1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM030.07A is suitable for the determination of N0A449280 (Figure 1) in air. The limit of quantification (LOQ) of the method has been established at 0.015 μg/m^3 (or 0.000015 μg/L), equivalent to 0.0027 μg of each isomer fortified onto the Tenax air sampling tubes. The calculation to determine the fortification level is shown below. According to guidelines SANCO/825/00 rev. 8.1, the LOQ must be equal to or lower than the concentration C which is defined as:

According to guidelines SANCO/825/00 rev. 7, the LOQ must be equal to or lower than the concentration C which is defined as:

$C = (AOEL_{systemic} \times 0.1 \times 60)/ 20$ mg/m^3

0.1 = safety factor

60 = body weight in kg

20 = air intake (volume per day in m^3)

The projected $AOEL_{systemic}$ for N0A449280 is = 0.005 mg/kg bw/day.

$C = (0.005 \times 0.1 \times 60) \div 20$ mg/m^3

Therefore C = 0.0015 mg/m^3 ≡ 0.0015 μg/L

LOQ ≤ 0.1 C

LOQ ≤ 0.1 x 0.0015 μg/L = 0.00015 μg/L

In this instance an LOQ of 0.01C was achievable = 0.000015 μg/L (0.015 ng/L)

Fortification level of adsorbent tubes:

Fortification amount (μg) = Sampling time (mins) x air flow (L/min) x conc^n in air (μg/L)

Sampling time = 6 hours (360 mins)

Air flow = 0.5 L/min

Fortification level to achieve LOQ of 0.01C = 360 x 0.5 x 0.000015 = 0.0027 μg per tube

This method satisfies OECD guidance document ENV/JM/MONO(2007)17, EU guidelines SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 7.

1.2 Method Summary

Air is drawn through a Tenax OVS (Occupational Safety and Health Administration (OSHA) Versatile Sampler) tube containing two layers of Tenax adsorbent at a rate of 0.5 L/min for a period of up to six hours, using a pre-calibrated motorised pump. After this time period the Tenax adsorbent is removed from the tube with the upper and lower layers separated for analysis. NOA449280 is then desorbed by ultrasonication in acetone. An aliquot of the acetone solution is then diluted with ultra pure water. Final determination is by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS). The limit of quantification (LOQ) is 0.015 μg/m^3 (or 0.000015 μg/L), equivalent to 0.0027 μg of NOA449280 fortified onto the Tenax air sampling tubes.

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 a 200 μg/mL stock solution for NOA449280 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient NOA449280 analytical standard 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.

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/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 should be prepared in acetonitrile 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 with acetonitrile: 10 µg/mL, 1.0 µg/mL, 0.1 µg/mL and 0.01 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant suppression or enhancement of the instrument response for NOA449280 has been observed using the procedures described in Section 3 during method validation and non-matrix standards should normally be used for calibration.

Calibration standards of NOA449280 for analytical determination by LC-MS/MS should be prepared in methanol/ultra pure water (50/50, v/v) by dilution of the acetonitrile standard solutions prepared in Section 2.3.2.

To prepare e.g., a 0.0005 µg/mL calibration standard, transfer acetonitrile/ultra pure water (20/80, v/v) (approximately 9 mL) into a 10 mL volumetric flask and add 50 µL of a 0.1 µg/mL NOA449280 standard in acetonitrile. Adjust to the 10 mL mark with methanol/ultra pure water (50/50, 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 should be generated to quantify NOA449280 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volumes of NOA449280 in methanol/ultra pure water (50/50, v/v).

Any reagent effects observed may be compensated for by use of reagent matched standards at the discretion of the study director, or by dilution of the final sample with methanol/ultra pure water (50/50, 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 six months for NOA449280 in acetonitrile 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

	Acetone	Methanol	Acetonitrile	Acetic acid
Harmful Vapour	✓	✓	✓	✓
Highly Flammable	✓	✓	✓	×
Harmful by Skin Absorption	×	×	✓	✓
Irritant to eyes and respiratory tract, causes burns	×	×	×	✓
Syngenta Divisional Toxicity Class	SHC D, S	SHC C, S	SHC C, S	SHC-C, S
OES Short Term (mg/m^3)	3560	310	105	37
OES Long Term (mg/m^3)	1780	260	70	25

*N/A not known.

At present there are insufficient data available to assign a Syngenta Hazard Classification for NOA449280. It should be treated as a category SHC-D compound 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 Preparation of OVS XAD-2 Air Sampling Tubes

Remove the protective plastic end caps from the Tenax air sampling tubes, as required.

3.2 Fortification of Air Sampling Tubes

At least one untreated control Tenax tube and two Tenax tubes fortified with known amounts of NOA449280 in acetonitrile should be analysed with each batch of samples to enable verification of the method and recovery corrections to be made, if desired. The fortifications should be made directly onto the surface of the upper glass fibre filter, using the needle tip of a suitable syringe or Gilson Microman pipette. After fortification, the Tenax tubes should be left at room temperature for approximately 15-20 minutes to allow the solvent to evaporate. Not more than 50 µL should be applied in one application. Further applications may be made if required, providing the solvent from the initial application is allowed to evaporate first. The volume of standard solution applied should be minimised as far as possible by use of high concentration standards, prepared as in Section 2.3.

3.3 Air Sampling

a) A suitable electronic or bubble type flow meter should be used to calibrate an appropriate motorised pump to draw air through the Tenax tube at a rate of 0.5 L/min.

b) Connect the Tenax tubes to the pre-calibrated motorised pumps using plastic tubing. The tube should be connected with the end containing the lower adsorbent layer attached to the pump via the plastic tubing

c) Set the pumps to run for up to 6 hours at a rate of 0.5 L/min per tube.

d) After the sampling period turn off the pumps and disconnect the tubes.

3.4 Desorption of NOA449280 from Tenax Sorbent

a) Using forceps, remove the Teflon holding ring and the glass fibre filter and transfer to a 15 mL polypropylene centrifuge tube. Carefully transfer the upper Tenax adsorbent layer into the tube containing the Teflon ring and glass fibre filter. Cap the tube containing the remaining lower adsorbent layer and retain for analysis in the event that low recoveries of NOA449280 are observed.

b) Add acetone (10 mL) to the tube containing the NOA449280 adsorbent, Teflon ring and the glass fibre filter. Cap the tube and shake gently and then ultrasonicate the tube and contents for 5 minutes to desorb the NOA449280 from the Tenax adsorbent and glass fibre filter. Allow the contents of the tube to settle.

c) Transfer a 0.2 mL aliquot of the solution from section 3.4 (b) to a suitable test tube. Add ultra pure water (0.80 mL). Shake to mix thoroughly.

d) Make a 5-fold dilution of the sample from Section 3.4 (c) in a suitable autosampler vial with ultra pure water. For example transfer a 200 µL aliquot of the solution from section 3.4 (c) to an autosampler vial. Add ultra pure water (0.8 mL). Shake the vial to mix thoroughly. This gives a total dilution factor of 25.

e) Analyse samples by LC-MS/MS.

3.5 Experimental Precautions

a) Bottled HPLC grade ultra pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

b) To prevent contamination of the instrument and to minimise possible carry-over issues, it is recommended that samples should be diluted so that the final analyte concentration does not exceed 0.05 µg/mL. It may also be useful to include blank injections of acetonitrile/ultra pure water (50/50 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

3.6 Time Required for Analysis

The methodology is normally performed with a batch of 12 samples. One person can complete the analysis of 12 samples in 1 day (8 hour working 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.

4.0 FINAL DETERMINATION

The method has been developed 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	:	Agilent 1100 series quaternary pump model number G1311A
Degasser	:	Agilent 1100 series model number G1322A
Column Oven	:	Agilent 1100 series model number G1316A
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 for NOA449280

Column	:	ACE 5 C18 5 μm, 50 x 3.0 mm
Column Oven Temperature	:	40°C± 3°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 = Acetic acid (0.1%, v/v) in ultra pure water

Mobile Phase Composition

Time (min)	% Solvent 1	% Solvent 2	Flow (mL/min)
0.0	20	80	1.0
2.0	90	10	1.0
2.4	90	10	1.0
2.5	20	80	1.0
4.0	20	80	1.0

Notes : The column eluate is diverted to waste for the first 0.75 min 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. Under these conditions the retention time of NOA449280 is 2.2 minutes.

4.3 Mass Spectrometer Conditions for NOA449280

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	550°C
Ionspray voltage	:	5500V
Collision gas setting (CAD)	:	Nitrogen set at 4 (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 primary transition**	**NOA449280 confirmatory transition**
Q1 *m/z*	:	400	400
Q3 *m/z*	:	324	228
Dwell time	:	150 ms	150 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	130 V	130 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	29 V	50 V
Collision cell exit potential (CXP)	:	11 V	17 V

Typical chromatograms are shown in the Figures Section.

4.4 Confirmatory Procedures for NOA449280

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

Residues may be calculated using an external standardisation procedure.
NOA449280 residues may be calculated in μg/L or $\mu g/m^3$ 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 five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to NOA449280. 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 residues in the sample, expressed as µg/L, as follows

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

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

Note: 1 L is equivalent to 0.001 m^3.

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

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

5.2 Single Point Calibration Procedure

NOA449280 residues may be calculated in µg/L or µg/m^3 for each sample using a mean standard response from each of the injections bracketing the sample as follows.

f) Make repeated injections of a standard containing NOA449280 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.

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

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

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

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

Peak response for sample
Average peak response for bracketing standards
Concentration of standard (µg/mL)
Sample concentration (L/mL)

Note: 1 L is equivalent to 0.001 m^3.

If residues need to be corrected for average percentage recovery e.g. storage stability studies, then the equation below should be used. Note the total sample dilution factor should be taken into account during all calculations.

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

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 in acetonitrile) 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.

7.2 Reagent and Solvent Interference

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

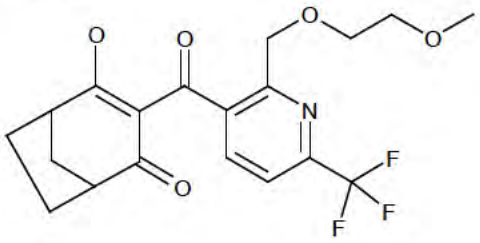
7.3 Labware Interference

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.

CHEMICAL STRUCTURES

Figure 1: NOA449280

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 Weight	:	399.39



APPENDIX 1 APPARATUS

Recommended Suppliers

Equipment	Description	Supplier
General glassware	General glassware	www.thermofisher.com/global/en/home.asp
Air sampling tubes	OVS XAD-2 / glass fibre adsorbent tubes (OSHA versatile sampler), part No. 226-56	www.skcltd.com
LC-MS/MS system	API 4000 equipped with a TurboIonSpray source	www.AppliedBiosytems.com
HPLC system	Agilent 1100 HPLC system equipped with quaternary pump, vacuum degasser and column compartment with column switching valve	www.Agilent.co.uk
Autosampler	CTC HTS PAL	www.CTC.ch
HPLC column	ACE 5 C18 5 µm, 50 x 3.0 mm	www.hichrom.co.uk
Nitrogen generator	Peak Scientific NM20ZA gas station	www.peakscientific.com

APPENDIX 2 REAGENTS

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	HPLC grade	www.thermofisher.com/global/en/home.asp
Acetonitrile	HPLC grade	www.thermofisher.com/global/en/home.asp
Methanol	HPLC grade	www.thermofisher.com/global/en/home.asp
Acetic acid	Analytical grade	www.sigmaaldrich.com
NOA449280 analytical standard	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

1. 0.1% acetic acid in ultra pure water: Add 1 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 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

Infuse a standard solution of NOA449280 (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 for NOA449280 under positive ionisation conditions.

Using the Analyst 1.4.1 software quantitative optimisation routine, tune the instrument for NOA449280, ensuring that the correct ions are selected (initial Q1 *m/z* = 400 and product ions *m/z* = 324 and *m/z* = 228 for NOA449280. Alternatively, the instrument ion optics and collision energy may be tuned manually for NOA449280, to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of NOA449280 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) are selected and subjected to further fragmentation by collisional activation. The most abundant daughter ions free from interference (*m/z* = 324 and *m/z* = 228) are then selected. The transition *m/z* 400 → *m/z* 324 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 → *m/z* 228 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.

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

APPENDIX 4 METHOD FLOW CHART

Remove the end caps from the Tenax OVS (Occupational Safety and Health Administration (OSHA) Versatile Sampler) sample tubes and fortify as necessary

↓

Connect the Tenax sample tubes to a suitable, pre-calibrated motorised pump.

↓

Draw air through the Tenax tubes at a rate of 0.5 L/min for a period of up to six hours, using a pre-calibrated motorised pump.

↓

Remove the Tenax adsorbent from the tube and separate the upper and lower layers for analysis

↓

Desorb NOA449280 by ultrasonication in acetone.

↓

Dilute an aliquot of the acetone extract with ultra pure water

↓

Analyse by LC-MS/MS