1.0 INTRODUCTION

1.1 Scope and Chemical Structures

Analytical method GRM023.05A is suitable for the determination of CSCD465008 and CSAA798670 (Figures 1 and 2 respectively) in soil. The limit of quantification (LOQ) of the method has been established at 0.0005 mg/kg.

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

Figure 1

Compound Code Number	**:**	CSCD465008 (also known as 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

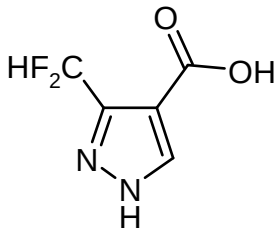
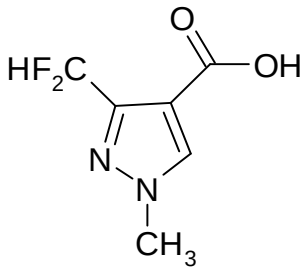


Figure 2

Compound Code Number	**:**	CSAA798670
Alternative Number		NOA449410
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



1.2 Method Summary

10 g soil samples are shaken at room temperature with 0.2% v/v formic acid in ultra pure water for 1 hour. Samples are centrifuged and an aliquot (20 mL, equivalent to 4 g) is acidified and taken through a solid phase extraction procedure, using Oasis HLB SPE cartridges. CSCD465008 and CSAA798670 are eluted from the cartridge with 50/50 v/v acetonitrile/ultra pure water. The eluates are evaporated to remove the acetonitrile and then diluted with ultra pure water. Final determination is by high performance liquid chromatography with triple quadrupole mass spectrometry detection (LC-MS/MS). The limit of quantification of the method is 0.0005 mg/kg.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock solutions

Weigh out accurately; using a five-figure balance, sufficient CSCD465008 and CSAA798670 analytical standards to allow dilution in acetonitrile to give 200 μg/mL stock solutions in volumetric flasks.

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 an appropriate storage vessel.

2.3.2 Fortification solutions

Sample fortification solutions containing CSCD465008 and CSAA798670 should be prepared by serial dilution in 50/50 v/v acetonitrile/ultra pure water. Mixed CSCD465008 and CSAA798670 standards may be prepared if required. It is recommended that the following solutions are prepared: 10.0 μg/mL, 1.0 μg/mL and 0.1 μg/mL. The preparation of LC-MS/MS calibration standards is discussed in Section 3.8.

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 prior to use.

An expiration date of six months for CSCD465008 and CSAA798670 is recommended unless additional data are generated to support a longer expiration date.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as ‘Hazards in the Chemical Laboratory’, edited by S G Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent hazards

	Acetonitrile	Concentrated hydrochloric acid	Methanol	Formic acid	Acetic acid
Harmful Vapour	✓	✓	✓	✓	✓
Highly Flammable	✓	✖	✓	✖	✖
Harmful by Skin Absorption	✓	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✖	✓	✓
Causes burns	✖	✓	✓	✓	✓
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	7	310	9	37
OES Long Term (mg/m^3)	70	N/A	260	N/A	25

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 CSCD465008 and CSAA798670. CSCD465008 and CSAA798670 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

The method is summarized in flow chart form in Appendix 8.

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) The samples should not be evaporated to dryness at any point, as low recovery of CSCD465008 in particular may occur

3.2 Sample Preparation

Samples should be prepared using an approved method of sample preparation for residue analysis.

3.3 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-weighed control soil sample, add the appropriate amount of standard solution containing CSCD465008 and CSAA798670 in 50/50 v/v acetonitrile/ultra pure water. Let each sample stand for at least five minutes after fortification to allow the spiking solution to soak into the matrix before proceeding with the extraction procedure. At least one untreated control and two fortified control samples should be analysed with each sample set.

3.4 Extraction

a) Weigh 10 g soil into a clean, polypropylene, disposable centrifuge bottle (250 mL size). Fortify samples as required at this point. Add 0.2% v/v formic acid in ultra pure water (50 mL) and cap the bottles securely. Shake on a mechanical shaker at a speed that visibly agitates the samples for 1 hour.

b) Centrifuge samples at 3500 rpm for 5 minutes (or at a speed that visibly separates the solid sample from the supernatant). The sample concentration is now 0.2 g/mL.

c) Transfer aliquots (20 mL, equivalent to 4 g) into polypropylene, disposable centrifuge tubes and acidify with concentrated hydrochloric acid (100 µL) so that pH is <2. The pH may be checked using suitable indicator paper. The low pH is required to retain CSCD465008 on the SPE cartridge. At pH> 2 the carboxylic acid functional group will be ionised and CSCD465008 and CSAA798670 will not be retained on the SPE cartridge, resulting in low recovery.

3.5 Solid Phase Extraction Procedure

a) 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 (e.g. IST Vacmaster). 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.

b) Using a suitable column connector, attach a column reservoir (20 mL capacity) fitted with a frit to prevent blockage of the SPE cartridge with any particulate material in the extract.

c) Load the soil extracts from Section 3.4 (c) onto the SPE cartridges via the 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.

d) 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.

e) Place suitable disposable, plastic, graduated centrifuge tubes (15 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.

f) Evaporate the collected eluates to 0.8 mL ± 0.1 mL under a stream of air 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 >10 % v/v acetonitrile in the sample will have an adverse effect on the chromatography of CSCD465008 and CSAA798670, with poor retention and peak shape.

g) Adjust the final volume to 1 mL with ultra pure water and mix sample thoroughly by ultra-sonication of the contents of centrifuge tube for a few seconds.

h) Transfer the samples into suitable autosampler vials ready for final determination by LC-MS/MS. The final sample concentration is 4 g/mL.

3.6 Time Required for Analysis

The methodology is normally performed with a batch of up to 20 samples. One person can complete the analysis of up to 20 samples in 2 days (8 working hour period).

3.7 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

3.8 Preparation of Calibration Standards for LC-MS/MS

No significant enhancement or suppression of the instrument response for CSCD465008 and CSAA798670 was observed using the procedures described during method development. Significant matrix effects were observed in the method validation for CSCD465008 however, and these were compensated for by application of a correction

factor in the calculation of results. A summary of the matrix effects is included in table 6 in Appendix 3.

Non-matrix calibration standards in ultra pure water should normally be used for quantification but any significant matrix effects observed may be compensated for by using e.g. matrix matched standards, at the discretion of the study director.

Further sample dilution with ultra pure water at Section 3.5 (g) may also be used to eliminate any observed matrix effects if instrument sensitivity allows.

To prepare a 0.002 µg/mL CSCD465008 and CSAA798670 non-matrix standard, add 200 µL of a 0.1 µg/mL CSCD465008 and CSAA798670 standard in 50/50 v/v acetonitrile/ultra pure water to ultra pure water in a 10 mL volumetric flask. Adjust to the 10 mL mark with ultra pure water. Stopper the flask securely and shake gently to mix thoroughly.

To prepare a 0.002 µg/mL matrix-matched standard, take an extra control sample through the analytical procedure as described from Section 3.4 to 3.5 (f). Add 20 µL of a 0.1 µg/mL CSCD465008 and CSAA798670 standard in 50/50 v/v acetonitrile/ultra pure water. Adjust final volume to 1 mL with ultra pure water. Cap the tube securely and ultrasonicate briefly to mix thoroughly for analysis by LC-MS/MS.

It is noted that in the method validation, the calibration standards were prepared in 50/50 v/v acetonitrile/ultra pure water. This is not recommended and calibration standards should normally be prepared in ultra pure water to ensure satisfactory chromatography and instrument sensitivity for CSCD465008 and CSAA798670.

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.

Final determination by LC-MS/MS with 2 transitions is considered to be highly specific and no further confirmatory conditions are included.

4.1 Instrument Description

HPLC system	:	Shimadzu Prominence
Pump	:	Shimadzu LC-20 AD
Degasser	:	Shimadzu DGU-20A5
Column Oven	:	Shimadzu CTO-20A
Detector	:	Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.1
Autosampler	:	Shimadzu SIL-HTC

4.2 Chromatography Conditions for CSCD465008 and CSAA798670

Column	:	Develosil RPAqueous-3 (3 µm) 150 mm x 3 mm
Guard column		Develosil RPAqueous guard column 10 x 4 mm
Column Oven Temperature	:	40°C
Injection volume	:	10 µL
Stop Time	:	7 minutes
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 = 0.1% v/v acetic acid in acetonitrile Solvent 2 = 0.1 % v/v acetic acid in ultra pure water

Isocratic Mobile Phase Conditions

Time (min)	% Solvent 1	% Solvent 2	Flow (mL/min)
0.0	20	80	0.5
7.0	20	80	0.5

Under these conditions the retention time of CSCD465008 is approximately 2.6 minutes and CSAA798670 is approximately 3.6 minutes.

Note : The column eluate is diverted to waste for the first 0.75 min to prevent a build up of inorganic salts from the sample contaminating the mass spectrometer front plate. A secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste is not necessary.

4.3 Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	500°C
Ionspray voltage	:	-4500V
Collision gas setting (CAD)	:	Nitrogen set at 4 (arbitrary units)
Gas 1 (GS1)	:	Air set at 45 (arbitrary units)
Gas 2 (GS2)	:	Air set at 40 (arbitrary units)
Interface heater (ihe)	:	Off
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	:	50 ms	50 ms	100 ms	100 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	-30 V	-30 V	-50 V	-50 V
Entrance potential (EP)	:	-10 V	-10 V	-10 V	-10 V
Collision energy (CE)	:	-12V	-30 V	-29 V	-24 V
Collision cell exit potential (CXP)	:	-5V	-5 V	-5 V	-5 V

Typical chromatograms for CSCD465008 and CSAA798670 in soil extracts are shown in Appendix 4.

5.0 CALCULATION OF RESULTS

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

5.1 Single Point Calibration Procedure

a) Make repeated injections of a mixed standard containing CSCD465008 and CSAA798670 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 mg/kg, using a mean standard response from each of the injections bracketing the sample as follows.

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

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 (g/mL)

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

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

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 mg/kg for each sample as follows.

a) Prepare mixed standard solutions of CSCD465008 and CSAA798670 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 the two analytes. 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 CSCD465008 and CSAA798670 in the sample, expressed as mg/kg, as follows

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

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in g/mL.

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

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

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 CSCD465008 and CSAA798670 in 50/50 v/v acetonitrile/ultra pure water) 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

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

7.2 Reagent and Solvent Interference

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

7.3 Labware Interference

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

8.0 METHOD VALIDATION

8.1 Extractability

CSCD465008 and CSAA798670 are efficiently extracted from soil under the conditions used in this method (Section 3) (Reference 3).

8.2 Recovery Data and Repeatability

Method validation has been carried out on the procedures described in Section 3. The method validation data are reported in eurofins-GAB GmbH report number S09-00917 (Reference 4), and a summary is included in Appendix 3.

8.3 Matrix Effects

Matrix effects (enhancement or suppression) on the instrument response were considered not to be significant during method devloepment and non-matrix calibration standards should normally be used for calibration. Matrix suppression effects were observed during method validation for CSCD465008 and recovery values were corrected for these effects by application of a correction factor in the calculation of results. The matrix effects for CSCD465008 and CSAA798670 are summarised in Table 6 in Appendix 3.

8.4 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 0.0005 mg/kg for CSCD465008 and CSAA798670.

8.5 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 for this procedure in the soil types tested during method validation is estimated at 0.00015 mg/kg for CSCD465008 and CSAA798670, using the primary and confirmatory transitions, based on 30% of the LOQ.

8.6 Detector Linearity

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

The linearity of the LC-MS/MS detector response for CSCD465008 and CSAA798670 was tested in the range from 0.6 to 100 ng/mL (equivalent to 6 pg to 1000 pg injected on column when using a 10 µL injection volume) and was found to be linear. This is equivalent to a range of 30% LOQ – 50 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.

CSCD465008 and CSAA798670 standards at 8 different concentration levels were injected and the response plotted against amount injected, using Microsoft Excel for both primary transition and confirmatory transitions.

Detector linearity graphs are presented in Appendix 5.

8.7 Extract Stability

Residues of CSCD465008 and CSAA798670 in final soil extracts in ultra pure water were found to be stable when stored for a period of 13 days at a nominal temperature of 4°C when analysed against a freshly prepared calibration standard (Reference 4). It can reasonably be assumed that other soil matrices will show similar stability.

9.0 LIMITATIONS

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

10.0 CONCLUSIONS

Method GRM023.05A has been demonstrated to be a reliable and accurate procedure for the determination of CSCD465008 and CSAA798670 in soil matrices, using commercially available laboratory equipment and reagents. The limit of quantification of the method is 0.0005 mg/kg for both analytes.

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

11.0 REFERENCES

1. Luxon S G (1992): Hazards in the Chemical Laboratory 5th Edition. The Royal Society of Chemistry. Thomas Graham House, The Science Park, Cambridge CB4 4WF, UK.ISBN 0-85186-229-2.

2. Cardone M J, Palermo P J and Sybrand L B: Potential error in single point ratio calculations based on linear calibration curves with a significant intercept. Anal Chem., 52 pp 1187-1191, 1980.

3. Fitzmaurice M and Mackenzie E (2009). SYN524464 : [14C]-SYN524464 - Rate of Degradation in Three Soils at 20°C. Battelle UK Ltd. Report Number NC/07/015.

4. Mewis A (2009): SYN524464 – Validation of a Method for the Determination of CSCD465008 and CSAA798670 in Soil. eurofins-GAB GmbH report number S09-00917.

APPENDIX 1 APPARATUS

UK suppliers

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

High density polypropylene centrifuge bottles (250 mL size), available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.

Mechanical shaker, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.

Laboratory centrifuge, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.

Polypropylene centrifuge tubes, 50 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.

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

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

Column reservoirs, 20 mL size, fitted with 2 frits (part no.12131017) available from Varian Ltd, 28 Manor Road, Walton-on-Thames, Surrey, KT12 2QF

Polypropylene centrifuge tubes, 15 mL capacity, 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.

Shimadzu Prominence HPLC system equipped with autosampler, dual piston 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.

Develosil RP Aqueous-3 HPLC column 3 µm, 150 x 3.0 mm (monomeric sorbent), available from Phenomenex, Queens Avenue, Hurdsfield Ind., Est., Macclesfield, Cheshire, SK10 2BN. Catalogue number CH0-6001.

Develosil RP Aqueous guard column 10 x 4 mm, available from Phenomenex, Queens Avenue, Hurdsfield Ind., Est., Macclesfield, Cheshire, SK10 2BN

Gas generator, e.g. 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, Liberty Lane, Hampton, NH 03842.

High density polypropylene centrifuge tubes (250 mL size), available from Fisher Scientific, Liberty Lane, Hampton, NH 03842.

Mechanical shaker, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842.

Laboratory centrifuge, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842.

Polypropylene centrifuge tubes, 50 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, 60 mg, 3 mL size, available from Waters Corporation, 34 Maple Street, Milford, Massachusetts, 01757-3696.

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

Column reservoirs, 20 mL size, fitted with 2 frits (part no. 12131017) available from Varian Inc., 2700 Mitchell Drive, Walnut Creek, CA 94598.

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.

Shimadzu Prominence HPLC system equipped with autosampler, dual piston pump, vacuum degasser and column compartment with column switching valve, available from Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD 21046.

Develosil RPAqueous-3 HPLC column, 3 μm, 150 x 3.0 mm (monomeric sorbent), available from Phenomenex, 411 Madrid Ave., Torrance, CA 90501-1430. Catalogue number CH0-6001.

Develosil RPAqueous guard column 10 x 4 mm, available from Phenomenex, 411 Madrid Ave., Torrance, CA 90501-1430

Gas generator, e.g. 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), acetonitrile and methanol available from Rathburn Chemicals Ltd., Walkerburn, EH43 6AU.

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

CSCD465008 and CSAA798670 analytical standard, available from GLP Testing Facility, Syngenta, CH-4333, Munchwilen, 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, concentrated hydrochloric acid and glacial acetic acid available from www.sigmaaldrich.com

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

Preparation of reagents

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

2. 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.

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

4. 0.1% v/v acetic acid in acetonitrile.
   Add concentrated acetic acid (1 mL) to 1 L acetonitrile in a 1L volumetric flask. Stopper flask securely and mix thoroughly by shaking.

Determination of LC-MS/MS matrix effects

The effect of soil matrices on the LC-MS/MS signal was assessed by preparing standards in the presence of soil matrix and comparing the peak areas of CSCD465008 and CSAA798670 against non-matrix standards at an equivalent concentration.

To prepare for example, a 0.002 µg/mL matrix-matched standard, take an extra control sample through the analytical procedure as described from Section 3.4 to 3.5 (f). Add 20 µL of a 0.1 µg/mL CSCD465008 and CSAA798670 standard in 50:50 v/v acetonitrile/ultra pure water. Adjust final volume to 1 mL with ultra pure water. Cap the tube securely and ultrasonicate briefly to mix thoroughly for analysis by LC-MS/MS.
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Table 6. : Matrix Effects CSCD465008.

Soil Type	CSCD465008 concentration - nominal (ng/mL)	Matrix effect (%)	
		m/z 161/141	*m/z* 161/66
Clay (Soil Type 6S)	2	-19	-20
	20	-27	-25
Loamy sand (Soil Type 2.2)	2	-18	-25
	20	-16	-15

Table 7. : Matrix Effects CSAA798670.

Soil Type	CSAA798670 concentration - nominal (ng/mL)	Matrix effect (%)	
		m/z 175/91	*m/z* 175/111
Clay (Soil Type 6S)	2	-2	1
	20	-5	-4
Loamy sand (Soil Type 2.2)	2	-10	-7
	20	-3	0

The matrix effects on the instrument response were considered to be significant for CSCD465008 in the soil types tested and these effects were compensated for application of a correction factor in the calculation of results. Matrix effects on the instrument response were considered not to be significant for CSAA798670 the in method validation and no correction for matrix effects was necessary.

No significant matrix effects were observed during method development however, and non-matrix standards should normally be used for quantification. Any matrix effects observed may be compensated for by use of matrix matched standards, at the discretion of the study director. Alternatively, where instrument sensitivity permits, samples may be further diluted with ultra pure water at point 3.5 (g).

APPENDIX 6 LC-MS/MS TUNING PROCEDURE

Calibration of instrument

The instrument must be mass-calibrated on a regular basis using polypropylene glycol (PPG) solutions according to the manufacturer's instructions. Calibrate both mass-resolving quadrupoles (Q1 and Q3).

Tuning instrument for CSCD465008 and CSAA798670

Infuse a standard solution of CSCD465008 and CSAA798670 (0.1 to 1.0 µg/mL in mobile phase, see section 4.2) directly into the mass spectrometer interface at a rate 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 *m/z* 161 for CSCD465008 and *m/z* 175 for CSCAA798670 under negative ionisation conditions.

Using the Analyst 1.4 software quantitative optimisation routine, tune the instrument for CSCD465008 and CSAA798670, ensuring that the correct ions are selected (initial Q1 *m/z* 161 and product ions *m/z* 141 and *m/z* 66 for CDCD465008, corresponding to loss of HF from the molecular ion and the pyrazole fragment respectively; initial Q1 *m/z* 175 and product ions *m/z* 111 and *m/z* 91 for CSCAA798670, corresponding to loss of HF and CO_2 from the molecular ion and a further loss of HF respectively. Alternatively, the instrument ion optics and collision energy may be tuned manually for CSCD465008 and CSAA798670, to ensure maximum sensitivity.

Note: If problems are encountered in tuning the instrument for these ions, the ions should be entered in the method as detailed in Section 4.3 and tuning performed manually.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of 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 CSCD465008 generated in the ion source are selected and subjected to further fragmentation by collisional activation. The most sensitive daughter ions (*m/z* 141 and *m/z* 66) are then selected and used for quantitative analysis.

In negative ionisation mode, anions of CSAA798670 generated in the ion source are selected and subjected to further fragmentation by collisional activation. The most sensitive daughter ions (*m/z* 111 and *m/z* 91) are then selected and used for quantitative analysis.

APPENDIX 8 METHOD FLOW CHART

Shake 10 g soil sample with 0.2% v/v formic acid in UPW (50 mL)

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Centrifuge

↓

Acidify 4 g (20mL) aliquots

↓

Oasis HLB SPE procedure

↓

Eliminate acetonitrile by evaporation

↓

Adjust to final volume (1 mL) with ultra-pure water

↓

Analyse by LC-MS/MS