2.0 INTRODUCTION

This final report describes the independent laboratory validation (ILV) of Syngenta Analytical Method GRM051.08C "Acibenzolar-S-methyl - Analytical Method GRM051.08C for the Determination of Acibenzolar-S-methyl and CGA210007 in Water by Direct Injection LC-MS/MS Analysis" (Reference 1) as performed by ADPEN Laboratories, Inc. (ADPEN). The method flow chart is presented in Appendix 1 and the analytical method is presented in Appendix 2.

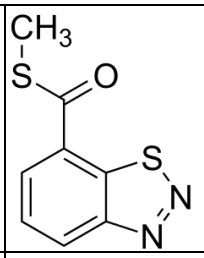
This study was designed to satisfy harmonized guideline requirements described in OECD Guidance Document ENV/JM/MONO(2007)17 (Reference 2), EPA Guideline OCSPP 850.6100 (2012) (Reference 3), EC SANCO/3029/99 Rev 4 (2000) (Reference 4), and EC SANCO/825/00 Rev 8.1 (2010) (Reference 5). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (Reference 6).

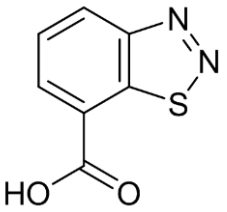
The method consists of fortifying an aliquot of the water sample and vialing for determination by LC-MS/MS.

3.0 MATERIALS AND METHODS

3.1 Test/Reference Substance

The test/reference substances were obtained from Syngenta Crop Protection, LLC. The following test/reference substances were used:

Compound Structure	
Product Code:	CGA245704
ADPEN Reference ID:	P5290
Common Name:	Acibenzolar-S-methyl
IUPAC Name:	benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester
Batch ID:	AMS 692/6
Batch No:	700096
CA Name:	1,2,3-benzothiadiazole-7-carbothioic acid, S-methyl ester
CA Reg. No.:	135158-54-2
Molecular Mass:	210.3 g/mol
Molecular Formula	C8H6N2OS2
Storage Conditions:	Ambient (< 30 °C), dark and dry
Purity (w/w):	99.7%
Expiration Date:	End of January 2016
Source:	Syngenta Crop Protection, LLC

Compound Structure	
Syngenta Code:	CGA210007
ADPEN Reference ID:	P5226
Batch ID:	JAK-XXI-66-1
Storage Conditions:	Refrigerator
Purity (w/w):	99.1%
Expiration Date:	January 31, 2016
Source:	Syngenta Crop Protection, LLC

The test/reference substances (analytical standards) used in this study were procured from the Sponsor and stored as directed. Characterization data for the test/reference standards are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificates of Analysis are included in Appendix 3.

3.2 Test System

The test systems evaluated in this study were surface and ground water. Control samples used in this study were characterized by AGVISE Laboratories of Northwood, North Dakota and reported to Syngenta Archive. GLP characterization results are presented in Appendix 4 and summarized below.

Sample ID	Water Type	Sample Description
RIMV00115-0001	Surface Water	Julian Surface Water
RIMV00115-0002	Ground Water	Summerfield Grnd.H_2O

Control water samples utilized for this study were sent from Syngenta to ADPEN on March 16, 2015 and June 2, 2015; received on March 17, 2015 and June 3, 2015. Upon receipt, the samples were logged in and stored in refrigerator E-57, which had an average temperature during the course of this study of 4 °C. Prior to analysis, the sample was sub-sampled and unique laboratory codes were assigned to each sub-sample and are cross-referenced to the Syngenta sample ID number on each page of the detailed residue reports. Sample extracts were stored in refrigerator E-20 while awaiting LC-MS/MS analysis. The average temperature during the course of this study for this refrigerator was 6 °C.

The control samples were checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in Syngenta Method GRM051.08C.

3.3 Equipment and Reagents

The equipment and reagents used for the independent laboratory validation were as outlined in the method. Identical or equivalent equipment was used, as permitted by the method.

3.3.1 Equipment

The recommended equipment and apparatus are listed in the Analytical Method GRM051.08C found in Appendix 2. Equipment with equivalent performance specifications may be substituted.

3.3.2 Reagents

All solvents and other reagents were of high purity (glass distilled/HPLC) grade solvents and analytical grade reagents. Particular care was taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated.

3.3.3 Preparation of Reagents

A list of reagents used in this method along with details of preparation of solutions is included in the Analytical Method GRM051.08C found in Appendix 2.

3.4 Preparation of Standard Solutions

Standard solutions were prepared and stored as recommended in the method.

3.4.1 Preparation of Stock Standard Solutions

Stock solutions of acibenzolar-S-methyl containing 99.9 µg/mL in acetonitrile (purity considered) and CGA210007 containing 99.2 µg/mL in acetonitrile (purity considered) were prepared by weighing 9.99 mg of Acibenzolar-S-methyl and 10.01 mg of CGA210007 into a separated 100 mL volumetric flasks adjusting the volume to 100 mL with acetonitrile.

Intermediate standard solutions of acibenzolar-S-methyl containing 9.99 µg/mL and CGA210007 containing 9.92 µg/mL, respectively, were prepared in acetonitrile from stock solutions and then further diluted 1 µg/mL, 0.1 µg/mL and 0.01 µg/mL in methanol. All standard solutions were stored in a freezer E-109 (-20 °C) in dark condition.

3.4.2 Preparation of Fortification Standard Solutions

Stock solutions of acibenzolar-S-methyl containing 99.9 µg/mL in acetonitrile (purity considered) and CGA210007 containing 99.2 µg/mL in acetonitrile (purity considered) were prepared by weighing 9.99 mg of Acibenzolar-S-Methyl and 10.01 mg of CGA210007 into a separated 100 mL volumetric flasks adjusting the volume to 100 mL with acetonitrile.

From each of stock solutions, fortification intermediate standard solutions of acibenzolar-S-methyl containing 9.99 µg/mL and CGA210007 containing 9.92 µg/mL, respectively, were prepared in acetonitrile and then further diluted to prepare each1 µg/mL, 0.1 µg/mL for 10× LOQ and 0.01 µg/mL for LOQ of acibenzolar-S-methyl and CGA210007 in acetonitrile. All fortification standard solutions were stored in freezer E-109 (-20 °C) in dark condition.

3.4.3 Preparation of Calibration Standard Solutions

Calibration standard solutions in solvent for HPLC-MS/MS analysis were prepared by volumetric dilution of the 0.01 µg/mL (10 ng/mL) standard solution into HPLC grade water containing 0.1% formic acid as follows:

Final Standard Concentration (ng/mL)	µL of Standard solution (10 ng/mL)	µL of Standard solution (1 ng/mL)	µL of Standard solution (0.1 ng/mL)	Solvent Volume (µL)
1.0000	100	-	-	900
0.6000	60	-	-	940
0.3000	30	-	-	970
0.1000	-	100	-	900
0.0500	-	50	-	950
0.0250	-	25	-	975
0.0125	-	-	125	875
0.0100	-	-	100	900

Calibration solutions in matrices for HPLC-MS/MS were prepared by volumetric dilution of the 0.01 µg/mL (10 ng/mL) standard solution into acidified control water matrices containing 0.1% formic acid as follows.

Final Standard Concentration (ng/mL)	µL of Standard solution (10 ng/mL)	µL of Standard solution (1 ng/mL)	µL of Standard solution (0.1 ng/mL)	Solvent Volume (µL)
1.0000	100	-	-	900
0.6000	60	-	-	940
0.3000	30	-	-	970
0.1000	-	100	-	900
0.0500	-	50	-	950
0.0250	-	25	-	975
0.0125	-	-	125	875
0.0100	-	-	100	900

3.5 ANALYTICAL PROCEDURE

Each validation set included a reagent blank, two control water samples, five control water samples fortified at LOQ, and five control samples fortified at 10× LOQ.

3.5.1 Modifications

Syngenta Method GRM051.08C was followed as written. Due to degradation of acibenzolar-S-methyl in the matrices for the storage stability test, prior to the fortification, untreated control samples, surface water and ground water were treated with 0.1 % of formic acid to prepare acidic condition at > pH 5.

3.5.2 Fortifications

Acidified untreated control surface and ground water samples containing 0.1% formic acid were fortified using microliter amounts of the fortification standard to 0.01 µg/mL for LOQ and 0.1 µg/mL for 10× LOQ concentrations as per method.

Fortifications used in this method validation are as follows:

Matrix	Fortification Volume (µL)	Fortification Conc. (µg/mL)	Final Volume (mL)	Final Conc. (µg/L)	Replicates
Surface water	100	0.01	20	0.05	5
	100	0.1	20	0.5	5
Ground water	100	0.01	20	0.05	5
	100	0.1	20	0.5	5

3.5.3 Extraction Procedure

1. Acidified untreated control water samples containing 0.1% formic acid were fortified using 0.1 mL of the fortification standard solutions, 0.01 µg/mL for LOQ (0.05 ppb) and 0.1 µg/mL for 10×LOQ (0.5 ppb) fortification as per the method.
2. Sample was shaken vigorously for 10 seconds to mix well using a vortex.
3. Samples were centrifuged or filtered through a 0.45 µm syringe filter if particles were visible before analysis.
4. An aliquot (approximately 1.5 mL) was transferred from the 50 mL sample to an auto sampler vial.
5. Final determination was done by LC-MS/MS.
6. All remained extracts were stored in refrigerator (0 to ≤ 15 °C) until storage stability test.

3.6 Instrumentation/Operating Conditions

3.6.1 Chromatographic Conditions

HPLC Instrument:	Agilent 1290 UPLC[1]			
Column:	Waters X-Select CSH C18 50×3 mm, 2.5 µm			
Column temperature:	40°C			
Injection volume:	100 µL			
Flow rate:	500 µL/min			
Mobile phase A:	0.1% Formic Acid in HPLC Water			
Mobile phase B:	Methanol			
Gradient Step Table:	Step	Time (min)	A (%)	B (%)
	0	0.00	80.0	20.0
	1	2.00	10.0	90.0
	2	4.50	10.0	90.0
	3	4.60	80.0	20.0
	4	6.00	80.0	20.0

3.6.2 Mass Spectrometer Conditions

Mass Spectrometer:	ABSciex QTrap 5500 (Instrument #28)
Ion Mode:	Turbo Spray
Polarity:	Positive (Acibenzolar-S-methyl), Negative (CGA210007)
Curtain gas (CUR):	15.00
Temperature (TEM):	550 °C
Collision gas setting (CAD):	Medium
GS1:	40.0
GS2:	40.0
Entrance potential (EP):	10.0
Scan type:	MRM

MRM Conditions	Q1 *m/z*	Q3 *m/z*	Retention Time (min)	Dwell time	DP	CE	CXP
Quantification Ions							
Acibenzolar-S-methyl	210.97	135.90	3.15	250	51	39	6
CGA210007	178.83	107.00	2.74	250	−80	−26	−11
Confirmation Ions							
Acibenzolar-S-methyl	210.97	91.00	3.15	250	51	27	14
CGA210007	178.83	57.00	2.74	250	−80	−52	−5

[1] The chromatographic system is a UPLC instrument; however, the method operates under HPLC conditions.

3.7 Data Acquisition

Peak integration and peak area count quantitation were performed by Analyst (version 1.6.2) data handling software. A best-fit, linear regression equation was derived and used in conjunction with the analyte response in each sample to calculate the concentration of the analyte. The square of correlation coefficients (R^2) for the calibration curves for each analytical set was greater than 0.99. Recovery results were computed for each sample.

A statistical treatment of the data includes the calculation of averages, standard deviations, relative standard deviations. Mean percent recoveries were calculated in LIMS and reported in Microsoft® Office Excel spreadsheets. Standard deviations and relative standard deviations were calculated and reported in Microsoft® Office Excel spreadsheets.

Example calculations for a control surface water sample fortified with acibenzolar-S-methyl is presented in Appendix 5.

4.0 RESULTS AND DISCUSSION

4.1 Method Establishment/Pre-Validation Evaluation

Initially, the mass spectrometer was optimized by infusing analyte standards to determine the optimum instrument operation parameters. Using these optimized instrument parameters, the retention times of the analytes, instrument detection limits and response linearity were established by injecting a series of calibration reference standards.

Prior to analysis of actual validation samples, a reagent blank and untreated control samples were analyzed to determine if interferences were present near the retention time of the analytes. The results of these evaluations indicated the selected control samples contained no detectable residues of Acibenzolar-S-Methyl and CGA210007 and had no peaks which might interfere with targeted analyte responses.

4.2 Independent Laboratory Results

Due to matrix effects and uncertain stability of acibenzolar-S-methyl in fortified control samples and calibration standard samples, the first trial results did not meet protocol acceptance criteria.

The method was successfully validated on the second trial for surface and ground water at the limit of quantification (LOQ) and at 10× LOQ, using the method as written with only the addition of 0.1 % of formic acid to standards and samples to improve analyte stability. Fresh matrix matched standard calibration were also prepared to minimize any impact from stability. Acceptable recoveries (70–110%) were observed at the limit of quantification and at 10× LOQ.

APPENDIX 1 Method Flow Chart