1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM010.04A is designed and developed in order to analyze cyprodinil (company code CGA219417) and its metabolite CGA249287 in surface water system. The chemical structures and properties are presented in Figure 1. The final determination is conducted using LC-MS/MS technology and both primary and confirmatory transitions. The method limit of quantification (LOQ) is at 0.1 ppb (0.1 µg/L) for CGA219417 and CGA249287 in surface water system.

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

1.2 Method Summary

A portion of sub-sample (10 mL) is transferred into a Polypropylene centrifuge tube (50 mL). The sample is directly injected onto a LC-MS/MS system and analyzed for the residues of CGA219417 and CGA249287. The LOQ of this method is 0.1 ppb (0.1 µg/L) for the analytes in surface 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.

Stock Solutions

Prepare a 100 µg/mL stock solution for CGA219417 and CGA249287 by one of the following methods:

Weigh out accurately, using a five figure balance, 10.00 mg (corrected for impurity) of each analytical standard into an amber "Class A" volumetric flask (100 mL). Dilute to the mark with acetonitrile to give 100 µg/mL individual stock solutions of CGA219417 and CGA249287.

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.

Preparation of Fortification Standard Solutions

Prepare an intermediate combined standard solution containing CGA219417 and CGA249287 (1 µg/mL) by mixing 1 mL of each stock solution in a volumetric flask (100 mL) and diluting with acetonitrile to the mark. Prepare the first level combined fortification standard (0.1 µg/mL) by mixing 10 mL of the intermediate standard solution (1 µg/mL) with 90 mL of ultrapure water in a 100 mL volumetric flask. Prepare the second level combined fortification standard (0.01 µg/mL) by mixing 10 mL of the first level combined fortification standard (0.1 µg/mL) with 90 mL of ultrapure water in a 100 mL volutric flask. It is strongly recommended that such two combined fortification standard solutions are prepared and used for fortification.

Preparation of Calibration Standards for LC-MS/MS

Prepare the calibration standard solutions by serial dilutions of the first level combined fortification standard solution (0.1 µg/mL) with ultrapure water. For example, mix 1 mL of the 0.1 µg/mL combined fortification standard with 49 mL of ultrapure water in a volumetric flask (50 mL) to yield 2 ng/mL combined calibration standard containing CGA219417 and CGA249287. Dilute the calibration standard solution further with ultrapure water to yield lower concentrations. It is strongly recommended that the following combined calibration standards are prepared: 0.02 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL and 2 ng/mL for CGA219417 and CGA249287.

Matrix-Match Calibration Standard Solutions

Matrix-match standards are needed for this method if significant matrix effect is observed. In case matrix-match standard solutions are needed, the first level combined fortification standard (0.1 µg/mL) is used to prerepare intermediate standards at 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL and 20 ng/mL in ultrapure water. Mix 1 mL of each intermediate standard with 9 mL of the control surface water sample (Section 3.2) to yield 0.02 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL and 2 ng/mL matrix-match standard solutions.

Calibration Curves

Calibration curves should be constructed for quantitation of CGA219417 and CGA249287 in unknown samples. At least five levels of calibration standard solutions over an appropriate concentration range should be prepared and a weighing factor of 1/x should be used.

2.4 Standard Solution Storage and Expiration

Stock solutions must be stored refrigerated when not used. All standard solutions should be stored in a refrigerator when not in use to prevent decomposition and/or concentration of the standards. Standard solutions should be allowed to equilibrate to room temperature prior to use. Note: Check the injection standard stability against the fortification standards from time to time.

An expiration date of 3 months for CGA219417 and CGA249287 is recommended unless additional data are generated to support a longer expiration date.

2.5 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	Methanol
Harmful Vapour	✓	✓
Highly Flammable	✓	✓
Harmful by Skin Absorption	✓	✓
Irritant to respiratory system and eyes	✓	✓
Causes severe burns	*	*
Syngenta Hazard Category (SHC)	SHC-C, S	SHC-C, S
OES Short Term (mg/m^3)	105	310
OES Long Term (mg/m^3)	70	260

N/A not known

At present there are insufficient data available to assign a Syngenta Hazard Classification for CGA219417 and CGA249287. They should be treated as a category SHC-D compounds until further information indicates otherwise. 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 vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

3.1 Precautions

a) Bottled HPLC grade ultra pure water is used to prepare the LC mobile phase, which produces a lower backsurface 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 (1 ppb) and samples with expected residues greater than 1 ppb should be diluted with ultrapure water so that the final analyte concentration does not exceed 1 ppb (µg/L). It may also be useful to include blank injections of ultrapure water after high level samples to clear any observed carry-over greater than 10% of the LOQ;

3.2 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis. If water samples are received frozen, they should be allowed to defrost thoroughly before use. Water samples should be stored in the darkness in plastic containers rather than glass to prevent losses of CGA219417 and CGA249287 due to adsorption or photodegradation.

3.3 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), recovery samples should be included with each sample set. To each pre-measured control water sample (10 mL), add 100 µL of the combined fortification standard containing CGA219417 and CGA249287. At least one untreated control and two recovery samples should be analyzed with each sample set. For example, to prepare a recovery sample at LOQ (0.1 ppb), transfer 10 mL of the untreated sample (control) into a polypropylene centrifuge tube (50 mL) and add 100 µL of the second level (0.01 µg/mL) fortification standard to the sample. Do not add <0.1 mL or > 0.2 mL of fortification standard to samples.

3.4 Extraction

A summary of the method is included in flow-chart form in Appendix 3.

a) Measure a representative amount of water sample (10 mL) into a polypropylene centrifuge tube (50 mL).

b) Fortify untreated control samples, if needed, with known amount of the combined fortification standard solutions;

3.5 Final Fraction

a) Stopper the polypropylene centrifuge tube (50 mL) and shake the sample vigorously for 10 seconds to yield sample final fraction. Transfer an aliquot (~1.5 mL) from the sample final fraction into a suitable autosampler vial ready for final determination by LC-MS/MS. Centrifuge the sample, if particles are visible.

3.6 Time Required for Analysis

The methodology is normally performed with a batch of 80 samples. One skilled chemist can complete the analysis of 80 samples in one day (8 hour working period).

3.7 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks 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 following instrumentation and liquid chromatographic conditions are suitable for analysis of CGA219417 and CGA249287. Other instrumentation can also be used, though optimization may be required to achieve the desired separation and mass spectrometer

sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

Pump	:	Waters Acquity UPLC® system (I Class) with Sample Manager and Column Manager
Detector	:	Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer with Analyst TM software version 1.6.2

4.2 Chromatographic Conditions

Column	:	Phenomenex Synergi 4µ Hydro-RP 75 mm x 4.6 mm, i.d., 4 µm particle size
Column Oven Temperature	:	40°C
Injection volume	:	10 µL
Stop Time	:	10 minutes
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1: ultrapure water Solvent 2: MeOH

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	50	50	0.6
1	50	50	0.6
5	0	100	0.6
7.5	0	100	0.6
7.6	50	50	0.6
10	50	50	0.6

Note: Under these conditions the retention times of CGA219417 and CGA249287 are 6.0 minutes and 4.0 minutes, respectively.

Column Switching Valve Program

Time (min)	Valve Position
	To waste
1.5	To mass spectrometer

Notes : The column eluate may be diverted to waste for the first 1.5 minutes to prevent ionic material from the sample contaminating the mass spectrometer front plate, if required. A

secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste has been found to be unnecessary. It is not necessary to reduce the flow rate into the mass spectrometer when using the API 4000.

4.3 Mass Spectrometer Conditions for CGA219417 and CGA249287

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 20 (arbitrary units)
Temperature (TEM)	:	500°C
Ionspray voltage	:	5000
Collision gas setting (CAD)	:	Nitrogen set at 12 (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		CGA219417 primary transition	CGA219417 confirmatory transition	CGA249287 primary transition	CGA249287 confirmatory transition
Q1 *m/z*	:	226.1	226.1	150.1	150.1
Q3 *m/z*	:	93.1	77.0	133.0	66.9
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)	:	115 V	115 V	92 V	92 V
Entrance potential (EP)	:	10 V	10 V	10 V	10 V
Collision energy (CE)	:	48 V	67 V	28	45
Collision cell exit potential (CXP)	:	8 V	5 V	10	10

Typical chromatograms for water are shown in the Figures Section. **Since the MS/MS sensitivity is extremely important for this method, see Appendix 4 for mass spectrometer parameters and tuning details.**

4.4 Confirmatory Procedures for CGA219417 and CGA249287

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

The concentrations of each analyte in unknown samples may be calculated in ppb (µg/L) for each sample as follows:

a) Prepare calibration standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 10 x LOQ). At least five levels of concentrations within this range should be prepared;

b) Make an injection of each sample solution and measure the peak areas corresponding to CGA219417 and CGA249287. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions;

c) Generate calibration curve and 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 (peak areas), x is the standard amount (pg) injected, 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) Alternatively (depending on the regression analysis software available) a quadratic equation may be used to fit the data. In this case the following general equation should be re-arranged and used to calculate residues:

$$y = a + bx + cx^2$$

Where y is the instrument response value (peak areas) , x is the standard amount (pg) injected and a, b, c are constants.

f) Calculate the CGA219417 and CGA249287 residues in the sample, expressed as ppb (µg/L) as follows:

$$\text{Residue (ppb)} = \frac{\text{analyte found (pg)}}{\text{sample final fraction injected on column (uL)}}$$

Where analyte found (pg) is calculated from the standard calibration curve and sample final fraction injected on column is the injection volume.

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

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

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

$$\text{Corrected Residue (ppb)} = \frac{\text{residue (ppb)}}{\text{average percentage recovery}}$$

5.2 Single-Point Calibration Procedure

CGA219417 and CGA249287 may be calculated in ppb (µg/L) 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 CGA219417 and CGA249287 at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4.0. When a consistent response is obtained, measure the peak areas obtained for CGA219417 and CGA249287;

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to CGA219417 and CGA249287;

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

d) Calculate CGA219417 and CGA249287 in the sample, expressed as ppb (µg/L) using a mean standard response from each of the injections bracketing the sample as follows:

$$\text{Residue (ppb)} = \frac{\text{Peak area (SA)}}{\text{Peak area (STD)}} \times \frac{\text{Standard injected on column (pg)}}{\text{sample final fraction injected (uL)}}$$

Peak area (SA) = Peak area response for unknown sample

Peak area (STD) = Average peak response for bracketing standards

Note: Although single point calibration may be used for quantitation it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 2).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed as detailed in Sections 3.0 and 4.0 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 set of samples.

At least two recovery samples (control samples accurately fortified with known amounts of CGA219417 and CGA249287 should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found, if necessary. The fortification levels should be appropriate to the unknown analyte concentrations 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%.

Note: 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.

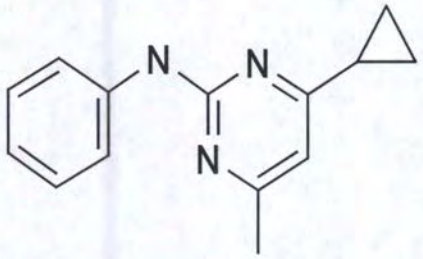
7.2 Reagent and Solvent Interference

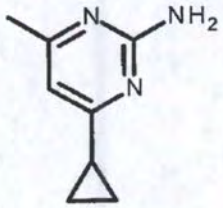
Using high purity solvents and reagents no interference has been found.

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 MeOH prior to use.

FIGURE 1 Chemical Structures

Compound	
Common Name:	Cyprodinil
Code Name:	CGA219417
IUPAC Name:	(4-Cyclopropyl-6-methyl-pyrimidin-2-yl)-phenyl-amine
CAS Number:	121552-61-2
Molecular Formula:	$C_{14}H_{15}N_3$
Molecular Weight:	225
Source:	Syngenta Production Chemistry

Compound	
Common Name:	None
Code Name:	CGA249287
IUPAC Name:	4-Cyclopropyl-6-methyl-pyridin-2-ylamine
CAS Number:	92238-61-4
Molecular Formula:	$C_8H_{11}N_3$
Molecular Weight:	149
Source:	Syngenta Product Safety Greensboro---Logistics and Support

APPENDIX 1 APPARATUS

Recommended Suppliers

Equipment	Description	Supplier
General glassware	General glassware	www.thermofisher.com/global/en/home.asp
Polypropylene Centrifuge Tube	50 mL capacity	available from Thermal Fisher Scientific, Liberty Lane, Hampton, NH 03842
Crimp cap autosampler vials and caps	2 mL capacity	available from Thermal Fisher Scientific, Liberty Lane, Hampton, NH 03842
LC-MS/MS system	API 4000 equipped with a TurboIonSpray source	www.AppliedBiosytems.com
HPLC system	Waters UPLC I-Class	www.waters.com
Autosampler	Waters UPLC I-Class	www.waters.com
HPLC column	Phenomenex Synergi 4µ Hydro-RP 75 mm x 4.6 mm, i.d., 4 µm particle size	www.phenomenex.com

APPENDIX 2 REAGENTS

Recommended Suppliers

Reagent	Description	Supplier
Ultrapure water	Optima® LC/MS	available from Thermal Fisher Scientific, Liberty Lane, Hampton, NH 03842
Acetonitrile	Optima® LC/MS	available from Thermal Fisher Scientific, Liberty Lane, Hampton, NH 03842
MeOH	Optima® LC/MS	available from Thermal Fisher Scientific, Liberty Lane, Hampton, NH 03842
CGA219417 and CGA249287 analytical standards	GLP certified	Product Safety, Syngenta Crop Protection, LLC. Box 18300, Greensboro, NC 27419-8300.

APPENDIX 3 METHOD FLOWCHART

Measure 10 mL portions of water sample into a 50 mL polypropylene centrifuge tube

↓

Fortify samples, if necessary

↓

Vial up and analyzed by LC-MS/MS

APPENDIX 4 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 CGA219417 and CGA249287

(1) Infuse a standard solution of CGA219417and CGA249287 (0.1 µg/mL) by an infusion pump into the UPLC column outlet line to the mass spectrometer interface via a T joint connector at a rate at of approximately 20 µL/min.

(2) Turn on the UPLC pump with an isocratic condition (50% ultrapure water and 50% MeOH) through the column specified in the method at a flow rate of 0.6 mL/min to the mass spectrometer interface.

(3) Using the instrument Analyst software quantitative optimization routine, tune the instrument for CGA219417 and CGA249287, ensuring that the correct ions are selected, i.e. for CGA219417 initial Q1 *m/z* = 226.1 and product ion *m/z* = 93.1 for primary transition and initial Q1 *m/z* = 77.0 for confirmatory; for CGA249827 initial Q1 *m/z* = 150.1 and product ion *m/z* = 133.0 for primary transition and initial Q1 *m/z* = 66.9 for confirmatory.

(4) Manually optimize the MS parameters, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP). In addition, manually maximize the MS sensitivity by adjusting source temperature, flow rates of curtain gas, collision gas, source gases 1 and 2 and ionSpray voltage.

(5) Finally adjust interface probe position and capillary position for CGA219417and CGA249287 in positive ionization mode.