Isocycloseram

SYN547407 - Analytical Method GRM072.12A for the Determination of SYN547407 and its Metabolites SYN549107, SYN549431 and SYN550738 in Soil

Analytical Method

TEST GUIDELINE(S): EPA 850.6100
EC SANCO/3029/99 rev 4
EC SANCO/825/00 rev 8.1

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM072.12A is suitable for the determination of SYN547407 (Figure 1) and its metabolites SYN549107 (Figure 2), SYN549431 (Figure 3), and SYN550738 (Figure 4) in soil. The limit of quantification (LOQ) of the method has been established at 0.001 mg/kg (or 0.001 ppm, 1 ppb).

This method satisfies US EPA guideline OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

1.2 Stereospecificity

SYN547407 is comprised of a diastereomeric pair; this method does not resolve the diastereomers or the R and S enantiomers of each diastereomeric pair and will detect and quantify all enantiomers of SYN547407 as a single chromatographic peak. SYN549107, SYN549431 and SYN550738 have one chiral centre comprised of R and S enantiomers; this method does not resolve the enantiomers of each metabolite and will detect and quantify each individual compound as a single chromatographic peak.

1.3 Method Summary

Soil samples (10g) are extracted by shaking with acetonitrile/ultra-pure water (80/20 v/v) followed by centrifugation and decanting of the supernatant. The sample is extracted a second time with acetonitrile/ ultra-pure water /acetic acid (80:20:0.1 v/v/v) followed by centrifugation and decanting of the supernatant. The supernatants are combined and then an aliquot of the combined extracts is centrifuged once more. An aliquot is then diluted 1:1 with ultra-pure water and final determination is by ultra-performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS). Note: The recommended instrumentation for analysis is AB Sciex Q Trap® 6500 or instrumentation with equivalent sensitivity.

The limit of quantification of the method is 0.001 mg/kg (0.001 ppm, 1 ppb).

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 1000 μg/mL stock solutions for SYN547407, SYN549107, SYN549431 and SYN550738 by one of the following methods.

Note: the amount weighed out must be corrected for the purity of the analytical standard as indicated on the certificate of analysis and also any salt content, where the analytical standard is received as a salt e.g. Na^+, Cl^- etc..

Weigh out accurately, using a four figure balance, sufficient SYN547407, SYN549107, SYN549431 and SYN550738 analytical standard into separate volumetric flasks (10 mL size). Dilute to the mark with acetonitrile to give an 1000 μg/mL stock solutions of SYN547407, SYN549107, SYN549431 and SYN550738.

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 and any salt content where the analytical standard is received as a salt e.g. Na^+, Cl^- etc.

$$V = \frac{W \times P}{C} \times 1000$$

P	=	Standard purity (including correction for salt content where the analytical standard is received as a salt e.g. Na^+, Cl^- etc.) 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

The standard material is weighed into a volumetric flask (10 mL size) and the required volume of acetonitrile is added. Note that the amount of solvent added must still allow for the volumetric flask to be sealed and mixed.

2.3.2 Fortification Solutions

Mixed sample fortification solutions containing SYN547407, SYN549107, SYN549431 and SYN550738 should be prepared by serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL. Individual standards of SYN547407, SYN549107, SYN549431 and SYN550738 may be prepared if desired.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant suppression or enhancement of the instrument response for SYN547407, SYN549107, SYN549431 and SYN550738 have been observed in the soil types tested using the procedures described in Section 3 during method validation. However, to compensate for possible matrix effects matrix matched standards should normally be used for calibration.

Solvent based calibration standards should be prepared by serial dilution of stock or fortification solutions to a final solution of acetonitrile/ultra-pure water (50/50 v/v).

For example, to prepare a mixed standard solution containing SYN547407, SYN549107, SYN549431 and SYN550738 at 10 µg/mL, transfer an aliquot (0.1 mL) of each 1000 µg/mL stock standard solution into a 10 mL volumetric flask and dilute to 10 mL mark using acetonitrile/ultra-pure water (50/50 v/v).

Matrix matched calibration standards should be prepared by serial dilution of mixed standard solutions in acetonitrile/water (50:50, v/v) into a final solution of representative matrix blank extract which was generated by completing the procedure described in section 3.3 with untreated matrix.

Recommended calibration range for analysis using instrumentation described in Section 4 over an appropriate range: 0.015 ng/mL to 10 ng/mL (0.45 pg to 300 pg on column based on a 30µL injection volume).

A calibration curve should be generated to quantify SYN547407, SYN549107, SYN549431 and SYN550738 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volumes of SYN547407, SYN549107, SYN549431 and SYN550738 in matrix.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored refrigerated in the dark 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.

A stability of 27 days was demonstrated for fortification solutions during method validation (Reference 1).

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 3).

Solvent and Reagent Hazards

	Acetonitrile	Methanol	Acetic acid
Harmful Vapour	✓	✓	✓
Highly Flammable	✓	✓	✗
Harmful by Skin Absorption	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓
Causes severe burns	✗	✗	✓
OES Short Term (mg/m^3)	105	310	37
OES Long Term (mg/m^3)	70	260	25

Suitable personal protective equipment should be worn when handling chemicals and reagents. The appropriate SDS should be consulted for each reagent and a local risk assessment should be carried out. In all cases avoid breathing vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

3.2 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 SYN547407, SYN549107, SYN549431 and SYN550738 in acetonitrile. Let each sample stand for at least five minutes after fortification to allow the spiking solution to soak into the soil before proceeding with the extraction. At least one untreated control and two fortified control samples should be analysed with each sample set.

3.3 Extraction (Mechanical Shake)

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

a) For extraction weigh 10 g of soil into a 250 mL polypropylene bottle; fortification solution is added at this step if required and the sample left for 5 min

b) Add 50 mL of acetonitrile/water (80:20 v/v).

c) Samples were shaken for 1 hour using a flatbed shaker at a rate of 250 rpm.

d) Centrifuge samples at 3500 rpm for 3 minutes.

e) Decant the supernatant into a 100 mL volumetric flask.

f) Extract the remaining soil with 50 mL of acetonitrile/ultra-pure water/acetic acid (80:20:0.1 v/v/v) by shaking the sample as above for 1 hour.

g) Centrifuge samples at 3500 rpm for 3 minutes.

h) Decant the supernatant again into the same 100 mL volumetric flask as above. Adjusted the volume to 100 mL with acetonitrile/water (80:20, v/v) and the solution was homogenised by shaking by hand.

i) Transfer an aliquot of the combined solution into a 50 mL centrifuge tube and centrifuged for 5 min at 10000 rpm.

j) Dilute 500 µL of the supernatant with 500 µL of ultra-pure water in a HPLC vial and analyze by LC-MS/MS. If further concentration is required and/or clean-up due to matrix, proceed to Section 3.4.

3.4 Optional (Solid Phase Extraction Procedure)

Note: SPE should only be performed if concentration of sample results in significant matrix effect that impacts residue determination using matrix-match calibration.

a) Take one Oasis HLB cartridge (3 mL, 60 mg) for each sample to be analyzed 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) Transfer 5 mL of soil extract to a 15 mL conical polypropylene tube (Falcon Tube) and add 10 mL of ultra-pure water.

c) Load diluted sample (15 mL) into SPE cartridge using a pipette or by attaching a column reservoir. Allow sample to percolate through under gravity or draw through under slight 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) Rinse the empty sample container with 3 mL of ultra-pure water/acetonitrile (70/30 v/v), transfer to SPE and allow to percolate through under gravity or draw through under slight vacuum to the level of the top frit at the same rate, again discarding the column eluate. Apply high vacuum for 3 minutes to remove any remaining water.

e) Elute SYN547407, SYN549107, SYN549431 and SYN550738 from SPE cartridge into a 15 mL tube using 5 mL acetonitrile under gravity or slight vacuum. Elute any remaining volume using high vacuum for 10 seconds.

f) Evaporate samples to 0.5 mL under a gentle stream of nitrogen or air in a sample concentrator with a heating block set to 40°C. Reconstitute to 1 mL using 0.5 mL of ultra-pure water, ultrasonicate/vortex to mix thoroughly. If sample is allowed to evaporate to dryness or near dryness, low recoveries may be observed.

k) Transfer the samples into suitable autosampler vials ready for final determination by LC-MS/MS. The final sample concentration is 0.3 g/mL.

3.5 Experimental Precautions

a) To prevent contamination of the instrument and to minimise possible carry-over issues, it is recommended that high level recoveries (>0.1 mg/kg) and samples with expected residues greater than 0.1 mg/kg should be diluted so that the final analyte concentration does not exceed 0.008 μg/mL.

3.6 Time Required for Analysis

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

3.7 Method Stopping Points

Stopping point were not tested during method validation, sample preparation should therefore be finalised within on working day.

4.0 FINAL DETERMINATION

The method has been developed for use on an Applied Biosystems QTRAP® 6500. 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

LC System	:	Sciex QTRAP 6500+
Detector	:	Agilent Infinity II
Gas Supply	:	House Supply

4.2 Chromatography Conditions for SYN547407, SYN549107, SYN549431 and SYN550738

Column	:	Kinetex C18 (50 mm x 2.1 mm, 2.6 µm, 00B-4462-AN, Phenomenex) with pre-column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)
Alternate Column	:	ACE Excel C18-Amide (50 mm x 2.1 mm, 2 µm, EXL10120502U, MAC-MOD)
Column Oven Temperature	:	30 °C
Injection volume	:	30 µL
Stop Time	:	8 min
Injection protocol	:	Analyse calibration standard after 3 - 5 sample injections
Mobile phase	:	(A) Ultra-Pure Water + 0.1% Acetic Acid (B) Acetonitrile + 0.1 % Acetic Acid

Mobile Phase Composition

Time (mins)	% solvent A	% solvent B	Flow rate (mL/min)
0	60	40	0.6
1.0	60	40	0.6
6.0	5	95	0.6
7.0	5	95	0.6
7.1	60	40	0.6
8.0	60	40	0.6

Valve Switching program

Time (mins)	
0	To waste
2.7	To mass spectrometer
5.5	To waste

Under these conditions, SYN549431, SYN547407, SYN549107 and SYN550738 eluted after 3.5, 3.9, 4.3 and 5.2 minutes respectively.

4.3 Mass Spectrometer Conditions for SYN547407 and SYN549431

Interface	:	TurboIonSpray
Polarity	:	Positive (+)
Curtain gas (CUR)	:	30 (arbitrary units)
Temperature (TEM)	:	550 °C
Ionspray voltage	:	4500 V in positive mode; -4500 V in negative mode
Collision gas setting (CAD)	:	8 (arbitrary units)
Gas 1 (GS1)	:	30 (arbitrary units)
Gas 2 (GS2)	:	60 (arbitrary units)
Interface heater (ihe)	:	MRM
Scan type	:	TurboIonSpray

MRM Conditions		**SYN547407 primary transition**	**SYN547407 confirmatory transition**	**SYN549431 primary transition**	**SYN549431 confirmatory transition**
Q1 *m/z*	:	548	548	435	435
Q3 *m/z*	:	418	160	392	160
Dwell time	:	25 ms	25 ms	25 ms	25 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	60 V	60V	60 V	60V
Entrance potential (EP)	:	14 V	14V	14 V	14V
Collision energy (CE)	:	30 V	60 V	30 V	45 V
Collision cell exit potential (CXP)	:	15 V	15 V	15 V	15 V

Typical chromatograms are shown in the Figures Section.

4.4 Mass Spectrometer Conditions for SYN549107 and SYN550738

Interface	:	TurboIonSpray
Polarity	:	Period 1: 0.0 - 4.5 min: Positive/negative switching mode (+/-) Period 2: 4.5 - 8.0 min: Negative mode (-)
Curtain gas (CUR)	:	30 (arbitrary units)
Temperature (TEM)	:	550 °C
Ionspray voltage	:	4500 V in positive mode; -4500 V in negative mode
Collision gas setting (CAD)	:	8 (arbitrary units)
Gas 1 (GS1)	:	30 (arbitrary units)
Gas 2 (GS2)	:	60 (arbitrary units)
Interface heater (ihe)	:	MRM
Scan type	:	TurboIonSpray

MRM Conditions		**SYN549107 primary transition**	**SYN549107 confirmatory transition**	**SYN550738 primary transition**	**SYN550738 confirmatory transition**
Q1 *m/z*	:	434	434	448	448
Q3 *m/z*	:	238	354	378	69
Dwell time	:	100 ms	100 ms	100 ms	100 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	-60 V	-60 V	-60 V	-60 V
Entrance potential (EP)	:	-14 V	-14 V	-14 V	-14 V
Collision energy (CE)	:	-35 V	-30 V	-18 V	-60 V
Collision cell exit potential (CXP)	:	-15 V	-15 V	-15 V	-15 V

Typical chromatograms are shown in the Figures Section.

4.5 Confirmatory Procedures for SYN547407, SYN549107, SYN549431 and SYN550738

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

SYN547407, SYN549107, SYN549431 and SYN550738 residues may be calculated in mg/kg for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (30% LOQ to at least 20% above the highest fortified level as a minimum). 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 SYN547407, SYN549107, SYN549431 and SYN550738. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of five 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 SYN547407, SYN549107, SYN549431 and SYN550738 residues in the sample, expressed as mg/kg, as follows

$$\text{Residue (mg/kg)} = \frac{c_1 \cdot c_{sample} \cdot V_{ex} \cdot V_{end}}{c_2 \cdot W \cdot V_{ali} \cdot 1000}$$

With:

c_1	Nominal concentration of bracketing matrix standards (ng/mL)
c_2	Average calculated concentration of matrix standards bracketed between samples, obtained from the matrix calibration function (ng/mL)

c_{sample}	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)
V_{ex}	Extraction volume (100 mL)
V_{end}	Final volume (1 mL)
W_{Sample}	weight (10 g)
V_{ali}	Aliquot (0.5 mL)
1000	Conversion from ng/g to mg/kg

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

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

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 SYN547407, SYN549107, SYN549431 and SYN550738 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 prior to use.

8.0 METHOD VALIDATION

8.1 Extractability

SYN547407, SYN549107, SYN549431 and SYN550738 have been shown to be efficiently extracted in a validation study using the conditions described in Section 3 of the analytical procedure (Reference 2).

8.2 Hydrolysis

Hydrolysis is not required for the determination of residues of SYN547407, SYN549107, SYN549431 and SYN550738 in soil.

8.3 Derivatization

Derivatization is not required for the determination of residues of SYN547407, SYN549107, SYN549431 and SYN550738 in soil.

8.4 Recovery Data and Repeatability

Method validation has been carried out on the procedures described in Sections 3 and 4. The method validation data are summarized in Tables 2 - 9.

8.5 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 obtained.

The limit of quantification has been set at 0.001 mg/kg (0.001 ppm, 1 ppb).

8.6 Limit of Detection (LOD)

The limit of detection of the method is defined as 30 % of the LOQ
(LOD = 0.0003 mg/kg).

Note that the LOD may vary between runs and from instrument to instrument.

The LOD was determined as 0.45 pg injected on column, equivalent to 0.015 ng/mL when using a 30 µL injection volume. LOD is based on the lowest concentration sample injected.

8.7 Matrix Effects

No significant matrix effects was observed for all analytes in the soils types tested during method validation. The method includes procedures to reduce matrix effects as far as practically possible, however matrix effects in some matrices may be still observed. Therefore matrix matched standards should be used to compensate for possible the matrix effects at the discretion of the study director. A summary of the matrix effects is included in Table 10.

8.8 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 SYN547407, SYN549107, SYN549431 and SYN550738 was tested in the range from 0.045 pg to 300 pg injected on column (equivalent to 0.015 ng/mL to 10 ng/mL standards when using a 30 µL injection volume. This range corresponds to equivalent sample concentrations of 0.0003 mg/kg to 0.2 mg/kg) and was found to be linear. This is equivalent to 30% LOQ to >120% of the highest validated level.

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.

Inject standards in the range of 30% LOQ – 20% above highest concentration required and plot the peak area against the standard concentration, using Microsoft Excel for both primary and confirmatory transitions for SYN547407, SYN549107, SYN549431 and SYN550738.

Detector linearity graphs are presented in the Figures Section.

8.9 Extract Stability

Final soil extracts in acetonitrile/ultra-pure water (50/50 v/v) retained in vials and stored at 1 °C to 10 °C in the dark were suitable for SYN547407, SYN549107, SYN549431 and SYN550738 residue analysis, for storage periods of up to 8 days.

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 method provided successful recovery tests at the relevant levels validate the suitability of the method.

10.0 CONCLUSIONS

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of SYN547407, SYN549107, SYN549431 and SYN550738 residues in soil. Only commercially available laboratory equipment and reagents are required. The analysis of 12 soil samples for SYN547407, SYN549107, SYN549431 and SYN550738 can be completed by one person in 1 day (8 working hour period). Untreated and fortified samples should be analysed with each set of samples to demonstrate absence of any interference and adequate recovery, if possible. The limit of quantification of the method is 0.001 mg/kg (0.001 ppm, 1 ppb).

This method satisfies US EPA guideline EPA OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

TABLE 1: Characterisation Data for Soil Samples used in Method Validation

Soil Type	pH (0.01 M $CaCl_2$)	Sand Content (% w/w)	Silt Content (% w/w)	Clay Content (% w/w)	Organic Carbon (%)
LUFA F2.1 (Loamy sand)	5.32	86.3	10.8	2.9	0.58
LUFA 5M (Sandy loam)	7.34	57.4	32.3	10.3	1.11

Determination of Matrix Effect

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 SYN547407, SYN549107, SYN549431 and SYN550738 against non-matrix standards at an equivalent concentration.

Note: % matrix effects determined as (B/A x 100) – 100 where A is non-matrix standard response and B is (reagent) matrix-matched standard response

TABLE 10: LC-MS/MS Matrix Effects

	Matrix Effect	
Analyte	LUFA F5M	LUFA F2.1
SYN547407 *m/z* 548 → 418	- 4	+ 3
SYN547407 *m/z* 548→ 160	- 5	+ 3
SYN549107 *m/z* 434 → 238	+ 2	+ 14
SYN549107 *m/z* 434 → 354	+ 3	+ 13
SYN549431 *m/z* 435 → 392	- 1	+ 8
SYN549431 *m/z* 435 → 160	- 5	+ 8
SYN550738 *m/z* 448 → 378	- 11	- 16
SYN550738 *m/z* 448 → 69	- 11	- 14

The magnitude of the matrix effects were considered not to be significant in the soils tested however, matrix standards should be used for calibration to compensate for possible matrix effects. Non-matrix matched standards may be used, at the discretion of the study director. Alternatively, where instrument sensitivity permits, samples may be further diluted in acetonitrile/ultra-pure water (50/50 v/v) to reduce or eliminate matrix effects.

TABLE 11: Stability of Fortification solutions at 1 – 10 °C.

Analyte	Solvent of fortification solution	Standard conc. of fortification solution† (µg/mL)	Storage period (Days)	Difference (%) of stored fortification solution compared to freshly prepared fortification solution
SYN547407 (548→418 m/z)	Acetonitrile	0.1	27	2
		1		1
SYN549431 (435→392 m/z)	Acetonitrile	0.1	27	2
		1		1
SYN549107 (434→238 m/z)	Acetonitrile	0.1	27	2
		1		0
SYN550738 (448→378 m/z)	Acetonitrile	0.1	27	0
		1		2

†Fortification solutions were diluted with H_2O/ACN (50:50, v/v) to a final concentration of 1.0 ng/mL for analysis

TABLE 12: Stability of Calibration solution at 1 – 10 °C.

Analyte	Solvent of calibration solution	Standard conc. of calibration solution(µg/mL) †	Storage period (Days)	Difference (%) of stored calibration solution compared to freshly prepared calibration solution
SYN547407 (548→418 m/z)	Acetonitrile	10	27	3
SYN549431 (435→392 m/z)	Acetonitrile	10	27	3
SYN549107 (434→238 m/z)	Acetonitrile	10	27	3
SYN550738 (448→378 m/z)	Acetonitrile	10	27	6

†Calibration solution was diluted with H_2O/ACN (50:50, v/v) to a final concentration of 1.0 ng/mL for analysis

CHEMICAL STRUCTURES

FIGURE 1: SYN547407

Alternative compound code number	:	CSCV765754
CAS Number	:	Not in registry
IUPAC Name	:	4-(5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl)-N-(2-ethyl-3-oxo-1,2-oxazolidin-4-yl)-2-methylbenzamide
Molecular Formula	:	$C_{23}H_{19}O_4N_3Cl_2F_4$
Molecular Weight	:	548.3

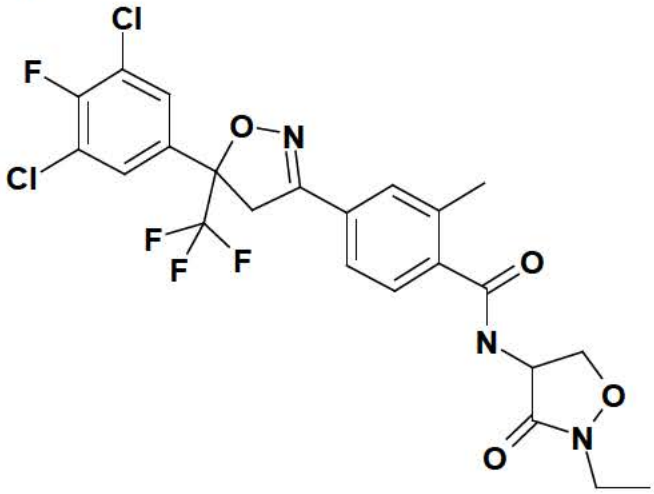


FIGURE 2: SYN549107

Alternative compound code number	:	CSCD747695
CAS Number	:	Not in registry
IUPAC Name	:	4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4H-isoxazol-3-yl]-2-methyl-benzoic acid
Molecular Formula	:	$C_{18}H_{11}Cl_2F_4NO_3$
Molecular Weight	:	436.2

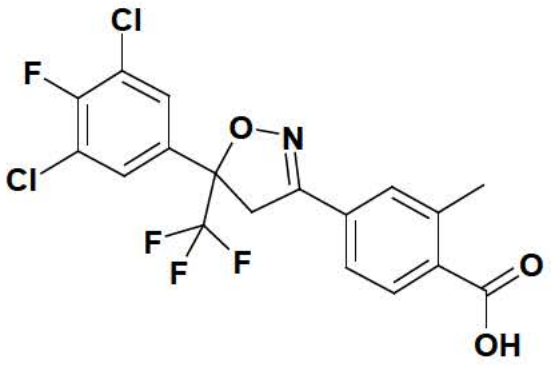


FIGURE 3: SYN549431

Alternative compound code number	:	CSDK352552
CAS Number	:	Not in registry
IUPAC Name	:	4-[5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl]-2-methylbenzamide
Molecular Formula	:	$C_{18}H_{12}Cl_2F_4N_2O_2$
Molecular Weight	:	435.2

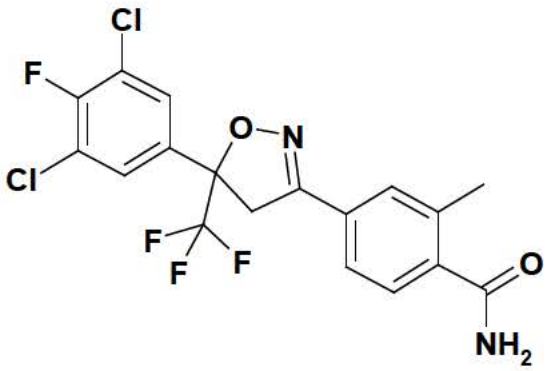
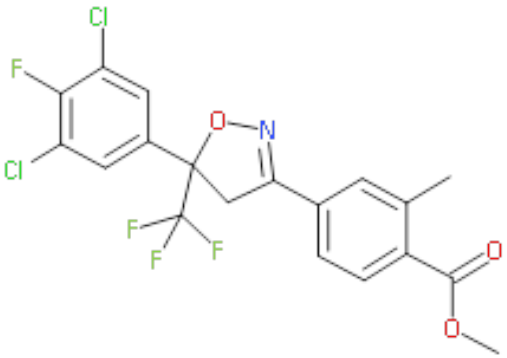


FIGURE 4: SYN550738

Alternative compound code number	**:**	CSDK444059
CAS Number	**:**	Not in registry
IUPAC Name	**:**	Methyl 4-[5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl]-2-methylbenzoate
Molecular Formula	:	$C_{19}H_{13}Cl_2F_4NO_3$
Molecular Weight	**:**	450.2



APPENDIX 1 Apparatus

Recommended Suppliers

Laboratory balance (Sartorius, Kern)
Volumetric pipettes (Brand, Eppendorf)
Vortex mixer (Scientific industries)
Horizontal flatbed shaker (Bühler)
Centrifuge (Hettich, Thermo)
Sample concentrator (Stuart)
LC column Kinetex C18 (50 mm x 2.1 mm, 2.6 µm, Part number 00B-4462-AN, Phenomenex) with pre-column (KJ0-4282, Phenomenex) with 4 mm C18 cartridge (AJ0-4287, Phenomenex)
LC column (alternate) ACE Excel C18-Amide (50 mm x 2.1 mm, 2 µm, EXL10120502U, MAC-MOD)
API 6500+ LC-MS/MS system (Sciex) with Agilent 1290 Infinity II

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Water	HPLC grade	Merck
Acetonitrile	HPLC grade	Prolabo
Acetic Acid	p.a.	Thermo Fisher
Demineralised Water	-	Prepared at laboratory
Analytical standards SYN547407 SYN549431 SYN549107 SYN550738	GLP certified	Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) Acetonitrile/ultra-pure water (80/20 v/v) – prepared by e.g. mixing 800 mL of acetonitrile with 200 mL of ultra-pure water

b) Acetonitrile/ultra-pure water / acetic acid (80/20/0.1 v/v/v) – prepared by e.g. mixing 800 mL of acetonitrile with 200 mL of ultra-pure water then addition of 1 mL acetic acid. (Extraction Solvent)

c) 0.1% acetic acid in ultra-pure water – add 1.0 mL concentrated acetic acid to 1L ultra-pure water. (Mobile Phase)

d) 0.1% acetic acid in Acetonitrile – add 1.0 mL concentrated acetic acid to 1L acetonitrile. (Mobile Phase)

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 SYN547407, SYN549107, SYN549431 and SYN550738

Note: SYN547407, SYN549107, SYN549431 and SYN550738 have 2 chlorine atoms. Chlorine has two isotopic forms ^{35}Cl and ^{37}Cl and therefore the mass spectrum shows ions at M (^{35}Cl x 2), M+2 (^{35}Cl + ^{37}Cl) and M+4 (^{37}Cl x2). In this method the instrument has been tuned on the ^{35}Cl isotope only for all analytes.

Infuse standard solutions of SYN547407, SYN549107, SYN549431 and SYN550738 (0.01 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 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 molecular ion signal at *m/z* 548 for SYN547407, and *m/z* 435 for SYN549431 in positive ionization mode. Tuning for SYN549107 *m/z* 434 and SYN550738 *m/z* 448 is performed in negative ionization mode.

Using the Analyst software quantitative optimization routine, tune the instrument for SYN547407, SYN549107, SYN549431 and SYN550738, ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of SYN547407, SYN549107, SYN549431 and SYN550738 standards using mobile phase 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.

For SYN547407, in positive ionization mode, the protonated molecular ion generated in the ion source (*m/z* 548) is selected and subjected to further fragmentation by collisional activation. The two most sensitive product ions (*m/z* 418 and *m/z* 160) are then selected and used for quantitative analysis.

Analyte	Fragment assignment
SYN547407 m/z 548 → 418	Amide cleavage
SYN547407 m/z 548 → 160	C7H6ClFO fragment

For SYN549431, in positive ionization mode, the protonated molecular ion generated in the ion source (*m/z* 435) is selected and subjected to further fragmentation by collisional activation. The two most sensitive product ions (*m/z* 160 and *m/z* 392) are then selected and used for quantitative analysis.

Analyte	Fragment assignment
SYN549431 m/z 435 → 160	
SYN549431 m/z 435 → 392	

For SYN549107, in negative ionization mode, the protonated molecular ion generated in the ion source (*m/z* 434) is selected and subjected to further fragmentation by collisional activation. The two most sensitive product ions (*m/z* 237 and *m/z* 354) are then selected and used for quantitative analysis.

Analyte	Fragment assignment
SYN549107 m/z 434 → 237	
SYN549107 m/z 434 → 354	

For SYN550738, in negative ionization mode, the protonated molecular ion generated in the ion source (*m/z* 448) is selected and subjected to further fragmentation by collisional activation. The two most sensitive product ions (*m/z* 378 and *m/z* 69) are then selected and used for quantitative analysis.

Analyte	Fragment assignment
SYN549107 m/z 448 → 378	
SYN549107 m/z 448 → 69	

APPENDIX 4 Method Flow Chart

Weigh soil sample (10 g) into a polypropylene bottle

↓

Fortify sample as appropriate for procedural recoveries

↓

Add extraction solvent acetonitrile/water (80:20 v/v)

↓

Extract by shaking (60 minutes) on a horizontal flatbed shaker

↓

Centrifuge sample at 3500 rpm for 3 minutes and decant supernatant into volumetric flask

↓

Add extraction solvent acetonitrile / ultra pure water / acetic acid (80:20:0.1 v/v/v) to remaining soil

↓

Extract by shaking (60 minutes) on a horizontal flatbed shaker

↓

Centrifuge sample at 3500 rpm for 3 minutes

↓

Combine extracts and fill up to 100 mL if required with acetonitrile/ultra pure water (80/20, v/v)

↓

Transfer aliquot of combined solution in 50 mL centrifuge tube

↓

Centrifuge sample at 10000 rpm for 5 minutes

↓

Dilute 500 µL of the supernatant with 500 µL of ultra-pure water in a HPLC vial

↓

Analyse by LC-MS/MS