1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM057.04A is suitable for the determination of Azoxystrobin (Figure 1) and Z-isomer R230310 (Figure 2) in surface and ground water. The limit of quantitation (LOQ) of the method has been established at a 0.1 µg/L (0.1 ppb). Analytical method GRM057.04A supersedes method RAM 235/01. GRM057.04A has the addition of Z-isomer R230310 at a limit of quantitation of 0.1 µg/L (0.1 ppb) and increased specificity/selectivity by LC-MS/MS.

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, EPA OPPTS 850.7100(1996) and EC Guidance Documents SANCO/3029/99 rev 4(2000) and SANCO/825/00 rev 8.1(2010).

1.2 Method Summary

Surface and ground water samples are analyzed directly by LC-MS/MS after dilution with acetonitrile/ultra pure water (50/50 v/v) using non-matrix matched external calibration.

The LOQ of the method is 0.1 µg/L (0.1 ppb) for surface and ground water.

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 100 µg/mL stock solutions for azoxystrobin and R230310 by one of the following methods:

Weigh out accurately, using a five figure balance, sufficient azoxystrobin and R230310 analytical standard into an amber "Class A" volumetric flask (100-mL). Dilute to the mark with acetonitrile and mix well to give 100 µg/mL stock solutions of azoxystrobin and R230310. Standards should be prepared in amber bottles and stored under refrigeration.

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 containing azoxystrobin and R230310 should be prepared by serial dilution in acetonitrile from the stock solution. It is recommended that the following solutions are prepared: 1.0 µg/mL, 0.10 µg/mL and 0.01 µg/mL for fortification purposes. Mixed standards of azoxystrobin and R230310 may be prepared if desired.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant matrix effects, suppression or enhancement of the instrument response for azoxystobin and R230310 has been observed in the water types tested using the procedures described in Section 3 during method validation and non-matrix matched calibration standards should be used for quantification.

A calibration curve should be generated to quantify azoxystrobin and R230310 residues. Standards over an appropriate concentration range should be prepared with a minimum of five levels. Calibration standards are prepared by serial dilution using acetonitrile/ultra pure water (50/50 v/v). Using the instrumentation described in Section 4 a calibration range from 0.005 pg/µL to 2 pg/µL was found to be linear.

Any observed matrix effects may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with acetonitrile/ultra pure water (50/50 v/v) should instrument sensitivity permit.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figures section.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in amber bottles and refrigerated 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 azoxystrobin and R230310 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

	Acetonitrile	Acetic acid
Harmful Vapor	✓	✓
Highly Flammable	✓	✖
Harmful by Skin Absorption	✓	✓
Irritant to respiratory system and eyes	✓	✓
Causes severe burns	✖	✓
Syngenta Hazard Category (SHC)	C, S	SHC-C, S
OES Short Term (mg/m^3)	105	37
OES Long Term (mg/m^3)	70	25

N/A not known

Azoxystrobin is rated SHC-C. 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 vapor. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form as shown in Appendix 4. In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included in each sample set. At least one untreated control and two control samples fortified with known amounts of azoxystrobin and R230310 should be analyzed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

3.1 Sample Preparation

a) If water samples are received frozen they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to subsequent aliquot for further treatment or analysis.

b) Accurately transfer 1.0 mL of the water sample to be analyzed into a 15 mL polypropylene falcon tube. Sample fortification is carried out at this time, if required.

c) Dilute to appropriate volume with acetonitrile/ultra pure water (50/50 v/v). Using the instrumentation described in section 4.0 the method LOQ (0.1 ppb) can be diluted 1 to 10 while maintaining acceptable signal/noise ratio.

d) Vial and submit samples for determination by LC-MS/MS.

Significant matrix effects can be compensated by addition of control matrix to the calibration standards. A fixed volume of 100 µL of control water can be added to each known volume of standard. This amount represents the same amount of matrix present after a 1 to 10 dilution at the method LOQ (0.1 ppb) when a final volume of 1 mL is maintained. Addition of any solvent should remain the same for each standard and sample.

3.2 Experimental Precautions

a) Bottled HPLC/OPTIMA grade ultra pure water and acetonitrile 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 minimize 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 0.005 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.

3.3 Time Required for Analysis

The methodology is normally performed with a batch of 36 samples. One skilled analyst can complete the analysis of 1-2 sample sets in 1 day (8 hour working period).

3.4 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekends 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 AB Sciex 5500 instrument. The system is controlled and data is processed by AB Sciex Analyst™ Software. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimization 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-MS/MS

LC System	:	Shimadzu UFLC XR
Detector	:	Applied Biosystems API 5500 QTRAP with Analyst Software (version 1.5.1)

4.2 Chromatography Conditions

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
Detector:	Applied BioSystem API QTRAP 5500
Retention Time:	Azoxystrobin:1.28 minutes, R230310: 1.02 minutes
Mobile Phase A:	OPTIMA Grade Water in 0.1% Acetic Acid
Mobile Phase B:	OPTIMA Grade Acetonitrile in 0.1% Acetic Acid

Isocratic Flow:

Time	A%	B%
0.0	50	50
3.0	50	50

4.2 Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	500 °C
Ionspray voltage	:	2500 V
Collision gas setting (CAD)	:	Nitrogen set at Medium (arbitrary units)
Gas 1 (GS1)	:	Air set at 50 (arbitrary units)
Gas 2 (GS2)	:	Air set at 50 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		Azoxystrobin Primary Transition	Azoxystrobin Confirmatory Transition	R230310 Primary Transition	R230310 Confirmatory Transition
Q1 *m/z*	:	404	404	404	404
Q3 *m/z*	:	372	329	372	329
Dwell time	:	50 ms	50 ms	50 ms	50 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	50V	50V	50V	50V
Entrance potential (EP)	:	10V	10V	10V	10V
Collision energy (CE)	:	18V	42V	18V	42V
Collision cell exit potential (CXP)	:	10V	10V	10V	10V

Typical chromatograms for surface and ground water are shown in the Figures Section. Chromatograms for other water types are similar.

4.3 Confirmatory Procedures for Azoxystrobin and R230310

Final determination by LC-MS/MS with two transitions (primary and confirmatory) 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 of azoxystrobin and R230310 may be calculated in ppb 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 levels).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to respective target ions. Calibration standard solutions should be interspersed throughout the analysis and/or every fourth or fifth injection.

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 (slope) 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 residues of interest in a sample, expressed as µg/L, as follows:

$$Residue\ (\mu g/L\ or\ ppb) = \frac{Analyte\ Found\ (pg)}{Water\ Sample\ Injected\ (mg\ or\ \mu L)}$$

Where on-column *Analyte Found (pg)* is calculated from the standard calibration curve and on-column *Water Sample (matrix) Injected* is calculated as follows:

$$Water\ Sample\ Injected\ (mg\ or\ \mu L) = Sample\ Volume\ (mL) \times \frac{Injection\ Volume\ (\mu L)}{Sample\ Final\ Volume\ (mL)}$$

f) Determine the recovery by first subtracting the residue found in the control sample, if any, from the residue found in the recovery sample. Calculate the recovery as a percentage (%) by the equation:

$$Recovery\ (\%) = \frac{(Residue\ in\ Recovery\ Sample) - (Residue\ in\ Control)}{Amount\ Fortified} \times 100\%$$

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

$$Corrected\ Residue\ (\mu g/L\ or\ ppb) = \frac{Residue\ (\mu g/L\ or\ ppb)}{Average\ Precent\ Recovery}$$

5.2 Single Point Calibration Procedure

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).

Azoxystrobin and R230310 residues may be calculated in µg/L (ppb) for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard containing azoxystrobin and R230310 at an appropriate concentration using LC-MS/MS conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for azoxystrobin and R230310.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to azoxystrobin and R230310.

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

d) Calculate the azoxystrobin and R230310 residues in the sample, expressed as µg/L (ppb) using a mean standard response from each of the injections bracketing the sample as follows:

$$Residue\ (\mu g/L\ or\ ppb) = \frac{PK\ area\ (SA)}{PK\ area\ (STD)} \times \frac{Standard\ Conc.}{Sample\ Conc.}$$

PK area (SA) = Peak response for sample
PK area (STD) = Average peak response for bracketing standards
Standard Conc. = Concentration of standard (µg/mL)
Sample Conc. = Sample concentration (L/mL)

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

$$Corrected\ Residue\ (\mu g/L\ or\ ppb) = \frac{Residue\ (\mu g/L\ or\ ppb)}{Average\ Precent\ Recovery}$$

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed 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 analyzed with each batch of samples. Control samples from the same matrix are recommended to monitor any instrumental matrix effects present.

At least two recovery samples (control samples accurately fortified with known amounts of analyte), including one at the method LOQ and one at the expected residue level, should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found in the sample. The fortification levels should be appropriate to the residue levels expected in the sample.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of $\leq 20\%$.

When 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.

TABLE 6 LC-MS/MS Matrix Effects

	% Matrix Effect	
Analyte	Surface Water	Groundwater
Azoxystrobin; *m/z = 404 → 372*	-10	-7
Azoxystrobin; *m/z = 404 → 329*	-9	-7
R230310; *m/z = 404 → 372*	-15	-12
R230310; *m/z = 404 → 329*	-15	-12

Determination of LC-MS/MS Matrix Effects

The effect of different water types on the LC-MS/MS signal was assessed by preparing standards in the presence of water matrix and comparing the peak areas of azoxystrobin and R230310 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 matrix-matched standard response

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

CHEMICAL STRUCTURES

FIGURE 1 Azoxystrobin

Compound Code Number : Azoxystrobin
CAS Name : methyl (*E*)-2-[[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy]-α-(methoxymethylene)benzeneacetate
IUPAC Name : methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate
Molecular Formula : $C_{22}H_{17}N_3O_5$
Molecular Weight : 403.4

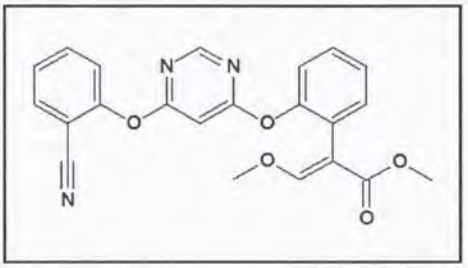
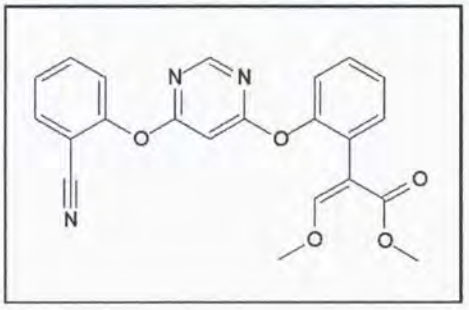


FIGURE 2 R230310 Z-Isomer

Compound Code Number : R230310
CAS Name : benzeneacetic acid, 2-[[6-(2-cyanophenoxy)-4-pyrimidinyl]oxy]-α-(methoxymethylene)-, methyl ester
IUPAC Name : methyl (*Z*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate
Molecular Formula : $C_{22}H_{17}N_3O_5$
Molecular Weight : 403.4



APPENDIX 1 Apparatus

Recommended Suppliers

Equipment	Description	Supplier
General lab glassware	General lab glassware	www.thermoscientific.com
General lab plastic-ware	General lab plastic-ware	www.thermoscientific.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
HPLC column	ACE 3 C18, 3.0 x 150mm	www.ace-hplc.com

APPENDIX 2 Reagents/Chemicals

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	Optima/HPLC grade	www.thermoscientific.com
Methanol	Optima/HPLC grade	www.thermoscientific.com
Acetonitrile	Optima/HPLC grade	www.thermoscientific.com
Ammonium Acetate	A.C.S. grade	www.thermoscientific.com
General Lab Chemicals	A.C.S. grade	www.thermoscientific.com
azoxystrobin/R230310 analytical standards	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) 0.1% Acetic Acid in Water (v/v) prepared by adding 1.0 ml acetic acid and diluting to 1,000 mL using Optima/HPLC Grade water.

b) 0.1% Acetic Acid in Acetonitrile (v/v) prepared by adding 1.0 ml acetic acid and diluting to 1,000 mL using Optima/HPLC Grade acetonitrile.

c) 50/50 Acetonitrile/Water (v/v) prepared by diluting 500mL of acetonitrile to 1,000 mL using Optima/HPLC Grade water.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polytyrosine-1,3,6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for azoxystrobin & R230310

Infuse a standard solution of azoxystrobin and R230310 (0.1 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 and temperature, 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* 404 for azoxystrobin in positive ionization mode and *m/z* 404 for R230310 in positive ionization mode. Both compounds will have the same molecular mass.

Using the Analyst™ Software optimization routine, tune the instrument for azoxystrobin and R230310, ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy for each compound 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 a azoxystrobin and R230310 standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, temperature, gas flows, and voltages) and the collision gas pressure for maximum sensitivity.

For Azoxystrobin and its Z-isomer R230310, in positive ionization mode, the protonated molecular ion generated in the ion source (*m/z* 404) is selected and subjected to further fragmentation by collision induced fragmentation. The product ion (*m/z* 372) is selected and used for quantitative analysis as the primary transition. Product ion *m/z* 329 is used for confirmatory purposes.

Final determination by LC-MS/MS is considered to be highly specific.

APPENDIX 4 Method Flow Chart for LC-MS/MS

Transfer sample (1.0 mL) to a 50 mL poly tube

↓

Fortify recovery sample, if needed

↓

Dilute as necessary with acetonitrile/ultra pure water (50/50 v/v)

↓

Mix well by agitation (vortex/shake)

↓

Vial and analyze by LC-MS/MS