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Isocycloseram

SYN547407 – Independent Laboratory Validation of Residue Method GRM072.17A for the Determination of SYN547407 and Metabolites SYN549107, SYN549431, SYN550455 and SYN551485 in Water

Final Report Amendment 1

TEST GUIDELINE(S): EPA 850.6100

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2.0 INTRODUCTION

This report provides the results and procedures for the independent laboratory validation (ILV) study conducted for the Syngenta analytical method entitled SYN547407 - Residue Method GRM072.17A for the Determination of SYN547407 and Metabolites SYN549107, SYN549431, SYN550455 and SYN551485 in Water [1]. Successful method validation was achieved by obtaining satisfactory procedural recoveries of parent, SYN547407, and degradation products SYN549107, SYN549431, SYN550455 and SYN551485 in fortified surface water and ground water samples. Fortification of the water was made at level of 0.05 ppb for the method limit of quantitation (LOQ), and 0.5 ppb for 10× of the LOQ. The mean recovery for each matrix, at each fortification level, was within the acceptance levels (70-120%) with a relative standard deviation (RSD) of ≤ 20%.

The following sections discuss and summarize analytical standards and reagent preparations, sample receipt, storage, sample preparation, sample analysis and results.

The independent method validation documented in this final report is designed to conform to OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2]. In addition, this study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 [3].

Final Report Amendment 1 was issued to correct the study title on pages 3 and 4 of this report.

3.0 MATERIALS AND METHODS

3.1 Reagents

3.1.1 Mobile Phase Preparation

Mobile Phase A: Added 1 mL of formic acid in 1 L of water and mixed well
Mobile Phase B: Mixed 1 mL formic acid and 1 L of acetonitrile.

3.1.2 Diluent for Standard Solutions

Mixed 500 mL of acetonitrile with 500 mL of water

3.2 Standards

3.2.1 Analytical Reference Standards

Analytical reference standards of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 were provided by Syngenta Crop Protection. Descriptions of each analytical reference standards including molecular structure, nomenclature and purity are presented in FIGURE 1 - FIGURE 5.

Upon receipt, the standards SYN547407, SYN549107, SYN549431, and SYN550455 were logged in and stored under refrigeration at 5 °C, and the standard SYN551485 was logged in and stored in a freezer at -20 ºC. Good Laboratory Practice (GLP) characterization for each standard has been generated and is kept on file at Syngenta Crop Protection, Inc.

3.2.1.1 Stock analytical standard solutions preparation

The 100 µg/mL stock solution for each of the analyte was prepared per the analytical method (GRM072.17A). Stock solutions were prepared by adding the appropriate volume of acetonitrile to a known amount of standard material. Further details on the preparation of stock solutions are presented in TABLE 1.

3.2.1.2 Fortification solutions preparation for the recovery samples

The combined fortification solutions containing SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 were prepared by serial dilution in acetonitrile as described in TABLE 2.

3.2.1.3 Calibration standard solutions preparation

Matrix matched calibration standards were not prepared since there was no significant matrix effect observed in the surface water or in the ground water samples. Solvent calibration standard solutions were prepared by performing appropriate dilutions with acetonitrile/ water (50/50 v/v) for both surface water and ground water sample analysis by performing serial dilution of the fortification solutions as per TABLE 3.

3.2.1.4 Instrument Conditions

LCMS parameters used for the analysis are summarized in FIGURE 7.

3.3 Sample Receipt, Storage

3.3.1 Sample Receipt and Storage Conditions

Bulk samples of surface water (approximately 0.5 L) and ground water (approximately 1.0 L) were shipped frozen overnight on dry ice to Primera, at the Cranbury, New Jersey location. All samples were logged in, and then stored frozen at -20 ºC upon arrival.

3.4 Analytical Methodology

3.4.1 Modifications

Minor modifications were made to the UHPLC conditions stated in the Syngenta analytical method GRM072.17A [1] during the tuning of the instrument and were documented in the study protocol [4]. The executed minor modifications included mobile phases, UHPLC gradient, and Dwell time, and the modifications were applied for the analyses of all samples in this study.

The mobile phase A was changed from 0.01%Acetic Acid in Water to 0.1%Formic Acid in Water and mobile phase B was changed from 0.01%Acetic Acid in Acetonitrile to 0.1%Formic Acid in Acetonitrile. The initial Pump B Conc. was changed from 10% to 0%. Dwell times of the transitions of SYN547407, SYN549431 and SYN551485 were changed from 25 msec to 50 msec (FIGURE 7).

3.4.2 Primary and Confirmatory Transitions

Instrument parameters and m/z ratios of the primary and confirmatory transition for each analyte is listed in FIGURE 7.

3.4.3 Sample Fortifications

Recovery samples were included in both surface water sample analysis and ground water analysis in order to verify method performance. Each analytical set contained five (5) replicates of recovery samples at LOQ level and 10× LOQ for each type of sample.

Water samples fortified at the LOQ (0.05 ppb) were prepared by adding 0.1 mL of the 0.25 ng/mL (SP-1) fortification solution into 0.5 mL of control water followed by adding 0.4 mL of acetonitrile.

Water samples fortified at 10× LOQ (0.5 ppb) were prepared by adding 0.1 mL of the 2.5 ng/mL (SP-2) fortification solution into 0.5 mL of control water followed by adding 0.4 mL of acetonitrile.

Two untreated control samples for each site of each sample type were also included in each analytical set.

3.4.4 Limit of Detection and Limit of Quantitation

The limit of detection (LOD) for the method is defined as the lowest analyte amount injected on column detectable above the mean amplitude of the background noise at the corresponding retention time. An estimate of the LOD can be taken as three times background noise. The limit of detection specified in the Syngenta analytical method GRM072.17A [1] has been used as 0.005 ng/mL for each of the analytes in the water types tested.

The LOQ for the method is defined as the lowest analyte concentration in a sample at which the methodology has been successfully validated with a mean recovery of 70 - 120% and a relative standard deviation of ≤ 20% has been obtained. The limit of quantification has been set at 0.05 ppb (ng/mL) for water analysis.

3.4.5 Control and Recovery Sample Preparation

All samples were prepared per analytical method (GRM072.17A) to obtain a homogeneous sample prior to analysis.

3.4.5.1 Water sample preparation

A summary sample preparation is included as a flow-chart form in FIGURE 6. The process was as follows:

1. Transferred 0.5 ml of the water sample to an autosampler vial.
2. Fortified each water sample with 0.1 ml of fortification solution in acetonitrile.
3. Adjusted final volume to 1.0 ml using acetonitrile.
4. Final sample solution was acetonitrile/water (50:50 v/v) and ready for final determination by LC-MS/MS.

System conditions for analysis of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 are presented in FIGURE 7.

3.5 Data Acquisition, Calculations and Statistics

3.5.1 LC-MS/MS Methods for Surface Water and Ground Water Analysis

All data from the mass spectrometer were recorded and processed using the computer program Analyst® instrument control and data processing software version 1.6.2. The program and computer system control the mass spectrometer, acquire the signal, measure the peak area for the analyte derived from its respective ions, and manage the data.

3.5.1.1 Data acquisition, calculations, and statistics for water samples

Data acquisition was conducted by a calibration curve for each analyte. The peak area versus calibration standard concentration (ng/mL) for each of the analytes SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 was plotted, and the linear regression curve statistics were calculated. A weighted ($1/x^2$) linear regression was used to fit the calibration data. The analyte concentration for each sample was interpolated from the calibration curve. The correlation coefficient (r) from the calibration curve generated for each reported analytical set was at least 0.996.

The concentrations of the fortified samples were calculated by fitting the respective peak area response for each sample to its concentration from the calibration curve. Percent (%) recovery calculations were performed as instructed in the method (GRM072.17A) using Microsoft® Excel 2016 using full precision of the cell, resulting in minor differences, in some instances, when calculating the percent recovery in the tables. See FIGURE 8 for a set of example calculations for the water samples.

4.0 RESULTS AND DISCUSSION

4.1 Method Establishment/Pre-Validation Evaluation

The mass spectrometer was optimized using MRM mode. Using the instrument parameters as described in the protocol, the retention times of the analytes, instrument detection limits, and response linearity were established by injecting a series of calibration standards.

4.2 Method Performance

Syngenta method number GRM072.17A was followed as written except for minor modification to the LC-MS/MS conditions which were followed as written in the study protocol and are briefly described in section 3.4.1. The ILV was successful as the mean recovery for each matrix at each fortification level was between 70-120% with an RSD of ⩽20%.

4.2.1 Detector Linearity

The linearity of the detector response was assessed using a calibration curve generated with each analysis set, for each analyte. It was demonstrated that the LC-MS detector response for analytes SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 for primary transition has correlation coefficients of 0.9996, 0.9991, 0.9978, 0.9984, and 0.9998, respectively within the calibration range from 0.005 ng/mL to 5 ng/mL.

4.2.2 Independent Laboratory Results- Primary Transition Results

The method for the primary transition was successfully validated on the first attempt for both surface water and ground water at the LOQ concentration level and at the 10× LOQ concentration level, using the method as written with minor modifications.

4.4 Communications

No communication was held between the performing laboratory (PASC) and sponsor (Syngenta) regarding the ILV procedure or progress, until the ILV was completed.

4.5 Deviations

N/A.

4.6 Circumstances Affecting Data

No circumstances occurred during this validation that affects the quality or integrity of the data.

5.0 SUMMARY AND CONCLUSIONS

The analytical method entitled SYN547407 - Residue Method GRM072.17A for the Determination of SYN547407 and Metabolites SYN549107, SYN549431, SYN550455 and SYN551485 in Water [1] was successfully validated on the first attempt using fortified surface water and ground water samples. The fortified replicates (n=5) at each fortification level (LOQ and 10X LOQ) demonstrated acceptable recoveries with acceptable standard deviations. In conclusion, the analytical method GRM072.17A is validated for analysis of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in Water at the performing laboratory, Primera Analytical Solutions Corp., Cranbury, New Jersey facility.

This independent method validation study to evaluate surface water and ground water samples satisfies the guideline requirements described in EPA 850.6100.

FIGURE 1 Analytical Reference Standard - SYN547407

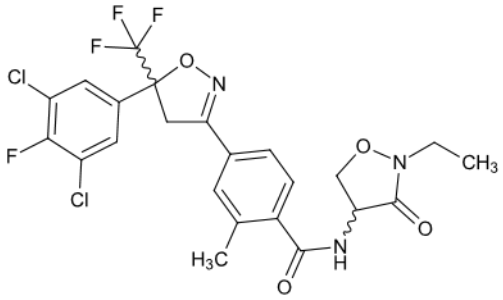
Code Name: SYN547407 Common Name: Isocycloseram IUPAC Name: 4-[(5RS)-5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydroisoxazol-3-yl]-N-[(4RS)-2-ethyl-3-oxoisoxazolidin-4-yl]-2-methylbenzamide CAS Number: 2061933-85-3 Batch ID: AMS 1521/3 Purity: 98.6% (w/w) Expiry/ Recertification Date: February, 2024 Source: Syngenta Crop Protection, LLC.	

FIGURE 2 Analytical Reference Standard - SYN549107

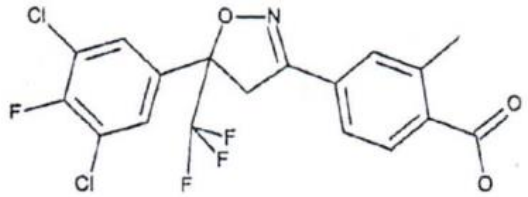
Code Name: SYN549107 Common Name: NA IUPAC Name: 4-(5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl)-2-methylbenzoic acid CAS Number: NA Batch ID: MES 421/3 Purity: 93% (w/w) Expiry/ Recertification Date: November, 2022 Source: Syngenta Crop Protection, LLC.	

FIGURE 3 Analytical Reference Standard - SYN549431

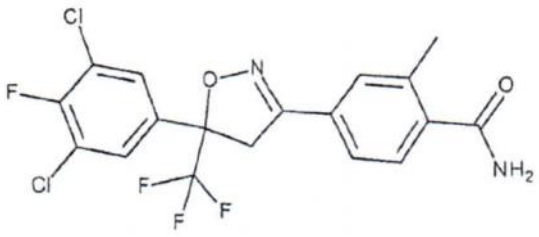
Code Name: SYN549431 Common Name: NA IUPAC Name: 4-(5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl]-2-methylbenzamide CAS Number: NA Batch ID: MES 431/3 Purity: 98% (w/w) Expiry/ Recertification Date: February, 2024 Source: Syngenta Crop Protection, LLC.	

FIGURE 4 Analytical Reference Standard - SYN550455

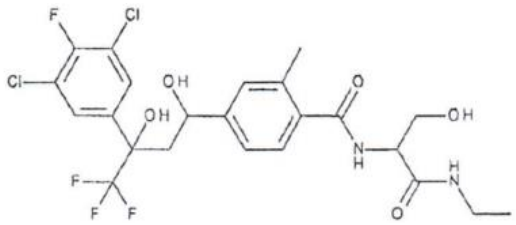
Code Name: SYN550455 Common Name: NA IUPAC Name: 4-[(3-(3,5-dichloro-4-fluorophenyl)-4,4,4-trifluoro-1,3-dihydrobutyl]-N-[1-ethylamino)-3-hydroxy-1-oxopropan-2-yl]-2-methylbenzamide CAS Number: NA Batch ID: MES 490/2 Purity: 97% (w/w) Expiry/ Recertification Date: November, 2022 Source: Syngenta Crop Protection, LLC.	

FIGURE 5 Internal Standard - SYN551485

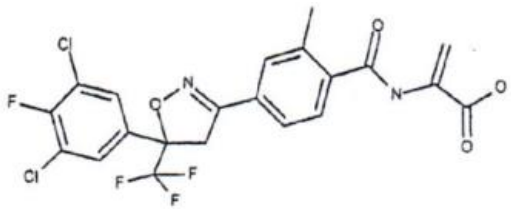
Code Name: SYN551485 Common Name: NA IUPAC Name: 2-{4-[5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3yl]-2-methylbenzamido}prop-2-enoic acid CAS Number: NA Batch ID: BPS 1661/3 Purity: 96% (w/w) Expiry/ Recertification Date: June, 2022 Source: Syngenta Crop Protection, LLC.	

FIGURE 6 Flow Diagram for Water Sample Preparation

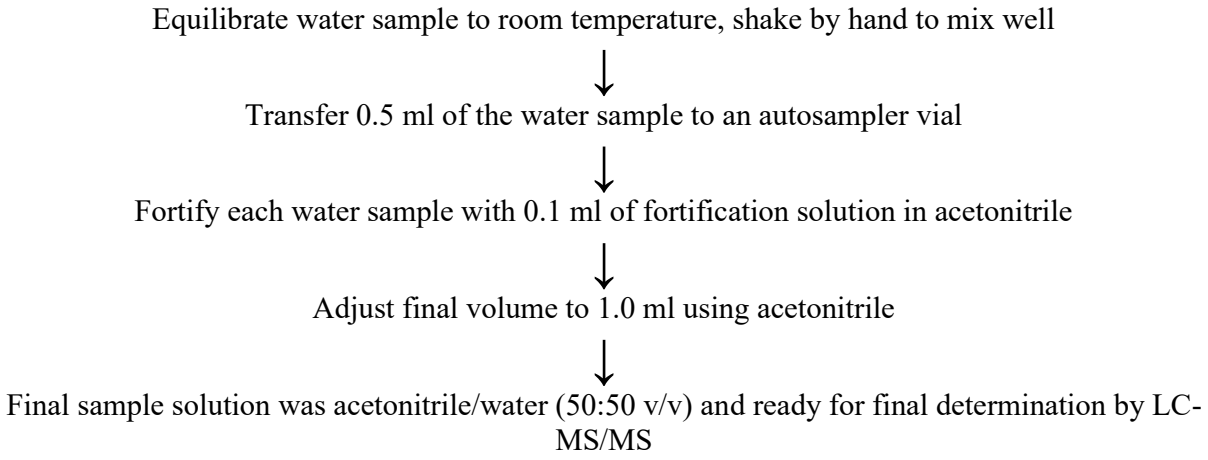


FIGURE 7 System conditions for analysis of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in Water Samples

Chromatographic conditions

Chromatographic System:	Shimadzu LC-30AD UHPLC
Column:	Phenomenex Kinetex C18 50 x 4.6 mm 2.6 µm
Column Temp.:	Set at 30 °C
Mobile Phase A:	0.1% FA in H_2O
Mobile Phase B:	0.1% FA in ACN
Flow Rate:	1.0 mL/min
Initial Pump B Conc.	0%
Run Time:	4.5 Minutes
Sample Tray Temp:	Set at 5 °C
Injection Volume:	30 µL
Strong Wash Solution:	ACN

UHPLC Gradient

Time (min)	Module	MP-A Conc.	MP-B Conc.
0.50	Pumps	100%	0%
1.20	Pumps	20%	80
3.00	Pumps	0%	100
3.50	Pumps	0%	100
4.00	Pumps	100%	0
4.50	System Controller	N/A	N/A

Mass Spectrometer parameters

Mass Spectrometer:	AB Sciex 6500 Triple Quad Mass Spectrometer
Ionization:	Positive Ion Electrospray (ESI+)
Scan Mode:	MRM
Ion Spray Voltage:	5500 V Positive Mode / -4500 V Negative Mode
Turbo Ion Spray Temp.:	600 °C
Curtain Gas Type:	Nitrogen Setting: 20
CAD Gas Type:	Nitrogen Setting: 9
Nebulizing Gas (Gas1) Type:	Nitrogen Setting: 60
Auxiliary Gas (Gas 2) Type:	Nitrogen Setting: 50
MS Acquisition Time:	4.5 Minutes

Compound ID	Transitions	Mode	Dwell Time (msec)	DP (V)	EP (V)	CE (eV)	CXP (V)
SYN547407 Primary	548 / 418	POS (+)	50	40	10	37	14
SYN547407 Confirmatory	548 / 160	POS (+)	50	40	10	55	10
SYN549107 Primary	434 / 354	NEG (-)	25	-91	-10	-30	-10
SYN549107 Confirmatory	434 / 238	NEG (-)	25	-91	-10	-30	-10
SYN549431 Primary	435 / 392	POS (+)	50	60	14	33	10
SYN549431Confirmatory	435 / 160	POS (+)	50	60	14	45	10
SYN550455 Primary	553 / 277	NEG (-)	25	-160	-10	-25	-10
SYN550455 Confirmatory	553 / 247	NEG (-)	25	-160	-10	-40	-11
SYN551485 Primary	503 / 390	NEG (-)	50	-60	-10	-25	-10
SYN551485 Confirmatory	503 / 431	NEG (-)	50	-60	-10	-20	-10

FIGURE 8 Recovery calculation for SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in Water Samples

The analysis sequences on the LC-MS/MS included an injection of a reagent blank solution, followed by calibration solutions at the following concentrations: 0.005 ng/mL, 0.01 ng/mL, 0.025 ng/mL, 0.10 ng/mL, 0.25 ng/mL, 1.00 ng/mL, 3.00 ng/mL, and 5.00 ng/mL. The linearity of the instrument response (peak area of each analyte standard response) against the analyte concentration was assessed to determine if it was acceptable across the calibration concentration range.

Subsequent recovery sample injections were conducted along with control blank with intermittent injection of each calibrant. The linearity and residue calculations are as follows,

Linear response:

Linear response of each analyte in water was calculated as follows:

a) Standard solutions were prepared at eight (8) levels

b) Each calibrant solution was injected twice (first as subsequent injection of STD-1 to STD-8) and, then intermittently during recovey sample injections), the areas of the standard peaks were measured.

c) Calibration curve parameters were generated using averaged calibrant standard peak area in linear regression with weighting factor of $1/x^2$.

d) The following equation was used to calculate linear regression,

$$y = mx + c$$

Where y is the instrument response (Sample response), x is the sample concentration, m is the linear term (slope) of the calibration curve and c is the constant term (intercept) value.

Quantitation of Analyte Recovery samples:

Analyte response in each recovery sample was calculated in ng/mL for each sample using respective sample peak area response

$$\textbf{Analyte Amount Detected (ng/mL)} = \frac{(\text{Sample Peak Area} - \text{Intercept})}{\text{Slope}}$$

Example Instrument Response Calculations:

An example calculation is presented for LOQ level recovery sample (Surface Water_LOQ_1) fortified at 0.05 ng/mL of SYN549107 in surface water [20210401_ILV_Negative.dab]. The relevant calibration curve is presented in **APPENDIX 6**, and the raw data transcript is presented in **APPENDIX 7**. Please note that some values reported for the calibration in **APPENDIX 7** have been rounded to fewer significant figures than were used in the calculations below.

The peak area = 27115

The equation for the calibration curve of SYN549107 in water:

$$y = 1050000\,x + 875$$

$$\text{SYN549107 concentration (ng/mL)} = \frac{(27115 - 875)}{1050000}$$

$$= 0.024990476$$

Calculation of the % Recovery:
No analyte has been detected in respective control samples. Therefore,

$$\text{Recovery} = \frac{\text{ppb found in recovery sample}}{\text{ppb fortified in recovery sample}} \times 100\%$$

Since the water sample was subjected to × 2 dilution (Section 3.4.4.1, steps 2 and 3), and the fortification level for water sample at LOQ is 0.05 ppb,

$$\text{Recovery} = \frac{(024990476 \times 2)}{0.05} \times 100\%$$

$$= 99.96190476$$

(The slight difference between % recovery calculated here and, SYN549107 LOQ replicate 1 in TABLE 4 is due to the use of a fewer number of significant figures in rounding of the values reported in instrument results)