≤ 20% has been achieved. 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 µg/L for SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670.

8.4 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.

The limit of detection of this method for each analyte was estimated and are presented in Tables 2 – 9.

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 was tested and was found to be linear for:

- SYN508210 and SYN508211 at concentrations ranging from 0.0125 to 0.5 µg/L (equivalent to 0.625 to 25 pg of SYN508210 and SYN508211 injected on to the column, based on a 50 µL injection);

- CSCC210616 at concentrations ranging from 0.025 to 1 µg/L (equivalent to 1.25 to 50 pg of CSCC210616 injected on to the column, based on a 50 µL injection);

- CSCD465008 and CSAA798670 at concentrations ranging from 1.25 to 50 µg/L (equivalent to 12.5 to 500 pg for CSCD465008 and CSAA798670 injected on to the column, based on a 10 µL injection).

The linear range for each analyte covers the range from 50% of the LOQ to 20 x LOQ. 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 at at least 5 different concentration levels were injected and the response plotted against the amount injected, using Microsoft Excel 2003 for both primary transitions and confirmatory transitions.

Linearity data are presented in Tables 26 – 30. Detector linearity graphs are shown in Figures 56 - 65.

8.6 Final Extract stability

Residues of SYN508210 and SYN508211 in 50/50 v/v drinking water/methanol and in final surface water extracts post SPE (in 95/5 v/v ultra pure water/acetonitrile) have been shown to be stable when stored refrigerated at 0 - 9 ^{o}C for up to 8 days and 9 days respectively when reanalysed against a freshly prepared calibration standard.

Residues of CSCC210616 in groundwater and in final surface water extracts post SPE (in 90/10 v/v ultra pure water/acetonitrile) have been shown to be stable when stored refrigerated at 0 - 9 ^{o}C for up to 12 days and 11 days respectively when reanalysed against a freshly prepared calibration standard.

Residues of CSCS465008 and CSAA798670 in final surface water extracts post SPE (in ultra pure water) have been shown to be stable when stored refrigerated at 0 - 9 ^{o}C for up to 16 days when reanalysed against a freshly prepared calibration standard.

Summaries of the stability data are presented in Tables 31 - 38.

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 types not tested in this study, 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 SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 residues in water. Only commercially available laboratory equipment and reagents are required. The analysis of a batch of 20 samples 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 0.05µg/L.

This method complies with OECD guidance document ENV/JM/MONO(2007)17, US EPA guidelines EPA OPPTS 860.1340 and OPPTS 850.7100 and EU guidelines SANCO/3029/99 rev. 4, 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 (1980): Potential error in single point ratio calculations based on linear calibration curves with a significant intercept. Anal Chem., 52 pp 1187-1191
3. Oppilliart S. (2009), Eurofins|ADME Bioanalyses Validation Report TK0009674-REG SYN524464 - Validation of the Analytical Method GRM023.06A for the Determination of Residues of SYN508210 and SYN508211 and the Metabolites CSCC210616, CSCD465008 and CSAA798670 in Water.

CHEMICAL STRUCTURES

Figure 1

Compound Code Number	**:**	SYN508210
CAS Number	**:**	599197-38-3
IUPAC Name	**:**	2′-[(1*RS*,2*SR*)-1,1′-bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide
Molecular Formula	**:**	$C_{18}H_{19}F_2N_3O$
Molecular Weight	**:**	331.4

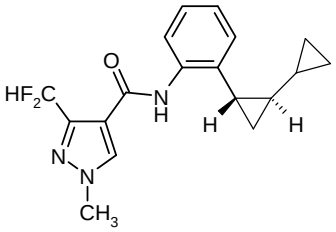


Figure 2

Compound Code Number	**:**	SYN508211
CAS Number	**:**	599194-51-1
IUPAC Name	**:**	2′-[(1*RS*,2*RS*)-1,1′-bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide
Molecular Formula	**:**	$C_{18}H_{19}F_2N_3O$
Molecular Weight	**:**	331.4

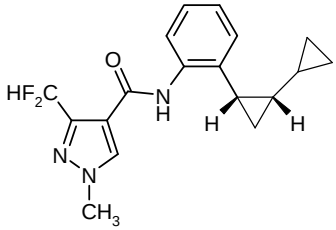


Figure 3

Compound Code Number	**:**	CSCC210616
Alternative Compound Code Number	**:**	SYN508272
CAS Number	**:**	Not in registry
IUPAC Name	**:**	3- Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid amide
Molecular Formula	**:**	$C_6H_7F_2N_3O$
Molecular Weight	**:**	175.1

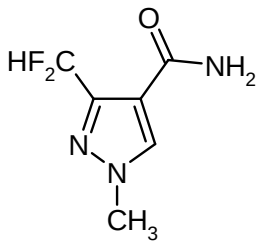


Figure 4

Compound Code Number	**:**	CSCD465008
CAS Number	**:**	Not in registry
IUPAC Name	**:**	3-(Difluoromethyl)-1H-pyrazole-4-carboxylic acid
Molecular Formula	**:**	$C_5H_4F_2N_2O_2$
Molecular Weight	**:**	162.1

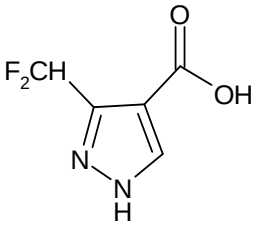
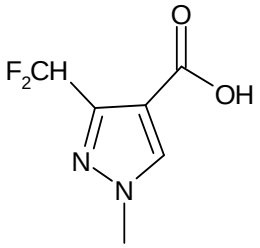


Figure 5

Compound Code Number	**:**	CSAA798670
CAS Number	**:**	Not in registry
IUPAC Name	**:**	3-(Difluoromethyl)-1-methyl-1H-pyrazole-4-carboxylic acid
Molecular Formula	**:**	$C_6H_6F_2N_2O_2$
Molecular Weight	**:**	176.1



APPENDIX 1 APPARATUS

UK suppliers

General glassware, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough Leicestershire LE11 5RG..

Polypropylene centrifuge tubes, 50 mL and 15 mL capacity, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG.

Isolute® Vacmaster-20® sample processing station, available from Argonaut Technologies, Tir-y-Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan, CF8 8AU.

Column connectors, available from Argonaut Technologies, Tir-y-Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan, CF8 8AU.

Column reservoirs, 70 mL size, available from Agilent Technologies UK Limited Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire SK8 3GR.

Polyethylene 70 μm frits for 70 mL reservoir, available from Agilent Technologies UK Limited Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire SK8 3GR.

Oasis HLB solid phase extraction cartridges 60 mg, 3 mL size, available from available from Waters Ltd, 730-740 Centennial Court, Elstree, Hertfordshire WD6 3SZ.

Plastic disposable pipettes, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.

Disposable borosilicate glass test tubes, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG

Ultrasonic bath e.g. Ultrawave U300/D, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.,.

Crimp cap autosampler vials and caps, available from Agilent Technologies UK Limited Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire. SK8 3GR.

API 4000 LC-MS/MS system equipped with a TurboIonSpray source, available from Applied Biosystems, 120 Birchwood Boulevard, Warrington, Cheshire WA3 7PB.

Perkin Elmer series 200 HPLC system equipped with a quarternary pump, vacuum degasser and column compartment with column switching valve, available from www.perkinelmer.co.uk

Shimadzu LC20AD HPLC system equipped with autosampler, quaternary pump, vacuum degasser and column compartment with column switching valve, available from Shimadzu UK Limited, Mill Court, Featherstone Road, Wolverton Mill South, Milton Keynes MK12 5RD.

HPLC column, ACE C18 5 µm, 100 x 3.0 mm , available from Hichrom Ltd., 1 The Markham centre, Station road, Theale, Reading, RG47 4PE Berkshire or www.hichrom.co.uk.

Develosil RPAqueous-3 HPLC column 3 µm, 150 x 3.0 mm, available from Hichrom Limited, 1 The Markham Centre, Station Road, Theale, Reading, Berkshire RG7 4PE or www.hichrom.co.uk.

Peak Scientific NM20ZA gas station, available from Peak Scientific Instruments Ltd., Fountain Crescent, Inchinnan Business Park, Inchinnan, Renfrew PA9 4RE.

US suppliers

General glassware, available from Fisher Scientific Fisher Scientific, Liberty Lane, Hampton NH 03842.

Polypropylene centrifuge tubes, 50 mL and 15 mL capacity available from Fisher Scientific, Liberty Lane, Hampton NH 03842.

Isolute® Vacmaster-20® sample processing station, available from Argonaut Technologies - Order Processing, 1101 Chess Drive, Foster City, CA 94404.

Oasis™ HLB solid phase extraction columns, 3 mL 60 mg size, available from Waters Corporation, 34 Maple Street, Milford, Massachusetts, 01757-3696.

Column connectors, available from Argonaut Technologies, Order Processing, 1101 Chess Drive, Foster City, CA 94404.

Column reservoirs, 70 mL size, available from available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304.

Polyethylene 70 µm frits for 30 mL reservoir, available Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304.

Plastic disposable pipettes, available from Fisher Scientific, Liberty Lane, Hampton NH 03842.

Disposable borosilicate glass test tubes, available from Fisher Scientific Liberty Lane, Hampton, NH 03842,.

Ultrasonic bath e.g. Ultrawave U300/D, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842,.
Crimp cap auto sampler vials and caps, available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304.

API 4000 LC-MS/MS system equipped with a TurboIonSpray source, available from Applied Biosystems, 850 Lincoln Center, Foster City, CA 94404-1128.

Perkin Elmer series 200 HPLC system equipped with autosampler, quaternary pump, vacuum degasser and column compartment with column switching valve, available from www.perkinelmer.com

Shimadzu LC20AD HPLC system equipped with autosampler, quaternary pump, vacuum degasser and column compartment with column switching valve, available from Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD 21046, U.S.A.

HPLC column, ACE C18 5 µm, 100 x 3.0 mm, available from www.hichrom.co.uk.

Develosil RPAqueous-3 HPLC column 3 µm, 150 x 3.0 mm, available from www.Hichrom.co.uk

Peak Scientific NM20ZA gas station, available from Peak Scientific Instruments, 1300 West Belmont Ave., Chicago IL 60657.

APPENDIX 2 REAGENTS

UK suppliers

Solvents: Ultra pure water (HPLC grade), methanol and acetonitrile available from Rathburn Chemicals Ltd., Walkerburn, Scotland EH43 6AU

Analytical grade concentrated formic acid, 37% hydrochloric acid and glacial acetic acid available from Sigma-Aldrich, The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT or www.sigmaaldrich.com

SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 analytical standards available from Syngenta, GLP Testing Facility, Syngenta,CH-4333 Munchweilen, Switzerland.

US suppliers

Solvents: Analytical grade acetonitrile and methanol available from B & J Brand Solvents, from Scientific Products Division of Baxter Healthcare Corporation, USA.

Ultra pure HPLC grade water from e.g. Fluka via Sigma-Aldrich www.sigmaaldrich.com

Analytical grade concentrated formic acid, 37% hydrochloric acid and glacial acetic acid available from www.sigmaaldrich.com

SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 analytical standards, available from Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

1. 50:50 v/v acetonitrile:ultra pure water
   Add 500 mL of acetonitrile to 500 mL ultra pure water in a 1 L volumetric flask. Stopper flask securely and mix thoroughly by shaking.

2. 0.2% v/v acetic acid in ultra pure water.
   Add concentrated acetic acid (2 mL) to 1 L ultra pure water in a 1 L volumetric flask. Stopper flask securely and mix thoroughly by shaking.

3. 0.1% v/v formic acid in ultra pure water.
   Add concentrated formic acid (1 mL) to 1 L ultra pure water in a 1 L volumetric flask. Stopper flask securely and mix thoroughly by shaking.

APPENDIX 3 API4000 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 SYN508210, SYN508211, CSCD465008 and CSAA798670 in Negative Ionisation Mode

Infuse separate standard solutions of SYN508210, SYN508211, CSCD465008 and CSAA798670 (0.1 µg/mL and 0.01 µg/mL in mobile phase) directly into the mass spectrometer interface at a rate at of about 10 µL/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 under negative ionisation conditions.

Using the Analyst 1.4.2 software quantitative optimisation routine, tune the instrument for SYN508210, SYN508211 CSCD465008 and CSAA798670, ensuring that the correct ions are selected. Alternatively, the instrument ion optics and collision energy may be tuned manually to ensure maximum sensitivity.

Analyte	Parent ion	Daughter ion Primary transition	Daughter ion Confirmatory transition
SYN508210	*m/z* 330	*m/z* 131	*m/z* 91
SYN508211	*m/z* 330	*m/z* 131	*m/z* 91
CSCD465008	*m/z* 161	*m/z* 141	*m/z* 66
CSAA798670	*m/z* 175	*m/z* 91	*m/z* 111

Note: If problems are encountered in tuning the instrument for these ions, the ions should be entered in the method as detailed above and tuning performed manually.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of SYN508210, SYN508211, CSCD465008 and CSAA798670 standards in mobile phase and 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

In negative ionisation mode, anions of SYN508210, SYN508211, CSCD465008 and CSAA798670 generated in the ion source are selected and subjected to further fragmentation by collisional activation. The most sensitive daughter ions are then selected and used for quantitative analysis.

The fragment *m/z* 131 for SYN508210 and SYN508211 corresponds to 1-methyl-3-(difluoromethyl)-1H-pyrazole-4-carboxamide and *m/z* 91 corresponds to loss of 2 x HF from the previous daughter ion *m/z* 131.

The fragment *m/z* 141 for CSCD465008 corresponds to loss of HF from the deprotonated parent molecule and *m/z* 66 corresponds to the pyrazole fragment.

The fragment *m/z* 91 for CSAA798670 corresponds to loss of 2x HF from the -methyl-3-(difluoromethyl)-1H-pyrazole-4-carboxamide and *m/z* 111 corresponds to loss of HF from the 1-methyl-3-(difluoromethyl)-1H-pyrazole-4-carboxamide fragment.

Tuning instrument for CSCC210616 in Positive Ionisation Mode

Infuse a standard solution CSCC210616 (0.1 to 1.0 µg/mL in mobile phase, see section 4.4) directly into the mass spectrometer interface at a rate at of about 10 µL/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 under positive ionisation conditions.

Using the Analyst 1.4.2 software quantitative optimisation routine, tune the instrument for CSCC210616 ensuring that the correct ions are selected. Alternatively, the instrument ion optics and collision energy may be tuned manually for CSCC210616 to ensure maximum sensitivity.

Analyte	Parent ion	Daughter ion Primary transition	Daughter ion Confirmatory transition
CSCC210616	*m/z* 176	*m/z* 136	*m/z* 156

Note: If problems are encountered in tuning the instrument for these ions, the ions should be entered in the method as detailed above and tuning performed manually.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of CSCC210616 standards in mobile phase and 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.

In positive ionisation mode, cations of CSCC210616 generated in the ion source are selected and subjected to further fragmentation by collisional activation. The most sensitive daughter ions are then selected and used for quantitative analysis.

The fragments *m/z* 136 and 156 correspond to loss of 2 x HF and 1 x HF from protonated CSCC210616 respectively.

APPENDIX 4 METHOD FLOWCHART

Direct injection analysis for SYN508210 and SYN508211:

Measure water sample (20 mL) into centrifuge tube

↓

Dilute with MeOH (20 mL)

↓

Analyse by LC-MS/MS using ACE C18 5µm 100 mm x 3mm HPLC column

Direct injection analysis for CSCC210616:

Analyse by LC-MS/MS using Develosil RPAqueous-3 150mm x 3mm HPLC column

SPE procedure for SYN508210 and SYN508211 and CSCC210616:

Measure water sample (50 mL) into centrifuge tube

↓

Oasis HLB procedure

↓

Elute with MeCN. Adjust volume to 5 mL

↓

Dilute aliquots as required with UPW and analyse by LC-MS/MS using ACE C18 5µm 100 mm x 3mm HPLC column for SYN508210 and SYN508211 and Develosil RPAqueous-3 150mm x 3mm for CSCC210616

SPE procedure for CSCD465008 and CSAA708670:

Measure water sample (50 mL) into centrifuge tube and acidify with conc. HCl to pH 1

↓

Oasis HLB procedure

↓

Elute with MeCN.

↓

Evaporate to approximately 0.5 mL and adjust to final volume of 1 mL with UPW.

↓

Analyse by LC-MS/MS using Develosil RPAqueous-3 150mm x 3mm HPLC column for CSCD465008 and CSAA798670