2.0 INTRODUCTION

Described in this report is the independent laboratory validation (ILV) of Syngenta Analytical Method GRM057.04A "Azoxystrobin – Residue Method (GRM057.04A) for the Determination of Azoxystrobin and Z-Isomer R230310 in Water by LC-MS/MS" (1) as performed by Smithers Viscient.

This study was designed to satisfy harmonized guideline requirements described in OCSPP 850.6100 (Data Reporting for Environmental Chemistry Methods). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (3).

The residue analytical method is suitable for the determination of Azoxystrobin and Z-Isomer R230310 in surface and ground water.

This method was validated by fortification of surface and ground water with both Azoxystrobin and Z-Isomer R230310 at concentrations of 0.100 and 1.00 µg/L. Recovery

samples were immediately diluted with 50:50 ultra-pure acetonitrile:ultra-pure reagent water. Calibration standards were also prepared in 50:50 ultra-pure acetonitrile:ultra-pure reagent water. All samples and standards were submitted for analysis by LC-MS/MS.

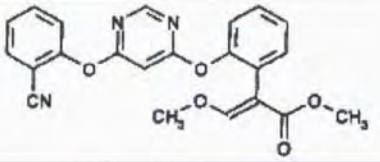
Exceptions to the method GRM057.04A as written, and that performed as Smithers Viscient are as follows:

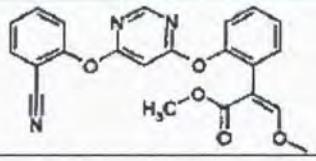
- An additional confirmation transition of 404.4/344.1 was analyzed for both Azoxystrobin & Z-Isomer.
- The instrument used for analysis was a LC-MS/MS 5000, as opposed to a LC-MS/MS 5500.

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	
Syngenta Code:	ICI5504
Common Name:	Azoxystrobin
CAS Name:	Methyl (E)-2-[[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy]-α-(methoxymethylene)benzeneacetate
CAS Number:	131860-33-8
Molecular Weight:	403.4
Standard Reference:	ASJ10008-04
Storage Conditions:	< 10°C
Purity:	99.7% ± 0.3%
Expiration Date:	End of August 2015

Compound Structure	
Syngenta Code:	R230310
Common Name:	Z-Isomer R230310
CAS Name:	Benzeneacetic acid, 2-[[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy]-a-(methoxymethylene)-, methyl ester
CAS Number:	143130-94-3
Molecular Weight:	403.4
Standard Reference:	601321
Storage Conditions:	< -10°C
Purity:	94% ± 5% (wt/wt)
Expiration Date:	End of December 2016

Characterization data for the test/reference standard are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificate of Analysis is included in Appendix 3.

The test/reference substance (analytical standard) used in this study was procured from the Sponsor and stored as directed on "Analytical Standards Chain of Custody" documents. All solutions made from the reference substances (analytical standards) were stored according to the method.

3.2 Test System

The test system evaluated in this study was surface and ground water. These matrices were chosen because it is representative of the matrices the method was designed for. Control samples used in this study were provided by the Sponsor. These control water samples were characterized by AGVISE Laboratories of Northwood, North Dakota and reported to Syngenta Archive under Syngenta Study Number TK0048240. GLP characterization results are presented in Table 1 and summarized below:

Sample ID	pH	Calcium (ppm)	Magnesium (ppm)	Sodium (ppm)	Hardness CaCO3 (mg/L)	TDS (ppm)	SAR
Surface	7.3	6.0	2.9	2.3	27	58	0.19
Ground	7.5	16	4.5	6.4	59	122	0.36

SAR (Sodium Adsorption Ratio)
TDS (Total Dissolved Solids)

The receipt and storage detail as per performing laboratory SOP.

These 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 GRM057.04A.

3.3 Equipment and Reagents

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

3.3.1 Equipment

1.	Instrument:	AB MDS Sciex 5000 Turbo V ESI with Shimadzu 20AD vacuum degasser, LC-20AD binary pump, CTO-20AC column compartment, and SIL-20ACHT autoinjector; Analyst 1.4.2 for data acquisition.
2.	Balance:	O'Haus G160
3.	Laboratory equipment:	Positive displacement pipets, disposable graduated glass pipets, volumetric flasks, polypropylene tubes, autosampler vials, and amber glass bottles with Teflon®-lined caps.

3.3.2 Reagents

Acetonitrile:	Burdick & Jackson, ultra-pure LC/MS grade
Acetic Acid	EMD, reagent grade
Purified reagent water:	Burdick & Jackson, ultra-pure LC/MS grade

3.3.3 Preparation of Reagents

A 50:50 ultra-pure acetonitrile:ultra-pure reagent water, v:v solution was prepared by mixing 350 mL of ultra-pure acetonitrile with 350 mL of ultra-pure reagent water. This reagent solution was mixed using a stir bar and stir plate and used to prepare the calibration standards and dilute the fortified recovery samples.

A 0.1% acetic acid in ultra-pure reagent water, v:v solution was prepared by mixed 1000 mL of ultra-pure reagent water with 1.0 mL of concentrated acetic acid. The mobile phase solution was mixed well and degassed under vacuum with sonication.

A 0.1% acetic acid in ultra-pure acetonitrile, v:v solution was prepared by mixed 1000 mL of ultra-pure acetonitrile with 1.0 mL of concentrated acetic acid. The mobile phase solution was mixed well and degassed under vacuum with sonication.

3.4 Preparation of Standard Solutions

Standard solutions were prepared and stored as recommended in the method. All primary and secondary stock solutions were stored refrigerated in glass amber bottles fitted with Teflon®-lined caps. All sub-stock solutions were prepared daily and discarded after use.

3.4.1 Stock Standard

A 1000 mg/L primary stock solution of Azoxystrobin was typically prepared by placing approximately 0.0252 g of the test substance (corrected for purity) into a 25.0-mL volumetric flask and bringing it to volume with acetonitrile.

Two secondary stock solutions of Azoxystrobin at 1.00 and 10.0 mg/L were prepared by placing 0.0500 mL and 0.500 mL, respectively, of the primary stock solution into separate 50.0-mL volumetric flasks and bringing to volume with acetonitrile. These secondary stock solutions were used to prepare additional sub-stock solutions.

A 1000 mg/L primary stock solution of Z-Isomer R230310 was typically prepared by placing approximately 0.0266 g of the test substance (corrected for purity) into a 25.0-mL volumetric flask and bringing it to volume with acetonitrile.

Two secondary stock solutions of Z-Isomer R230310 at 1.00 and 10.0 mg/L were prepared by placing 0.0500 mL and 0.500 mL, respectively, of the primary stock solution into separate 50.0-mL volumetric flasks and bringing to volume with acetonitrile. These secondary stock solutions were used to prepare additional sub-stock solutions.

3.4.2 Fortification Standard

A 0.0100 mg/L mixed sub-stock solution comprised of a mixture of Azoxystrobin and Z-Isomer R230310 was prepared by placing 0.100 mL of each 1.00 mg/L secondary stock solutions into a disposable glass vial and bringing to a final volume of 10.0 mL with acetonitrile. A 0.100 mg/L mixed sub-stock solution comprised of a mixture of Azoxystrobin and Z-Isomer R230310 was prepared by placing 0.100 mL of each 10.0 mg/L secondary stock solutions into a disposable glass vial and bringing to a final volume of 10.0 mL with acetonitrile. These mixed sub-stock solutions were used to prepare calibration standards and recovery samples for the ILV.

3.4.3 Calibration Standard

Calibration standards were prepared in 50:50 ultra-pure acetonitrile:ultra-pure reagent water at concentrations of 0.00500 and 0.0100 µg/L using the 0.0100 mg/L mix sub-stock solution, and at concentrations of 0.0500, 0.100, 0.500, and 2.00 µg/L using the 0.100 mg/L mixed sub-stock solution.

3.5 Analytical Procedures and Modifications

Analytical Method GRM057.04A was independently validated as written.

3.5.1 Modifications

Syngenta Method GRM057.04A was followed as written with exception:

- An additional confirmation transition of 404.4/344.1 was analyzed for both Azoxystrobin & Z-Isomer (intended for use as an alternative/additional confirmation transition).
- The instrument used for analysis was a LC-MS/MS 5000, as opposed to a LC-MS/MS 5500 (use of an LC-MS/MS 5500 was unavailable).

3.5.2 Fortifications

Untreated control surface and ground water samples were fortified using microliter amounts of the appropriate fortification standard to LOQ and 10X 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 & Ground	10.0	0.0100	1.00	LOQ (0.100)	5
Surface & Ground	10.0	0.100	1.00	10X LOQ (1.00)	5

3.5.3 Extraction Procedure

As indicated by method GRM057.04A the following extraction steps were performed:

Fortified recovery samples were prepared at each concentration level in 15 mL polypropylene tubes using the appropriate mixed sub-stock solutions according to table 3.5.2. Prior to use, surface and ground water solutions were allowed to thaw completely, followed by shaking to thoroughly ensure homogeneity of the matrix. Following fortification, samples were immediately diluted with 50:50 ultra-pure acetonitrile:ultra-pure reagent water to a final volume of 10.0 mL. An additional set of two (each), unfortified surface and ground water samples were also diluted as the fortified recovery samples to act as controls. Samples and calibration standards were transferred to amber autosampler vials for LC-MS/MS analysis.

3.6 Instrumentation

LC-MS/MS

LC System	:	Shimadzu 20AD HPLC
MS Detector	:	MDS Sciex 5000 with Analyst Software (version 1.4.2)

Flow Rate:	0.8 mL/min
Column:	ACE 5 C18, 3.0 x 50 mm
Column Oven Temp:	40°C
Injection Vol.	30µL
Run Time:	3.0 minute
Retention Time:	Azoxystrobin: approximately 1.47 minutes, R230310: approximately 1.18 minutes
Mobile Phase A:	0.1% acetic acid in ultra-pure reagent water

Mobile Phase B: 0.1% acetic acid in ultra-pure acetonitrile
Isocratic Flow:

Time	A%	B%
0.0	50	50
3.0	50	50

Mass Spectrometer Conditions

Interface	:	ESI
Polarity	:	Positive
Curtain gas (CUR)	:	25.00
Temperature (TEM)	:	450°C
Ionspray voltage	:	3000 V
Collision gas setting (CAD)	:	8.00
Gas 1 (GS1)	:	35.00
Gas 2 (GS2)	:	35.00
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		A Primary Transition	A Confirmatory #1 Transition	A Confirmatory #2 Transition	B Primary Transition	B Confirmatory #1 Transition	B Confirmatory #2 Transition
Q1 *m/z*	:	404.38	404.38	404.38	404.39	404.39	404.39
Q3 *m/z*	:	372.10	329.00	344.10	372.00	329.00	344.10
Dwell time (ms)	:	150	150	150	150	150	150
Resolution Q1	:	Unit	Unit	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	91.00	91.00	91.00	126.00	126.00	126.00
Entrance potential (EP)	:	10.00	10.00	10.00	10.00	10.00	10.00
Collision energy (CE)	:	21.00	42.00	35.00	17.00	42.00	35.00
Collision cell exit potential (CXP)	:	44.00	26.00	26.00	42.00	40.00	40.00

3.7 Data Acquisition

Peak integration and peak area count quantitation were performed by Analyst Software (version 1.4.2). A quadratic equation was derived and used in conjunction with the analyte response in each sample to calculate the concentration of 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, standard deviations, and relative standard deviations were calculated using a current Microsoft Office Excel package.

4.1 Method Establishment/Pre-Validation Evaluation

Initially, the mass spectrometer was optimized by infusing analyte standard(s) to determine the optimum instrument operation parameters. Using the instrument parameters as described in the method, 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 sample selected by the study sponsor were analyzed to determine if interferences were present near the retention time of the analyte. The results of these evaluations indicated that the selected control samples contained no detectable levels of Azoxystrobin or Z-Isomer R230310, therefore, minimum interferences were expected for the targeted analyte responses.

APPENDIX 4 Example Calculations

A calibration curve was constructed by plotting the analyte concentration (µg/L) in the calibration standards against the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the peak area of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \quad y = ax^2 + bx + c$$

$$(2) \quad DC(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

$$(3) \quad A = DC \text{ x } DF$$

where:

y	=	peak area for the test substance
a, b and c	=	regression constants
DC (x)	=	detected concentration (µg/L) in the sample
C	=	constant c minus the peak area ratio; C = (c - y)
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

$$(4) \; LOQ_{INST} = -b + \sqrt{b^2 - 4aC}$$

$$(5) \; LOQ = LOQ_{INST} \text{ x } DF_{CTRL}$$

where:

a, b, c	=	regression constants
C	=	regression constant c – $Area_{MIN}$; C = (c - $Area_{MIN}$)
$Area_{MIN}$	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis