1.0 EXECUTIVE SUMMARY

The purpose of this study was to develop and perform the validation of residue method GRM023.06A to demonstrate the applicability of this method in water.

The limit of quantification (LOQ) for SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 in water was 0.05 µg/L.

One reagent blank sample, control samples in duplicate, fortified samples in quintuplet at the limit of quantification (0.05 µg/L) and in quintuplet at ten times the LOQ (0.5 µg/L) in groundwater, surface water and drinking water were analysed.

Acceptable mean recoveries between 70% and 110% with a relative standard deviation lower than 20% were found for each transitions of:

- SYN508210 and SYN508211 (i.e. *m/z* 330.2 → 131.1 and *m/z* 330.2 → 91.1),
- CSCC210616 (i.e. *m/z* 176.0 → 136.1 and *m/z* 176.0 → 156.1),
- CSCD465008 (i.e. *m/z* 161.0 → 141.0 and *m/z* 161.0 → 65.9),
- CSAA798670 (i.e. *m/z* 175.0 → 91.1 and *m/z* 175.0 → 111.1).

The specificity of the method was proven for LC-MS/MS, since no significant interference higher than 30% of the LOQ was detected in any of the blank and unfortified water specimens.

No significant enhancement or suppression of detector response was observed in the presence of water at each transitions: the measured matrix effect was less than 10 %. It was therefore appropriate to use non-matrix-matched standards for calibration and quantification of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 in water.

The response of the LC-MS/MS was shown to be linear for all transitions of each analyte over a concentration range of:

- 0.0125 to 0.5 µg/L for SYN508210 and SYN508211,
- 0.025 to 1.0 µg/L for CSCC210616,
- 1.25 to 50 µg/L for CSCD465008 and CSAA798670.

The stability of each analyte in final extracts stored between 0 and 9°C was demonstrated after 8 to 16 days of storage, depending on the matrix and the experimental procedure followed, using the quantification transitions.

The repeatability and specificity of the method have been demonstrated, and it is therefore considered valid for the determination of residues of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 in water at the LOQ of 0.05 µg/L and over concentration ranges typical of those for which the method was used.

The method has been validated according to the OECD guidance document ENV/JM/MONO(2007)17, EU guidelines SANCO/3029/99 Rev. 4 and SANCO/825/00 Rev.

7. The method validation also complies with US EPA guidelines OPPTS 850.7100 and OPPTS 860.1340.

2.0 INTRODUCTION

Analytical method GRM023.06A has been developed by Syngenta and modified by Eurofins| ADME Bioanalyses laboratories and referenced as method AGR/MOA/SYN524464-1, for the determination of residues of SYN508210 and SYN508211 and its degradates CSCC210616, CSCD465008 and CSAA798670 in water at the LOQ of 0.05 µg/L. This study was conducted to validate the analytical method.

This study was conducted in compliance with OECD guidance document ENV/JM/MONO(2007)17, European guidelines SANCO/3029/99 Rev. 4, SANCO/825/00 Rev. 7 and US EPA guideline OPPTS 850.7100 and OPPTS 860.1340.

Specifically:

a) To establish that the method will produce recovery values which are within an acceptable range (i.e. mean recoveries between 70% and 110%, with a relative standard deviation within a run less than 20%), for each fortification level and overall.

b) To establish that the limit of quantification (LOQ) of the analytical method is 0.05 µg/L for SYN508210 and SYN508211 and its degradates CSCC210616, CSCD465008 and CSAA798670.

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

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

e) To assess suppression or enhancement of instrument response to each analytes in the presence of each different matrix (water from different origins).

f) To assess the stability of each analytes stored between 0 and 9°C in final extracts from drinking water.

g) To assess and report the limit of detection (LOD) for each analyte.

3.0 MATERIALS AND METHODS

3.1 Reference Items

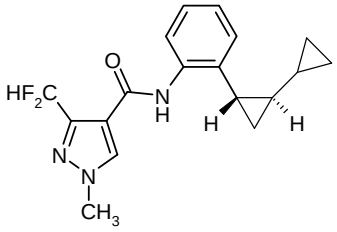
Compound Code Number : SYN508210

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



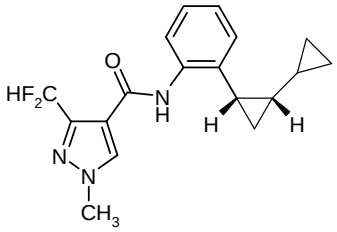
Compound Code Number : SYN508211

CAS Number : 599194-51-1

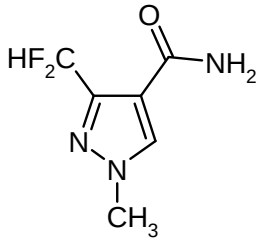
IUPAC Name : 2′-[(1*RS*,2*RS*)-1,1′-bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide

Molecular Formula : $C_{18}H_{19}F_2N_3O$

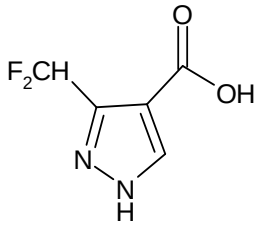
Molecular Weight : 331.4



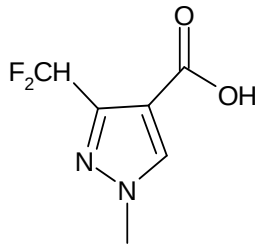
Compound Code Number	**:**	CSCC210616
Alternative Compound Code Number		SYN508272
CAS Number	**:**	Not in registry
IUPAC Name	**:**	3- Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid amide
Molecular Formula	**:**	$C_6H_7F_2N_3O$
Molecular Weight	**:**	175.1



Compound Code Number	**:**	CSCD465008
Alternative Compound Code Number		R958945
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



Compound Code Number :		CSAA798670
Alternative Compound Code Number		NOA449410 or CA4312
CAS Number	**:**	[176969-34-9]
IUPAC Name	**:**	3-(Difluoromethyl)-1-methyl-1H-pyrazole-4-carboxylic acid
Molecular Formula	**:**	$C_6H_6F_2N_2O_2$
Molecular Weight	**:**	176.1



The reference substances used for this validation study were the following:

Name:	SYN508210	SYN580211	CSCC210616	CSCD465008	CSAA798670
Supplier:	Syngenta	Syngenta	Syngenta	Syngenta	Syngenta
Batch:	KI-7193/2	KI-7245/6	MES 114/1	TE-6404/13	AMS 1234/2
Purity (%):	98.0	92.0	97.0	96.0	99.5
Expiration:	31 Oct 2010	31 Oct 2010	30 Jun 2010	31 Oct 2010	31 Jul 2011
Storage:	Between 0 and 9°C	Between 0 and 9°C	Between 0 and 9°C	Between 0 and 9°C	20°C ± 4°C

The certificates of analysis have been provided by the sponsor. The remaining reference items will be stored at Eurofins|ADME Bioanalyses as long as their qualities can be maintained.

3.2 Test System

The validation study was carried out using control water samples sampled or purchased locally as detailed in Table1.

3.3 Preparation and Stability of Analytical Standard Solutions

200 µg/mL stock solutions were prepared in acetonitrile. These solutions are assumed to be stable for 6 months when stored between 0 and 9°C.

Fortification solutions of SYN508210 and SYN508211 at 100 µg/L and 10 µg/L were prepared in acetonitrile from the primary stock solutions.

Fortification solutions of CSCC210616, CSCD465008 and CSAA798670 at 100 µg/L and 10 µg/L were prepared in ultra pure water from the primary stock solutions.

Calibration standards of SYN508210 and SYN508211 for analytical determination by LC-MS/MS were prepared over an appropriate range in acetonitrile/ultra pure water (50/50, v/v).

Calibration standards of CSCC210616, CSCD465008 and CSAA798670 for analytical determination by LC-MS/MS were prepared over an appropriate range in ultra pure water.

The analytical standards solutions were freshly prepared.

The analytical procedure is described in detail in Appendix 3.

3.4 Fortification Levels

Recovery of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 through the analytical procedure was assessed by fortifying five aliquots of water with each analyte at the LOQ (0.05 µg/L) and five aliquots of water with each analyte at ten times the LOQ (0.5 µg/L).

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 Detection (LOD) and Limit of Quantification (LOQ)

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.
For each analyte, the baseline noise of control samples was measured at the appropriate retention time. The mean baseline noise was calculated, multiplied by 3 and then compared to the height of the first standard of the calibration curve.

The following LOD was estimated for SYN508210 (by direct injection):

	SYN508210 Groundwater matrix		SYN508210 Surface water matrix		SYN508210 Drinking water matrix	
	m/z 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1
Baseline noise of both control samples (cps)	2.2 6.9	1.2 1.4	3.3 6.2	2 2.2	3.7 4.5	1.7 1.7
Mean baseline noise x 3 (cps)	14	4	14	6	12	5
Height of standard at 0.0125 µg/L (cps)	191	103	87	58	130	70
Estimated LOD (µg/L)	0.0018	0.0009	0.0041	0.0027	0.0024	0.0018

The following LOD was estimated for SYN508210 (by solid-phase extraction):

	SYN508210 Groundwater matrix		SYN508210 Surface water matrix		SYN508210 Drinking water matrix	
	m/z 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1
Baseline noise of both control samples (cps)	4.4 1.8	2 3.3	3.7 6.6	2.7 3.6	5.5 3.1	1.6 2.3
Mean baseline noise x 3 (cps)	9	8	15	9	13	6
Height of standard at 0.0125 µg/L (cps)	286	188	531	380	276	181
Estimated LOD (µg/L)	0.0008	0.0011	0.0007	0.0006	0.0012	0.0008

The following LOD was estimated for SYN508211 (by direct injection):

	SYN508211 Groundwater matrix		SYN508211 Surface water matrix		SYN508211 Drinking water matrix	
	m/z 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1
Baseline noise of both control samples (cps)	3.7 3.4	2.3 2.4	2.5 6.2	2.6 2.2	3.5 4.0	1.2 2.0
Mean baseline noise x 3 (cps)	11	7	13	7	11	5
Height of standard at 0.0125 µg/L (cps)	154	113	94	74	98	67
Estimated LOD (µg/L)	0.0017	0.0016	0.0035	0.0024	0.0029	0.0018

The following LOD was estimated for SYN508211 (by solid-phase extraction):

	SYN508211 Groundwater matrix		SYN508211 Surface water matrix		SYN508211 Drinking water matrix	
	m/z 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1	*m/z* 330 2 → 131 1	*m/z* 330 2 → 91 1
Baseline noise of both control samples (cps)	4.8 3.5	3.2 2.9	4 4.6	3.1 2.9	2.9 3.3	1.9 2.4
Mean baseline noise x 3 (cps)	12	9	13	9	9	6
Height of standard at 0.0125 µg/L (cps)	308	218	620	429	285	189
Estimated LOD (µg/L)	0.0010	0.0011	0.0005	0.0005	0.0008	0.0009

The following LOD was estimated for CSCC210616 (by direct injection):

	CSCC210616 Groundwater matrix		CSCC210616 Surface water matrix		CSCC210616 Drinking water matrix	
	m/z 176 0 → 136 1	*m/z* 176 0 → 156 1	*m/z* 176 0 → 136 1	*m/z* 176 0 → 156 1	*m/z* 176 0 → 136 1	*m/z* 176 0 → 156 1
Baseline noise of both control samples (cps)	9.4 3.1	8.4 4.4	5.4 7	6 4.2	7.5 6	6.4 4.4
Mean baseline noise x 3 (cps)	19	19	19	15	20	16
Height of standard at 0.025 µg/L (cps)	339	280	209	193	237	170
Estimated LOD (µg/L)	0.0014	0.0017	0.0022	0.0020	0.0021	0.0024

The following LOD was estimated for CSCC210616 (by solid-phase extraction):

	CSCC210616 Groundwater matrix		CSCC210616 Surface water matrix		CSCC210616 Drinking water matrix	
	m/z 176 0 → 136 1	*m/z* 176 0 → 156 1	*m/z* 176 0 → 136 1	*m/z* 176 0 → 156 1	*m/z* 176 0 → 136 1	*m/z* 176 0 → 156 1
Baseline noise of both control samples (cps)	7.4 3.8	21 23	6.7 4.1	12 24	4.2 8.1	20 28
Mean baseline noise x 3 (cps)	17	66	16	54	18	72
Height of standard at 0.025 µg/L (cps)	62	88	67	68	62	71
Estimated LOD (µg/L)	0.0068	0.0189	0.0061	0.0199	0.0075	0.0252

The following LOD was estimated for CSCD465008 (by solid-phase extraction):

	CSCD465008 Groundwater matrix		CSCD465008 Surface water matrix		CSCD465008 Drinking water matrix	
	m/z 161 0 → 141 0	*m/z* 161 0 → 65 9	*m/z* 161 0 → 141 0	*m/z* 161 0 → 65 9	*m/z* 161 0 → 141 0	*m/z* 161 0 → 65 9
Baseline noise of both control samples (cps)	18 4.1	3.8 nc	7.6 13	13 16	11 9.8	nc nc
Mean baseline noise x 3 (cps)	33	11	31	44	31	nc
Height of standard at 1.25 µg/L (cps)	773	216	1864	304	719	629
Estimated LOD (µg/L)	0.0011	0.0013	0.0004	0.0036	0.0011	nc

nc: not calculable

The following LOD was estimated for CSAA798670 (by solid-phase extraction):

	CSAA798670 Groundwater matrix		CSAA798670 Surface water matrix		CSAA798670 Drinking water matrix	
	m/z 175 0 → 91 1	*m/z* 175 0 → 111 1	*m/z* 175 0 → 91 1	*m/z* 175 0 → 111 1	*m/z* 175 0 → 91 1	*m/z* 175 0 → 111 1
Baseline noise of both control samples (cps)	8.2 7.3	6.6 2.9	6.8 5.8	3.8 3.8	3.5 5.5	1.8 1.5
Mean baseline noise x 3 (cps)	23	14	19	11	14	5
Height of standard at 1.25 µg/L (cps)	378	190	841	357	283	138
Estimated LOD (µg/L)	0.0015	0.0019	0.0006	0.0008	0.0012	0.0009

The LOQ of the method is defined as the lowest analyte concentration in a sample at which the methodology has been successfully validated. An LOQ of 0.05 µg/L was confirmed for SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 in water.

3.5.2 Sample Analysis

Samples were analysed according to the procedures described in analytical method GRM023.06A detailed in Appendix 3.

The principle of the method is as follows:

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

Alternatively, for analysis of SYN508210, SYN508211 and CSCC210616 the water samples were taken through a solid-phase extraction (SPE) procedure using Oasis HLB cartridges.

The SPE cartridges were washed with ultra pure water and the compounds were eluted with acetonitrile. The final volume was adjusted to 5 mL with acetonitrile. A 0.5 mL aliquot of the eluate was diluted with ultra pure water for analysis of SYN508210 and SYN508211. A 1 mL aliquot was diluted with ultra pure water for the analysis of CSCC210616.

For the analysis of CSCD465008 and CSAA798670, water samples were acidified then taken through a solid-phase extraction (SPE) procedure using Oasis HLB cartridges. The SPE cartridges were washed with ultra pure water and the compounds were eluted with 50/50 v/v acetonitrile/ultra pure water. The column eluates were evaporated to remove the acetonitrile and then diluted with ultra pure water.

For all analytes, final determination was by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

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 - 18).

3.5.3 Detector Linearity

Standard solutions containing:

- 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),

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 injected onto the column. The lowest concentration injected was at 50% of the LOQ of the method. The highest concentration injected was equivalent to 20×LOQ (see Tables 19 to 25).

3.5.4 Storage Stability of Sample Extracts

The stability of the final extracts of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 was assessed by storing the extracts between 0 and 9°C. The samples were initially analysed 1- 2 days after extraction and were re-analysed after 8 - 16 days of storage. The recovery data obtained are summarised in Tables 26 - 33 and detailed analytical results are presented in Tables 34 - 41.

3.5.5 Matrix Effects

The fortified samples were analysed using standard solutions prepared in acetonitrile/ultra pure water (50/50, v/v) for SYN508210 and SYN508211 and prepared in ultra pure water for CSCC210616, CSCD465008 and CSAA798670.
Each sample set included an appropriate matrix-matched standard, prepared in the presence of matrix. For each matrix, the response obtained from the matrix-matched standard was compared to the response obtained from the standard in solvent to allow calculation of any matrix effect (either suppression or enhancement of response). The results are presented in Table 42.

APPENDIX 3 ANALYTICAL METHOD DESCRIPTION

1. PREPARATION AND USE OF THE STANDARD SOLUTIONS

The standard solutions must be stored in a refrigerator or in a freezer protected from light when not in use and should be freshly prepared. The standard solutions used for this study were stored at 4°C (between 0 and 9°C).

1.1. Stock Solutions

Between 5 and 50 mg of SYN508210, SYN508211, CSCC210616, CSCD465008 or CSAA798670 were accurately weighed into separated amber volumetric flasks.
Adequate volume of acetonitrile 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 the SYN508210 and SYN508211 primary stock solutions were performed in acetonitrile to obtain solutions at 100 and 10 µg/L.
Appropriate serial dilutions of the CSCC210616, CSCD465008 and CSAA798670 primary stock solutions were performed in ultra pure water to obtain solutions at 100 and 10 µg/L.

1.3. Calibration Solutions of SYN508210 and SYN508211

Appropriate serial dilutions of the SYN508210 and SYN508211 primary stock solutions were performed in 50/50 v/v acetonitrile/ultra pure water to obtain solutions at 100 and 10 µg/L. Appropriate serial dilutions of these SYN508210 and SYN508211 solutions were performed in 50/50 v/v acetonitrile/ultra pure water at the following concentrations:

0.125 – 0.25 – 0.5 – 1.25 – 3.0 and 5.0 µg/L.

Calibration standards were prepared by ten-fold dilution of the above standards in 50/50 v/v acetonitrile/ultra pure water as no matrix effect was observed. The following calibration solutions were obtained:

0.0125 – 0.025 – 0.05 – 0.125 – 0.3 and 0.5 µg/L.

1.4. Calibration solutions of CSCC210616

Appropriate serial dilutions of the CSCC210616 primary stock solution were performed in ultra pure water to obtain solutions at 100 and 10 µg/L. Appropriate serial dilutions of these solutions were performed in ultra pure water at the following concentrations:

0.25 – 0.5 – 1.0 – 2.5 – 7.5 and 10.0 µg/L.

Calibration standards were prepared by ten-fold dilution of the above standards in ultra pure water as no matrix effect was observed. The following calibration solutions were obtained:

0.025 – 0.05 – 0.1 – 0.25 – 0.75 and 1.0 µg/L.

1.5. Calibration solutions of CSCD465008 and CSAA798670

Appropriate serial dilutions of the CSCD465008 and CSAA798670 primary stock solutions were performed in ultra pure water to obtain solutions at 10 and 1 µg/mL. Appropriate serial dilutions of the CSCD465008 and CSAA798670 solutions were performed in ultra pure water at the following concentrations:

12.5 – 25 – 50 – 100 – 350 and 500 µg/L.

Calibration standards were prepared by ten-fold dilution of the above standards in ultra pure water as no matrix effect was observed. The following calibration solutions were obtained:

1.25 – 2.5 – 5 – 10 – 35 and 50 µg/L.

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)
- HPLC pump: Series 200 (Perkin Elmer) and Shimadzu
- HPLC column: ACE C18 5 µm, 100 × 3 mm (AIT)
- HPLC column: Develosil RP Aqueous 3 µm, 150 × 3 mm (AIT)
- Cartridge Oasis HLB 60 mg/3 mL (Waters)
- Evaporating block equipped with a nitrogen flow system

- Vortex
- Precision balances
- Ultrasonic bath
- General glassware

2.2. Reagents

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

3. ANALYTICAL PROCEDURE

3.1. 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.

3.2. 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.

3.3. 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^{TM} 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.

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. 10 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 2 mL/min collecting the column eluates. Apply positive pressure or a high 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) Transfer a 1 mL aliquot into a suitable tube for analysis of CSCC210616 by LC-MS/MS. Add ultra pure water (9 mL) and ultrasonicate briefly to mix thoroughly. The final sample concentration is 1 mL/mL.

j) Transfer a 0.5 mL aliquot into a suitable tube for analysis of SYN508210 and SYN508211 by LC-MS/MS. Add ultra pure water (9.5 mL) and ultrasonicate briefly to mix thoroughly. The final sample concentration is 0.5 mL/mL.

k) Transfer the samples into suitable autosampler vials ready for final determination of SYN508210, SYN508211 and CSCC210616 by LC-MS/MS.

3.4. 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 3.7 (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.

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 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 ultra sonication of the contents of centrifuge tube.

i) Transfer the samples into suitable autosampler vials ready for final determination by LC-MS/MS. The final sample concentration is 50 mL/mL.

4. PARAMETERS FOR CHROMATOGRAPHIC ANALYSIS

4.1. Operating Indications for SYN508210 and SYN508211

The following parameters were used during the study. They may be adapted to the equipment used.

LC-MS/MS:

- Pump: Series 200, Perkin Elmer or Shimadzu
- Detector: API 4000, Sciex
- Data Acquisition: Analyst 1.4.2
- Column: ACE5 C18 5 µm, 100 × 3 mm
- Column temperature: 40 ± 5°C
- Retention time: SYN508210: approximately 6.7 – 6.9 minutes
  SYN508211: approximately 7.2 – 7.4 minutes
- Injection volume: 50 µL
- Injector temperature: 20 ± 5°C
- Flow: 0.6 mL/minute
- Mobile phase: Solvent A: methanol
  Solvent B: ultra pure water + 0.1% formic acid

- Gradient

Time (minute)	% A	% B
0.0	50	50
6.0	90	10
9.0	90	10
9.1	50	50
13.0	50	50

- Switch

Time (minute)	Position
0.0	waste
2.0	spectrometer

- Ionisation mode: Negative
- Scan Type: MRM
- Calibration range: 0.0125 to 0.5 µg/L

Analyte	**Parent ion (*m/z*)**	**Daughter ion (*m/z*)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
SYN508210 and SYN508211	330.2	131.1 (quantification)	-90	-10	-30	-10
		91.0 (confirmatory)	-90	-10	-46	-6

CAD (collision gas)	4
CUR (curtain gas)	25
GS1 (ion source gas 1)	50
GS2 (ion source gas 2)	60
IS (ion spray voltage)	-4500
TEM (°C)	550
RESOLUTION Q1	Unit
RESOLUTION Q3	Unit
Dwell (ms)	600

4.2. Operating indications for CSCC210616

The following parameters were used during the study. They may be adapted to the equipment used.

LC-MS/MS:

- Pump + Injector: Series 200, Perkin Elmer and Shimadzu
- Detector: API 4000, Sciex
- Data Acquisition: Analyst 1.4.2, Sciex
- Column: Develosil RP Aqueous 3 µm, 150 × 3 mm
- Column temperature: 40 ± 5°C
- Retention time: approximately 3.4 minutes
- Injection volume: 50 µL
- Injector temperature: 20 ± 5°C
- Flow: 0.5 mL/minute
- Mobile phase: 80/20 v/v ultra pure water + 0.1% formic acid/methanol
- Switch

Time (minute)	Position
0.0	waste
0.8	spectrometer

- Ionisation mode: Positive
- Scan Type: MRM
- Calibration range: 0.025 to 1 µg/L

Analyte	**Parent ion (*m/z*)**	**Daughter ion (*m/z*)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
CSCC210616	176.0	136.1 (quantification)	50	10	23	13
		156.1 (confirmatory)	50	10	14	14

CAD (collision gas)	4
CUR (curtain gas)	25
GS1 (ion source gas 1)	50
GS2 (ion source gas 2)	60
IS (ion spray voltage)	5500
TEM (°C)	500
RESOLUTION Q1	Unit
RESOLUTION Q3	Unit
Dwell (ms)	300

4.3. Operating indications for CSCD465008 and CSAA798670

The following parameters were used during the study. They may be adapted to the equipment used.

LC-MS/MS:

- Pump + Injector: Series 200, Perkin Elmer and Shimadzu
- Detector: API 4000, Sciex
- Data Acquisition: Analyst 1.4.2, Sciex
- Column: Develosil RP Aqueous 3 µm, 150 × 3 mm
- Column temperature: 40 ± 5°C
- Retention time: CSCD465008: approximately 1.7 - 2.4 minutes
  CSAA798670: approximately 1.8 - 3.4 minutes
- Injection volume: 10 µL
- Injector temperature: 20 ± 5°C
- Flow: 0.5 mL/minute
- Mobile phase: 80/20 v/v ultra pure water + 0.2% acetic acid/acetonitrile
- Switch

Time (minute)	Position
0.0	waste
0.8	spectrometer

- Ionisation mode: Negative
- Scan Type: MRM
- Calibration range: 1.25 to 50 µg/L

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
CSCD465008	161.0	141.0 (quantification)	-45	-10	-13	-11
		65.9 (confirmatory)	-45	-10	-30	-10
CSAA798670	175.0	91.1 (quantification)	-50	-10	-29	-13
		111.1 (confirmatory)	-50	-10	-23	-8

CAD (collision gas)	4
CUR (curtain gas)	25
GS1 (ion source gas 1)	50
GS2 (ion source gas 2)	60
IS (ion spray voltage)	-4500
TEM (°C)	550
RESOLUTION Q1	Unit
RESOLUTION Q3	Unit
Dwell CSCD465008 (ms)	100
Dwell CSAA798670 (ms)	300

4.4. Calibration

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

4.5. Result calculation

The chromatographic system was calibrated using a calibration curve of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 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 SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670
A = slope of the linear least squares fit of the calibration curve
C = concentration determined from standard curve (µg/L)
B = Y-intercept of the linear least squares fit of the calibration curve.

Note: for the solid phase extraction procedure only, SYN508210, SYN508211 were quantified using a 1/x weighing linear regression.

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

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

$$Residue\ (\mu g/L) = \frac{extract\ concentration\ (\mu g/L) \times final\ volume\ (mL)}{initial\ volume\ (mL)}$$

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 SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 found in test sample (µg/L).
S = concentration of SYN508210, SYN508211, CSCC210616, CSCD465008 and CSAA798670 added in test sample (µg/L).