1.0 INTRODUCTION

1.1 Scope

Analytical method GRM030.06A is suitable for the determination of NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 (Figures 1 to 7) in water. The limit of quantification (LOQ) of the method has been established at 0.01µg/L for NOA449280, SYN503780, CSCD656832 CSCD642512, CSAA806573 and CSCC163768.

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

1.2 Method Summary

10mL water sample are acidified to less than pH 2 by addition of concentrated formic acid. Samples are subjected to SPE clean-up using Strata-X 33 µm 60g/6mL cartridges. Analytes are eluted in methanol, acetonitrile and formic acid and evaporated to dryness. Residues are reconstituted in methanol/0.2% formic acid in water (10/90 v/v) and analysed by LC-MS/MS. The limit of quantification (LOQ) of the method has been established at 0.01µg/L for NOA449280 and its metabolites SYN503780, CSCD656832 CSCD642512, CSAA806573 and CSCC163768.

The limit of quantification of the method is 0.01 µg/L (0.01 ppb) for each analyte.

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

Prepare individual 0.50 to 200 µg/mL (1 mg/mL during the validation phase) stock solutions for NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512, CSAA806573 or CSAA589691 analytical standard into an amber "Class A" volumetric flask (50 mL). Dilute to the mark with methanol to give 50 - 200 µg/mL (1 mg/mL during the validation phase) stock solutions of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512, CSAA806573 or CSAA589691.

Alternatively, the appropriate volume of methanol 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 methanol 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 NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 should be prepared by serial dilution in methanol first and subsequently with methanol/0.2% formic acid in water (1/9, v/v). It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL. Mixed standards of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 may be prepared if desired.

It should be noted that during method development CSSD656832 and CSCD642512 were shown to be unstable at 0.1µg/mL concentration. It is therefore recommended that if a standard of CSCD642512 and CSSD656832 is required at this concentration is should be prepared fresh on the day of use from a higher concentration stock solution.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant matrix effects, suppression or enhancement of the instrument response for NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 has been observed in the water types tested using the procedures described in Section 3 during method validation and non-matrix calibration standards should normally be used for quantification.

Sample calibration solutions containing NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 should be prepared by serial dilution in methanol/0.2% formic acid in ultrapure water (1/9, v/v). The final calibration solutions are recommended to contain each analyte at concentration levels ranging from 0.015 ng/mL to 0.5 ng/mL.

A calibration curve may also be generated to quantify NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 standard in methanol/0.2% formic acid in ultrapure water (1/9, v/v).

Any matrix effects observed may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with methanol/0.2 % formic acid in ultrapure water (1/9, v/v) should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of four months (Reference 1) for NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 in methanol/0.2 % aqueous formic acid 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 2).

Solvent and Reagent hazards

	Methanol	Formic acid
Harmful Vapour	✓	✓
Highly Flammable	✓	✓
Harmful by Skin Absorption	✓	✓
Irritant to respiratory system and eyes	✓	✓
Causes severe burns	✗	✓
Syngenta Hazard Category (SHC)	SHC-C, S	SHC-C, S
OES Short Term (mg/m^3)	310	N/A
OES Long Term (mg/m^3)	260	9

N/A not known

At present there are insufficient data available to assign a Syngenta Hazard Classification to NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573. They should be treated as category SHC-D compounds until further information indicates otherwise. The Syngenta Hazard Category scale rates highly toxic chemicals as category SHC-E and non-toxic chemicals as category SHC-A. An additional hazard category of S indicates the compound is a severe skin and eye irritant.

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

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Preparation

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

b) Transfer 10 mL of the water sample to be analysed into a glass vial (~ 40 mL size). Sample fortification, if required, is to be carried out at this time.

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-measured control water sample, add the appropriate amount of standard solution containing NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 in methanol/0.2% formic acid in water (1/9, v/v). At least one untreated control and two control samples fortified with a known amount of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

c) Add 200 µL of concentrated formic acid and shake well to mix. Check pH of each sample is below pH 2 using an appropriate indicator paper.

3.2 Solid Phase Extraction Procedure.

a) Take one Strata-X 33µm SPE cartridge (60 mg, 6 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 to the level of the top frit, discarding the column eluate. Do not allow the cartridges to become dry.

b) Using a suitable column connector, a column reservoir (70 mL capacity) may be attached if desired. The reservoir should be fitted with a frit to prevent blockage of the SPE cartridge with any particulate material in the extract.

c) Load water samples from Section 3.1 (c) onto the SPE cartridges (a suitable column reservoir may be used if desired) and allow to percolate through under gravity 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, rinse sample vessels with 2 mL of 2% formic acid in ultra pure water, add to the top of the SPE cartridge and allow to percolate through under gravity to the level of the top frit, again discarding the column eluate. Remove the excess water by application of high vacuum for 10 seconds but do not dry for extended periods.

e) Place suitable collection tubes (e.g. 10 mL or 15 mL glass test tubes) under each port, as required, in the manifold rack. Elute the cartridges with 0.1% of formic acid in methanol (5 mL), under gravity at a rate of approximately 1 - 2 mL/min to the level of the top frit collecting the column eluate. Apply high vacuum for approximately 5 seconds to collect the excess solvent from the SPE cartridges. NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 are eluted in this step.

f) Evaporate the collected eluates to dryness under a stream of nitrogen in a sample concentrator with the water bath set at 40 ^{o}C.

g) Add 100µL of methanol and mix the sample thoroughly by ultrasonicating the contents of glass test tube briefly.

h) Add 900 µL of 0.2% formic acid in ultra pure water and mix well to prepare the final sample extract.

i) Just before chromatography, dilute an aliquot of the final sample 2-fold and transfer the sample to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 5 mL/mL or 0.005 L/mL.

j) If necessary dilute the final sample extract with expected higher concentrations of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 using further methanol/0.2% formic acid in water (1/9, v/v) to a suitable final volume to within the linear calibration range.

3.3 Experimental Precautions

a) The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Since the method is extremely sensitive co-eluted material at very low concentration level can significantly impact the chromatography. For instance even HPLC grade methanol can enhance the baseline to an unacceptable level. Therefore it is recommended to use methanol and water of highest available purity.

c) To achieve highest possible sensitivity a very well maintained and cleaned LC-MSMS system is essential. The curtain plate, the skimmer and the first quadrupole have to be kept very clean and polished where possible.

d) To prevent contamination of the instrument and to minimise possible carry-over issues, it is recommended that high level recoveries (>0.5 µg/L) and samples with expected residues greater than 0.5 µg/L should be diluted so that the final analyte concentration does not exceed 0.005 µg/mL. It may also be useful to include blank injections of methanol/0.2% formic acid in ultra pure water (5/95 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

e) CSAA806573 is not stable in the final extract solution (methanol/0.2% formic acid in ultra pure water (1/9, v/v), even when refrigerated. Ideally, this analyte should be analysed within one day after extraction. Acceptable recoveries (74 – 84 %) were obtained in method validation after storage of the groundwater samples for 7 days refrigerated but some degradation is apparent.

f) Calibration standard solutions have to be diluted freshly on each day before a new set of samples is analysed, as CSSD656832 and CSCD642512 were shown to be unstable at 0.1µg/mL concentration during method development.

3.4 Time Required for Analysis

The methodology is normally performed with a batch of 20 samples. One person can complete the analysis of 20 samples in 1 day (8 hour working period).

3.5 Method Stopping Points

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

4.0 FINAL DETERMINATION

The method has been developed and validated for use on an Applied Biosystems API4000. 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.

4.1 Instrument Description

Pump	:	model G1311A, Series 1200 - Agilent
Degasser	:	model G1322A, Series 1200 - Agilent
Column Oven	:	model G1316A including switching 6-port valve, Series 1200 - Agilent
Detector	:	model API 4000 - Applied Biosystems
Autosampler	:	model HTC Pal – CTC Analytics
Gas Supply	:	central gas supply of the laboratory

4.2 Chromatography Conditions

Guard column		C18, 4 mm x 2.0 mm, Phenomenex
Column	:	XTerra® MS C18, 4.6 mm x 50 mm, 3.5 µm, Waters
Column Oven Temperature	:	40 °C
Injection volume	:	50 µL
Stop Time	:	20 min
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	solvent 1: 0.2 % formic acid in ultra pure water solvent 2: methanol

Mobile Phase Composition

Time (min)	% Solvent 1	% Solvent 2	Flow, mL/min
0.0	90	10	0.75
5.0	5	95	0.75
12.0	5	95	0.75
12.1	90	10	0.75
20.0	90	10	0.75

Notes : The column eluate is diverted to waste for the first 2 minutes to prevent ionic material from the sample contaminating the mass spectrometer front plate. A secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste has been found to be unnecessary.

4.3 Mass Spectrometer Conditions for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573

Interface	:	TurboIonSpray
Polarity	:	Negative (positive for NOA449280)
Curtain gas (CUR)	:	Nitrogen set at 10 (psig)
Temperature (TEM)	:	500 °C
Ionspray voltage	:	- 4500 V
Collision gas setting (CAD)	:	Nitrogen set at 12 (psig)
Gas 1 (GS1)	:	Air set at 60 (psig)
Gas 2 (GS2)	:	Air set at 60 (psig)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**CSSS163768** (SYN504810) **primary transition**	**CSSS163768** (SYN504810) **confirmatory transition**	**CSAA806573** (NOA451778) **confirmatory transition**	**CSAA806573** (NOA451778) **primary transition**
Retention window (min)		0 - 4	0 - 4	4.16 - 4.67	4.16 - 4.67
Q1 *m/z*	:	233.91	233.91	219.93	219.93
Q3 *m/z*	:	146.00	189.90	156.00	175.80
Dwell time	:	500 ms	500 ms	500 ms	500 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	- 30 V	- 30 V	- 45 V	- 45 V
Entrance potential (EP)	:	- 10 V	- 10 V	- 10 V	- 10 V
Collision energy (CE)	:	- 24 V	- 16 V	- 30 V	- 16 V
Collision cell exit potential (CXP)	:	- 7 V	- 1 V	- 7 V	- 9 V

MRM Conditions	**SYN503780 confirmatory transition**	**SYN503780 primary transition**	**CSCD642512** (SYN545859) **confirmatory transition**	**CSCD642512**(SYN545859) **primary transition**
Retention window (min)	4.67 - 5.49	4.67 - 5.49	4.67 - 5.49	4.67 - 5.49

Q1 m/z	:	277.96	277.96	398.10	398.10
Q3 m/z	:	145.90	201.80	137.10	340.00
Dwell time	:	500 ms	500 ms	500 ms	500 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	- 50 V	- 50 V	- 65 V	- 65 V
Entrance potential (EP)	:	- 10 V	- 10 V	- 10 V	- 10 V
Collision energy (CE)	:	- 28 V	- 16 V	- 53 V	- 38 V
Collision cell exit potential (CXP)	:	- 11 V	- 9 V	- 7 V	- 7 V
MRM Conditions		**CSCD656832** (SYN545680) **confirmatory transition**	**CSCD656832** (SYN545680) **primary transition**	**NOA449280 primary transition**	**NOA449280 confirmatory transition**
Retention window (min)		4.67 - 5.49	4.67 - 5.49	4.67 - 5.49	4.67 - 5.49
Q1 m/z	:	205.98	205.98	400.00	400.00
Q3 m/z	:	74.20	142.00	228.00	324.00
Dwell time	:	500 ms	500 ms	500 ms	500 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	- 45 V	- 45 V	81 V	81 V
Entrance potential (EP)	:	- 10 V	- 10 V	10 V	10 V
Collision energy (CE)	:	- 46 V	- 30 V	29 V	49 V
Collision cell exit potential (CXP)	:	- 3 V	- 5 V	22 V	16 V

Typical chromatograms are shown in the Figures Section.

4.4 Confirmatory Procedures for NOA449280 and its metabolites SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573

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

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 residues may be calculated in µg/L for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 30% or 50% LOQ to 10 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit ("X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of m and c should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Calculate the NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 residues in the sample, expressed as µg/L, as follows

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

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample concentration is the final sample concentration in L/mL, accounting for any concentration in the SPE step where used.

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

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

5.2 Single Point Calibration Procedure

NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 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 a standard containing NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 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 NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573.

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

d) Calculate the NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 residues in the sample, expressed as µg/L using a mean standard response from each of the injections bracketing the sample as follows.

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

Peak response for sample

Average peak response for bracketing standards

Concentration of standard (µg/mL)

Sample concentration (L/mL)

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

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

Although single point calibration may be used to quantify residues it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 2).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analysed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analysed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 in methanol) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

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

Where the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

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

7.1 Matrix

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

7.2 Reagent and Solvent Interference

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

7.3 Limit of Detection (LOD)

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

The LOD was roughly estimated using the peak of a standard solution with the lowest concentration level of the calibration range. The primary MS/MS transitions were used to estimate the LOD.

The estimated LODs of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 were approximately 0.0003, 0.0007, 0.0005, 0.001, 0.0004 and 0.0008 µg/L, respectively.

7.4 Matrix Effects

No significant matrix effects were observed in the water types tested during method validation and non-matrix standards should generally be used for quantification. A summary of the matrix effects is included in Table 14. Although this peak enhancement was not significant it may indicate that the detection of the metabolites CSD656832 and CSAA806573 can be susceptible to co-eluted substances (matrix effect) and should be assessed. In case a matrix effect is observed matrix matched calibration should be applied.

7.5 Detector Linearity

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

The linearity of the LC-MS/MS detector response for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 was tested in the range from 0.75 pg to 25 pg injected on column (equivalent to 0.015 ng/mL to 0.5 ng/mL standards when using a 50 µL injection volume) and was found to be linear.

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

Standards at 8 different concentration levels in the range 0.015 – 0.5 ng/mL were injected and the response plotted against the amount injected, using Microsoft Excel for both primary and confirmatory transitions for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573.

A plot of the response factor versus the amount injected for all calibration points is also included.

Detector linearity graphs are presented in the Figures Section.

7.6 Extract Stability

Final water samples in methanol/0.2% formic acid in ultra pure water (1/9,v/v) retained in vials and stored at a temperature of approximately 4-8°C were suitable for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 residue analysis, for storage periods of up to 44 days (extracts from surface and drinking water) and up to 7 days in extracts from groundwater. Some degradation of CSAA806753 in surface and drinking water final extracts is apparent at the 44 days storage interval. It is recommended that samples containing CSAA806753 are analysed within a maximum 7 of days of preparation. A summary of the stability data is presented in Table 15 to Table 20.

8.0 LIMITATIONS

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

However, some conditions should be regarded:

- Robust and reliable elution recoveries from the cartridge Strata-X SPE were only obtained with 0.1 % of formic acid in methanol. Other solvent mixtures were found to elute less efficiently.
- The method is extremely sensitive to co-eluted material at very low concentration levels that can significantly impact the chromatography. For instance even HPLC grade methanol can enhance the baseline to an unacceptable level. Therefore it is recommended to use methanol and water of highest available purity.
- To achieve highest possible sensitivity a very well maintained and cleaned LC-MS/MS system is essential. The curtain plate, the skimmer and the first quadrupole have to be kept very clean and polished where possible.
- Although the effect was not significant under the conditions reported in this study, CSCD656832 and CSAA806573 appear to be susceptible to signal enhancement in the presence of certain co-eluted matrix.
- Ideally, the extracts should be analysed within one day after extraction and calibration standard solutions should be diluted freshly from standard stock solutions on each day before a new set of samples is going to be analysed.

CHEMICAL STRUCTURES

Figure 1 NOA449280

Compound Code Number : NOA449280
CAS Number : 352010-68-5
IUPAC Name : 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(triflouro-methyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one
Molecular Formula : $C_{19}H_{20}F_3NO_5$
Molecular Weight : 399.4

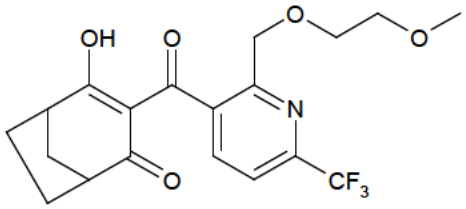


Figure 2 SYN503780

Compound Code Number : SYN503780
CAS Number : 380355-55-5
IUPAC Name : 2-(2-methoxy-ethoxymethyl)-6-triflouromethyl-nicotinic acid
Molecular Formula : $C_{11}H_{12}F_3NO_4$
Molecular Weight : 279.2

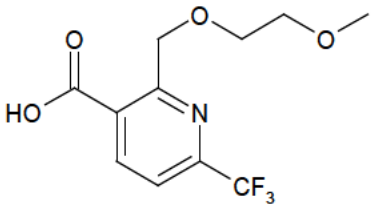


Figure 3 CSCC163768

Compound Code Number : CSCC163768
CAS Number : Not in registry
IUPAC Name : 6-(trifluoromethyl)pyridine-2,3-dicarboxylic acid
Molecular Formula : $C_8H_4F_3NO_4$
Molecular Weight : 235.1

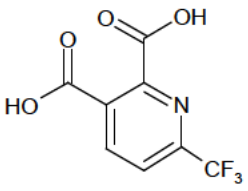


Figure 4 **CSCD656832**

Compound Code Number	:	CSCD656832
CAS Number	:	Not in registry
IUPAC Name	:	6-(trifluoromethyl)pyridin-3-ol-2-carboxylic acid
Molecular Formula	:	$C_7H_4F_3NO_3$
Molecular Weight	:	207.1

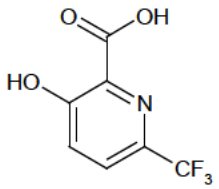


Figure 5 **CSCD642512**

Compound Code Number	:	CSCD642512
CAS Number	:	Not in registry
IUPAC Name	:	Not known
Molecular Formula	:	$C_{18}H_{16}F_3NO_6$
Molecular Weight	:	399.3

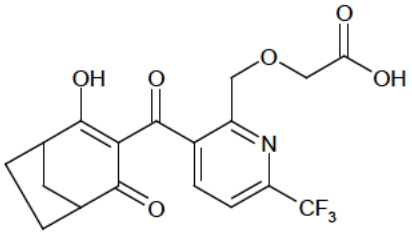
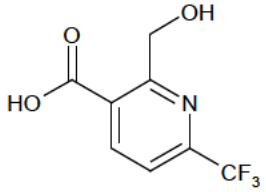


Figure 6 **CSAA806573**

Compound Code Number	:	CSAA806573
CAS Number	:	Not in registry
IUPAC Name	:	Not known
Molecular Formula	:	$C_8H_6F_3NO_3$
Molecular Weight	:	221.1



APPENDIX 1 APPARATUS

Recommended Suppliers

Equipment	Description	Supplier
General glassware	General glassware	www.vwr.de
Centrifuge tube	Glass tube, 15 mL with screw top	www.vwr.de
SPE cartridges	Phenomenex Strata-X 33 µm, 60 mg, 3 mL, polymeric reversed phase, (Part no. 8B-S100-UBJ)	www.phenomenex.com
LC-MS/MS system	API 4000 equipped with a TurboIonSpray source	www.AppliedBiosytems.com
HPLC system	Series 1200	www.home.agilent.com
Autosampler	HTC Pal	www.ctc.ch
Guard column	C18, 4 x 2.0 mm	www.phenomenex.com
HPLC column	XTerra®MS C18, 3.5 µm, 4.6 x 50 mm	www.waters.com
Nitrogen	From internal gas supply	

APPENDIX 2 REAGENTS

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	HPLC grade	Prepared in the lab using an ultra pure water unit (Synergy UV) from Millipore www.millipore.com
Acetonitrile	HPLC grade	www.lgcstandards.com/
Ethanol:	purity ≥ 99.8 %	www.carl-roth.de
Methanol	HPLC grade	www.carl-roth.de
Formic acid	Analtyical grade	www.vwr.de
NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 analytical standards	GLP certified	GLP Testing Facility, Syngenta, CH-4333, Munchweilen, Switzerland or Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) 2% formic acid:
20 mL of concentrated formic acid in 1 L of water

b) 0.2 % formic acid:
2 mL of concentrated formic acid in 1 L of water

c) 0.1 % formic acid:
0.5 mL of concentrated formic acid in 500 mL of methanol

d) Eluent for SPE:
500 mL of methanol, 500 mL of acetonitrile and 2 mL of concentrated formic acid

e) Dilution solution:
900 mL of 0.2 % aqueous formic acid and 100 mL of methanol

APPENDIX 3 LC-MS/MS TUNING PROCEDURE

Calibration of Instrument

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

Tuning instrument for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512, and CSAA806573

Infuse separate standard solutions of NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 (0.01 to 1.0 µg/mL in mobile phase, see section 4) directly into the mass spectrometer interface at a rate at of about 10-20 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* 400 for NOA449280 in positive ionisation mode, *m/z* 278 for SYN503780, *m/z* 234 for CSCC163768, *m/z* 206 for CSCD656832, *m/z* 398 for CSCD642512 and *m/z* 220 for CSAA806573 all in negative ionisation mode.

Using the Analyst 1.4 software quantitative optimisation routine, tune the instrument either positive ionisation mode for NOA449280 or negative ionisation for SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573, ensuring that the correct ions are selected:

Q1 *m/z* 400 and product ions *m/z* 324 and 228 for NOA449280,

Q1 *m/z* 278 and product ions *m/z* 202 and 146 for SYN503780,

Q1 *m/z* 234 and product ions *m/z* 190 and 146 for CSCC163768

Q1 *m/z* 206 and product ions *m/z* 142 and 74 for CSCD656832

Q1 *m/z* 398 and product ions *m/z* 340 and 137 for CSCD642512

Q1 *m/z* 220 and product ions *m/z* 176 and 156 for CSAA806573

Alternatively, the instrument ion optics and collision energy may be tuned manually for NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573, 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 NOA449280, SYN503780, CSCC163768, CSCD656832, CSCD642512 and CSAA806573 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, the protonated molecular ions generated in the ion source are selected and subjected to further fragmentation by collisional activation. For NOA449280 *m/z* 400 → *m/z* 228 is used as the primary transition for quantitative analysis,

and corresponds to loss of the fragments $CH_3O(CH_2)_2OH$ and $N{\equiv}CH\text{-}CF_3$ from the protonated molecular ion. The transition *m/z* 400 → *m/z* 324 may be used as a confirmatory transition, corresponding to loss of the fragment $CH_3O(CH_2)_2OH$.

In negative ionisation mode, the deprotonated molecular ions generated in the ion source are selected and subjected to further fragmentation by collisional activation. For SYN503780 the transition *m/z* 278 → *m/z* 202 is used as the primary transition for quantitative analysis and corresponds to loss of $CH_3\text{-}O\text{-}(CH_2)_2\text{-}O\text{-}$, with cleavage at the ether linkage on the pyridine ring side chain. The transition *m/z* 278 → *m/z* 146 corresponding to the trifluoromethyl-pyridine fragment may be used as a confirmatory transition.

For CSCC163768 the transition *m/z* 234 → *m/z* 146 is used as the primary transition for quantitative analysis and corresponds to loss of 2 x CO_2 from the deprotonated parent molecule. The transition *m/z* 234 → *m/z* 190 may be used as a confirmatory transition, and corresponds to the loss of CO_2 from the deprotonated parent molecule.

For CSCD656832 the transition *m/z* 206 → *m/z* 142 is used as the primary transition for quantitative analysis and corresponds to loss of CO_2 and HF from the parent molecule. The transition *m/z* 206 → *m/z* 74 may be used as a confirmatory transition.

For CSCD642512 the transition *m/z* 398 → *m/z* 340 is used as the primary transition for quantitative analysis and corresponds to loss of $\text{-}CH_2COOH$ from the parent molecule. The transition *m/z* 398 → *m/z* 137 may be used as a confirmatory transition, and corresponds to the pyridine fragment after loss of $\text{–}CH_2COOH$ from the parent molecule and cleavage at the pyridine-carbonyl bond.

For CSAA806573 the transition *m/z* 220 → *m/z* 176 is used as the primary transition for quantitative analysis and corresponds to loss of CO_2 from the deprotonated parent molecule. The transition *m/z* 220 → *m/z* 156 may be used as a confirmatory transition, and corresponds to loss of HF from the previous fragment *m/z* 176.

APPENDIX 4 METHOD FLOW CHART

Acidify 10 g of water with 200 µL of formic acid, mix well,
check if pH is 2

↓

Condition a Strata-X SPE cartridge with
2 mL methanol,
2 mL pure water

↓

Apply the acidified water sample onto the Strata-X SPE cartridge,
rinse vial with 2 mL of 2 % formic acid,
apply the rinseate,
discard eluates,
apply vacuum for 10 sec

↓

Elute from Strata-X SPE cartridge with 5 mL of elution solvent*,
evaporate with nitrogen to dryness on a water bath (40 °C)

↓

Re-constitute with 100 µL of methanol,
mix well (Vortex) and ultrasonicate for 5 min,
add 900 µL of 0.2 % formic acid in pure water
(final volume equivalent to 1 mL)

↓

Before chromatography dilute to at least a factor of 2 as follows:
to 100 µL of this final solution add 100 µL of methanol/0.2 % formic acid in ultra pure water (1+9, v+v)**

↓

Analyse by LC-MS-MS

*Elution solvent: 0.1 % formic acid in methanol

** Final dilution: If higher concentrations of an analyte are expected in the final extract dilute the final extract with methanol/0.2 % formic acid in ultra pure water (1+9, v+v) appropriately to fit the peak into the calibration range