1. INTRODUCTION

1.1 Scope and chemical structures

Analytical method GRM 030.01A is suitable for the determination of NOA449280 (Figure 1) and SYN503780 (Figure 2) in water. The limit of quantitation (LOQ) of the method has been established at 0.01 µg/L.

This method satisfies 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	**:**	NOA449280
CAS Number	**:**	352010-65-5
IUPAC Name	**:**	4-Hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-trifluoromethyl-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one
Molecular Formula	**:**	$C_{19}H_{20}F_3NO_5$
Molecular Mass	**:**	399.39

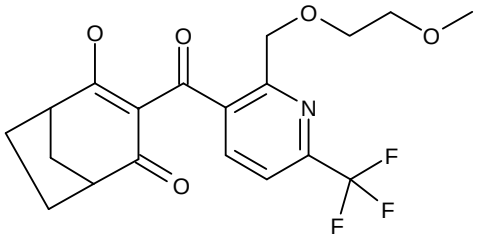
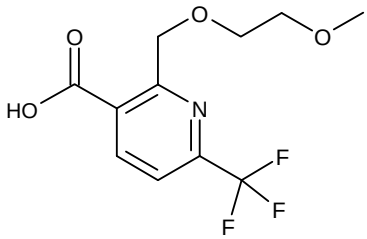


Figure 2

Compound Code Number	**:**	SYN503780
CAS Number	**:**	Not listed
IUPAC Name	**:**	2-(2-Methoxy-ethoxymethyl)-6-trifluoromethyl-nicotinic acid
Molecular Formula	**:**	$C_{11}H_{12}F_3NO_4$
Molecular Mass	**:**	279.22



1.2 Method summary

Acidified environmental water samples are concentrated using solid phase extraction (SPE). After elution with methanol, samples are evaporated to dryness and dissolved in acetonitrile:water 20:80 v/v and analysed by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS). The limit of quantification of the method is 0.01 μg/L.

2. 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.

Prepare individual 200 μg/mL stock solutions for NOA49280 and SYN503780 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient NOA449280 and SYN503780 analytical standards and carefully transfer into separate “Class A” volumetric flasks (50 mL). Dilute to the mark with acetonitrile to give a 200 μg/mL stock solutions of NOA449280 and SYN503780.

Alternatively, the appropriate volume of acetonitrile 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/L)
1000	Unit conversion factor

The standard material is weighed into a "Class A" volumetric flask.

Sample fortification solutions should be prepared in 70:30 v/v acetonitrile:ultra pure water from the primary stock solution in "Class A" volumetric flasks. It is recommended that, as a minimum, the following solutions are prepared by serial dilution: 10 μg/mL, 1.0 μg/mL, 0.1 μg/mL. Mixed NOA449280 and SYN503780 standards may be prepared if desired. The preparation of LC-MS/MS calibration standards is discussed in Section 3.6.

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 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	Methanol	Acetic acid	Formic acid
Harmful Vapour	✓	✓	✓	✓
Highly Flammable	✓	✓	✖	✖
Harmful by Skin Absorption	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓
Causes severe burns	✖	✖	✓	✓
Syngenta Hazard category	SHC-C, S	SHC-C, S	SHC-C, S	SHC-C,
OES Short Term (mg m^3)	105	310	37	9
OES Long Term (mg m^3)	70	260	25	N/A

N/A not known
In all cases avoid breathing vapour. Avoid contact with eyes and skin.

At present there are insufficient data available to assign a Syngenta Hazard Category for NOA449280 and SYN503780. They should be treated as category SHC-C 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 S designation indicates a skin irritant.

3. ANALYTICAL PROCEDURE

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

3.1 Modifications and potential problems

Bottled HPLC grade water is used to prepare aqueous mobile phase as this gives a reduced MS/MS background signal when compared to water from a laboratory water purification system.

3.2 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 polypropylene centrifuge tube (15 mL size). Sample fortification, if required, is to be carried out at this time. Cap the tubes securely and shake gently to mix.

At least one untreated control and two control samples fortified with a known amount of NOA449280 and SYN503780 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 concentrated formic acid (200 µL) to each sample. Cap the tubes and shake gently to ensure thorough mixing. Check that the pH is < pH 2 using suitable indicator paper.

3.3 Solid Phase Extraction

a) Take one Phenomenex Strata-X SPE cartridge (60 mg, 3 mL) 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 each cartridge 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) Load water samples from Section 3.2 (c) onto the SPE cartridges (a suitable column reservoir may be used if desired) 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. NOA449280 and SYN503780 are retained on the SPE cartridges.

c) On completion of loading, wash the empty sample tubes with ultra pure water containing 2% formic acid (2 mL) and add the rinse to the column reservoir. 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) Remove the column reservoir and column connector from the SPE cartridge if used. Briefly apply a high vacuum for approximately 5 - 10 seconds to remove excess water from the cartridges but do not dry for extended periods.

e) Place suitable collection tubes (e.g. 10 mL glass test tubes) under each port, as required, in the manifold rack. Elute the cartridges with methanol (3 mL), under gravity or draw through under low vacuum 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 and SYN503780 are eluted in this step.

f) Evaporate the samples to dryness under a stream of clean, dry air in a sample concentrator with the temperature set to 45 ºC. This should take approximately 20 minutes. Although there is no information to suggest that losses occur if samples are

left at dryness for extended periods it is recommended that samples are removed soon after the solvent has evaporated.

g) Add acetonitrile (200 µL) and ultrasonicate thoroughly. Add ultra pure water (0.8 mL) and again ultrasonicate thoroughly to ensure the sample is completely dissolved and thoroughly mixed.

h) Transfer an aliquot to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 10 mL/mL.

3.4 Time required for analysis

The methodology can be performed with a batch of up 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.

3.6 Preparation of calibration standards for LC-MS/MS

No significant suppression or enhancement of the instrument response for NOA449280 and SYN503780 has been observed in the water types tested using the above procedure in this laboratory. Non-matrix calibration standards should be prepared as described below.

To prepare e.g., an LOQ equivalent calibration standard (0.1 ng/mL), transfer ultra pure water (approximately 9 mL) into a 10 mL volumetric flask and add 10 µL of a 0.1 µg/mL mixed NOA449280 and SYN503780 standard in 70:30 v/v acetonitrile:ultra pure water. Adjust to the 10 mL mark with ultra pure water:acetonitrile 80:20 v/v). Stopper the flask securely and shake to mix thoroughly. Transfer an aliquot of the standard into a suitable autosampler vial ready for analysis by LC-MS/MS.

A calibration curve may also be generated to quantify NOA449280 and SYN503780 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volumes of NOA449280 and SYN503780 standard in 70:30 v/v acetonitrile:ultra pure water.

4. FINAL DETERMINATION

The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use. The method has been developed for use on the Applied Biosystems API 4000 LC-MS/MS.

4.1 Instrument description

Pump	: Agilent 1100 series quaternary pump model number G1311A
Degasser	: Agilent 1100 series model number G1322A
Column Oven	: Agilent 1100 series model number G1316A fitted with column switching valve
Detector	: Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.1
Autosampler	: Agilent 1100 series model number G1313A
Gas Supply	: Peak Scientific NM20ZA gas station

4.2 Chromatography conditions

Column	:	ACE 5 C18 5 µm 50 x 3.0 mm i.d. or Phenomenex Ultracarb ODS (30) 5 µm 50 mm × 3.2 mm i.d
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	4.0 minutes
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 acetonitrile Solvent 2 0.2% acetic acid in ultra pure water

Mobile phase composition

Time (min)	% Solvent 1	% Solvent 2	Flow, mL/min
0.0	20	80	1
2.0	80	20	1
3.0	80	20	1
3.1	20	80	1
4.0	20	80	1

Under these conditions the retention time of SYN503780 is 1.5 minutes and NOA449280 is 2.5 minutes using the ACE column or 1.9 minutes and 2.7 minutes respectively using the Phenomenex Ultracarb. Validation data presented were generated using the ACE column.

Note : It is not necessary to reduce the flow rate into the mass spectrometer when using the API 4000.

4.3 Mass spectrometer conditions

Interface	:	TurboIonSpray
Polarity	:	Period 1 (2.1 minutes): Negative Period 2 (1.4 minutes):Positive
Curtain gas (CUR)	:	Nitrogen set at 17 (arbitrary units)
Temperature (TEM)	:	550 °C
Ionspray voltage	:	-4500 V Period 1 4500 V Period 2
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)
Gas 1 (GS1)	:	Air set at 60 (arbitrary units)
Gas 2 (GS2)	:	Air set at 60 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**NOA449280**	**NOA449280 confirmatory**	**SYN503780**	**SYN503780 confirmatory**
Q1 *m/z*	:	400	400	278	278
Q3 *m/z*	:	324	228	202	146
Dwell time	:	150 ms	150 ms	150 ms	150 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	66 V	66 V	-40 V	-40 V
Entrance potential (EP)	:	10 V	10 V	-10 V	-10 V
Collision energy (CE)	:	31 V	53 V	-18 V	-26 V
Collision cell exit potential (CXP)	:	24 V	55 V	-11 V	-1 V

Typical chromatograms are shown in Appendix 4.

5. CALCULATION OF RESULTS

5.1 Single point calibration procedure

NOA449280 and SYN503780 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 and SYN503780 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 and SYN503780.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to NOA449280 and SYN503780.

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

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

$$\text{Residue}\ (\mu\text{g L}^{-1}) = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}$$

PK area (SA)	Peak response for sample
PK area (STD)	Average peak response for bracketing standards
Standard Conc.	Concentration of standard (µg/mL)
Sample Conc.	Sample concentration (L/mL)

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

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

.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).

5.2 Multi point calibration procedure

NOA449280 and SYN503780 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, 50% LOQ to 10 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 NOA449280 and SYN503780. 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 NOA449280 and SYN503780 residues in the sample, expressed as µg/L as follows

$$\text{Residue}\ (\mu\text{g L}^{-1}) = \frac{\text{Analyte found}\ (\mu\text{g/mL})}{\text{Sample conc.}\ (\text{L/mL})}$$

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

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

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

6. CONTROL AND RECOVERY SAMPLES

Control samples should be completed as detailed in Sections 3.2 - 3.3 for each set of samples analysed to verify that samples are free from NOA449280 and SYN503780 contamination. A minimum of one control should be analysed with each batch of samples.

At least two recovery samples (control samples accurately fortified with a known amount of NOA449280 and SYN503780 prior to extraction) should also be completed alongside each batch of samples. Provided the recovery values are acceptable they may be used to correct any NOA449280 and SYN503780 residues found. The recovery 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. SPECIFICITY

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

7.1 Matrix Interference

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

7.2 Reagent and solvent interference

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

7.3 Labware interference

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

APPENDIX 1 APPARATUS

UK suppliers

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

Plastic centrifuge tubes, 15 mL size, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

Strata-X solid phase extraction cartridges 60 mg, 3 mL size, available from available from Phenomenex, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10 2BN, UK.

Isolute Vacmaster-20^{TM} sample processing station, available from Argonaut Ltd., Tir-y-Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan CF8 8AU, UK.

Borosilicate glass disposable test tubes Available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK

Techne Dri-block 3D heating block, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK

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

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 UK.

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

Agilent 1100 HPLC system equipped with autosampler, quaternary pump, vacuum degasser and column compartment with column switching valve, available from Agilent Technologies UK Limited, Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire SK8 3GR.

CTC HTS PAL autosampler, available from Presearch Ltd, System House, 59-61 Knowlpiece, Hitchin, Herts SG4 0TY, UK.

HPLC column, ACE 5 C18 5 μm 50 mm × 3.0 mm i.d., available from Hichrom Ltd., 1 The Markham centre, Station road, Theale, Reading, RG47 4PE Berkshire, UK or www.hichrom.co.uk.

HPLC column, Phenomenex Ultracarb ODS (30) 5 μm 50 mm × 3.2 mm i.d. available from Phenomenex, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10 2BN, UK

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

US suppliers

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

Nalgene™ polypropylene centrifuge tubes, 15 mL capacity with 0.1 mL graduations. Available from Nalge Company, 75 Panorama Creek Drive, PO Box 20365, Rochester, NY 14602-0365.

Strata-X solid phase extraction cartridges 60 mg, 3 mL size, available from Phenomenex, 411 Madrid Ave., Torrance, CA 90501-1430, USA.

Isolute Vacmaster-20^{TM} sample processing station, available from Argonaut USA Ltd., PO Box 280 329, Lakewood, Colorado, 8022-0329.

Borosilicate glass disposable test tubes, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Techne Dri-Block heating block, available from Techne Incorporated, 3700 Brunswick Pike, Princeton, New Jersey 08540-6192.

Ultrasonic bath e.g. Ultrawave U300/D, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Crimp cap auto sampler vials and caps, available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304 USA.

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

Agilent 1100 HPLC system equipped with autosampler, quaternary pump, vacuum degasser and column compartment with column switching valve, available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304 USA.

CTC HTS PAL autosampler, available from LEAP Technologies Inc., P.O. Box 969, Carrboro,NC 27510 USA.

HPLC column, ACE 5 C18 5 μm 50 mm × 3.0 mm i.d, available from www.Hichrom.co.uk

HPLC column, Phenomenex Ultracarb ODS (30) 5 μm 50 mm × 3.2 mm i.d. available from Phenomenex, 411 Madrid Ave., Torrance, CA 90501-1430, USA.

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

APPENDIX 2 REAGENTS

UK suppliers

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

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

NOA449280 and SYN503780 analytical standards, available from GLP Testing Facility, Syngenta, CH-4333, Munchweilen, Switzerland.

US suppliers

Solvents: 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 and glacial acetic acid, available from www.sigmaaldrich.com

NOA449280 and SYN503780 analytical standards, available from Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of reagents

1. 2% formic acid in ultra pure water: Add 2 mL concentrated formic acid to ultra pure water in a 1 L volumetric flask. Adjust to the 1 L mark with ultra pure water. Stopper the flask securely and shake to mix thoroughly.

2. 0.2% acetic acid in ultra pure water: Add 2 mL glacial acetic acid to ultra pure water in a 1 L volumetric flask. Adjust to the 1 L mark with ultra pure water. Stopper the flask securely and shake to mix thoroughly.

APPENDIX 6 API4000 MS/MS TUNING PROCEDURE

Calibration of instrument

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

Tuning instrument for NOA449280 and SYN503780

Infuse a standard solution of NOA449280 and SYN503780 (1.0 to 10 µg/mL) in mobile phase (see section 4.2) 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* 400.1 for NOA449280 under positive ionisation conditions and
m/z 277.8 for SYN503780 under negative ionisation conditions.

Using the Analyst 1.4.1 software quantitative optimisation routine, tune the instrument for NOA449280 and SYN503780, ensuring that the correct ions are selected (initial Q1 *m/z* 400 for NOA449280 and *m/z* 278 for SYN503780. Product ions *m/z* 324.1 and *m/z* 228.1 for NOA449280 and *m/z* 202.0 and *m/z* 146.0 for SYN503780). Alternatively, the instrument ion optics and collision energy may be tuned manually for NOA449280 and SYN503780, to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of NOA449280 and SYN503780 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, protonated molecular ions of NOA449280 generated in the ion source (*m/z* 400.1) are selected and subjected to further fragmentation by collisional activation. The most abundant daughter ions free from interference (*m/z* 324.1 and *m/z* 228.1) are then selected. The transition *m/z* 400.1→ *m/z* 324.1 is used as the primary transition for quantitative analysis, corresponding to loss of CH_3-O-$(CH_2)_2$-O-, with cleavage at the ether linkage on the pyridine ring side chain. The transition *m/z* 400.1 → *m/z* 228.1 may be used as a confirmatory transition, and corresponds to cleavage at the carbonyl position, on the bicyclo[3.2.1]oct-3-en-2-one side of the molecule.

In negative ionisation mode, the deprotonated SYN503780 ion generated in the ion source (*m/z* 277.8) is selected and subjected to further fragmentation by collisional activation. The most abundant daughter ions free from interference (*m/z* 202.0 and *m/z* 146.0) are then selected. The transition *m/z* 277.8→ *m/z* 202.0 is used as the primary transition for quantitative analysis and corresponds to loss of CH_3-O-$(CH_2)_2$-O-, with cleavage at the ether linkage on the pyridine ring side chain. The transition *m/z* 277.8→ *m/z* 146.0 corresponding to the trifluoromethyl-pyridine fragment may be used as a confirmatory transition.

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

APPENDIX 8 METHOD FLOWCHART

Transfer water (1 mL) into a suitable autosampler vial

↓

Acidify with formic acid (200 µL)

↓

Concentrate samples using SPE (60 mg Strata-X).

↓

Elute SPE cartridges with methanol

↓

Evaporate samples and dissolve in acetonitrile:ultra pure water (20:80 v/v)

↓

Analysis by LC-MS/MS