1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM018.13A is based on Analytical Method GRM018.09A with inclusion of an additional procedure for analysis of CGA249257 and analysis of CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306 in water. See Figures 1 – 11 for the structures and relevant information of these analytes. The limit of quantification (LOQ) of the method has been established at 0.05 ppb (µg/L) for each of the analytes.

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

1.2 Method Summary

CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 are analyzed by direct injection determination of water samples using high performance liquid chromatography with triple quadrupoles mass spectrometric detection (LC-MS/MS). CGA249257 may be concentrated before injection for sensitivity issues. The LOQ of the method is 0.05 ppb (µg/L) for each of the analytes.

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 individual 50 - 100 µg/mL stock solutions for CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient for CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306 analytical standards into an amber "Class A" volumetric flask (50 mL). Dilute to the mark with 50/50 (v/v) acetonitrile/ultrapure water, except for CGA255548, CGA300407 and CGA249257 which are dissolve in acetonitrile only, and SYN510306 in 50/50 (v/v) MeOH/ultrapure water, to give 50 - 100 µg/mL individual stock solutions for CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306.

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 50/50 (v/v) acetonitrile/ultrapure water except for acetonitrile for CGA255548, CGA249257 and CGA300407 and 50/50 (v/v) MeOH/ultrapure water for SYN510306
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

Note: Fortification standard solutions must be prepared **freshly** from stock solutions prior to sample analysis of each time.

Transfer aliquots (1 mL) from each stock solution into a 100 mL volumetric flask and dilute to the mark with acetonitrile to yield a 1 µg/mL combined solution containing CGA215944, CGA363431, CGA359009, CGA363430, CGA215525, CGA300407, CGA294849, CGA371075, CGA255548, CGA249257 and SYN510306. Make serial dilutions from the 1

µg/mL combined solution with ultrapure water to yield combined fortification standard solutions. It is recommended that the following solutions are prepared: 20 ng/mL and 2 ng/mL in ultrapure water.

2.3.3 Preparation of Intermediate Standard Solutions

Note: Intermediate calibration standard solutions must be prepared **freshly** from the fresh 1 µg/mL combined solution (Section 2.3.2) prior to sample analysis of each time. Transfer appropriate amounts of the 1 µg/mL combined solution (Section 2.3.2) to volumetric flasks and dilute to the mark with ultrapure water to yield 1 ng/mL, 2 ng/mL, 4 ng/mL, 8 ng/mL, 20 ng/mL and 40 ng/mL combined intermediate standard solutions.

Since significant suppression or enhancement of the instrument responses has been observed in the water types tested using the procedures described in Section 3 during method validation, matrix-matched standards may normally be used for calibration in this method.

For analysis of CGA294849 and CGA371075, use the intermediate standards and make 1: 40 dilution with untreated control sample final fraction (Section 3.3c) to yield 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL matrix-matched standards.

For analysis of CGA215944, CGA215525, CGA300407, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306, use the intermediate standards and make 1: 40 dilution with 0.05% NH_4OH in untreated control sample final fraction to yield 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL matrix-matched standards.

For analysis of CGA249257, use the intermediate standards and make 1: 40 dilution with 90/10 (v/v) ultrapure water/MeOH to yield 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL standards.

Individual calibration curves should be generated to quantify for CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 residues. Any matrix effects observed may be compensated for by use of matrix-matched standards at the discretion of the study director, or reduced by dilution of the final sample with ultrapure water should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator (freezer condition for CGA300407) 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 0.5 months for stock solutions of CGA215944, CGA215525, CGA300407, CGA371075, CGA255548, CGA359009, CGA249257 and SYN510306 is recommended unless additional data are generated to support a longer expiration date. Stock solutions for CGA363431, CGA363430 and CGA294849 were found to be unstable for 14 days.

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	Methanol	Ammonia (29.4% (w/v))
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	SHC-C, S
OES Short Term (mg/m^3)	105	310	24
OES Long Term (mg/m^3)	70	260	17

N/A not known

CGA215944 has been designated Syngenta Hazard Category (SHC) SHC-D. At present there are insufficient data available to assign an SHC for CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306. 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 vapor. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

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

3.1 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis. Water samples should be filtered before analysis.

3.2 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), recovery samples should be prepared by adding appropriate amounts of fortification standards and are included with each sample set. To each pre-measured control water sample (40 mL), add 1 mL of the **freshly prepared** combined fortification standard solutions (Section 2.3.2). For example, add 1 mL of 2 ng/mL combined fortification standard to 40 mL of untreated control sample to yield a 0.05 ppb recovery sample. At least one untreated control and two fortified control samples should be analyzed with each sample set.

3.3 Procedure for Determinations

a) Measure a representative amount of water (40 mL) into a polypropylene centrifuge tube (50 mL size);

b) Prepare combined fortification standards from stock solutions and fortify the sample with appropriate volumes of the combined fortification standards as required at this point if necessary. Cap the tube securely and vortex mix for 30 seconds.

c) For analysis of CGA294849 and CGA371075, transfer an aliquot (1.0 mL) of the sample into a suitable injection vial or filter the aliquot using 0.22 µm PVDF filters (if there are visible particles) for LC-MS/MS final determination of CGA294849 and CGA371075. Prepare fresh matrix-matched calibration standards per Section 2.2.3 using untreated control sample and inject them along with samples for analysis of CGA294849 and CGA371075. See Sections 4.2 and 4.3 for LC-MS/MS analysis.

d) For analysis of CGA249257, transfer an aliquot (1.0 mL) of the sample into a suitable injection vial for LC-MS/MS final determination of CGA249257. If there are sensitivity issues, take an aliquot (10.0 mL) of the sample from Section 3.3 (a) and add MeOH (10 mL) to the sample. Evaporate the total volume to 1 mL or less under stream of N_2 or air at 40 - 50° C. Bring the volume back to 1.0 mL with ultrapure water, if necessary. Centrifuge the sample at 8000 rpm with refrigeration at 10°C for about 3 minutes. Transfer the supernatant to the injection vial for LC-MS/MS analysis. See Sections 4.6 and 4.8 for LC-MS/MS analysis.

e) Optional Analytical Procedures of CGA249257

1. Measure out a portion of water sample (10 mL) into a 50 mL centrifuge tube, and fortify the sample if necessary;

2. Add 10 mL of acetonitrile to the sample and vortex;

3. Add Quechers packet buffered AOAC method (Agilent part number: 5982-5755) or approximately 6g magnesium sulfate and 1.5g sodium acetate. Vortex and shake by hand venting occasionally **(Warning: sample generates a lot of heat and should be vented to avoid pressure buildup)** and then shake on mechanical shaker for approximately 5 minutes;

4. Centrifuge 5 minutes at 5000 rpm and 10°C. There will be two liquid layers as the Quechers packet contents cause water and acetonitrile to separate. Decant off the top acetonitrile layer into clean 100mL round bottom flask;

5. Add another 10 mL of acetonitrile to the sample tube containing water and Quechers material and repeat shake, vortex and decanting steps above combining acetonitrile layers;

6. Repeat step 5. The combined acetonitrile layers should total 30 mL of acetonitrile. (**Note: Water layer appears to shrink with each additional acetonitrile layer added while the total combined volume remains constant. As the acetonitrile combined layers always equals 30 mL after decanting, the water is not re-associating with the acetonitrile but appears to be absorbed more into the magnesium sulfate. If two layers are not observed on the third partition then it should be assumed that all visible liquid is the acetonitrile layer)**

7. Using a rotovap or N_2 stream at 40°C, evaporate acetonitrile layers to dryness. **Failing to remove all acetonitrile can cause chromatographic issues.**

8. Reconstitute low recovery samples to 1 mL with 10/90 (v/v) MeOH/ultrapure water and high recovery samples to 10 mL with 10/90 (v/v) MeOH/ultrapure water to make final concentrations of 0.5 ppb. Swirl thoroughly and briefly sonicate before vialing for LC-MS/MS analysis. See Sections 4.7 and 4.8 for LC-MS/MS confirmatory analysis.

f) For analysis of CGA215944, CGA215525, CGA363431, CGA359009, CGA300407, CGA255548, CGA363430 and SYN510306, transfer an aliquot of 20 mL of the sample into a second clean polypropylene centrifuge tube (50 mL size) and add 10 µL of NH_4OH aqueous solution (29% w/v) to the sample. Cap the tube securely and vortex mix for 30 seconds. Transfer an aliquot (~ 1 mL) from the basified sample into a separate injection for LC-MS/MS final determination of CGA215944, CGA215525, CGA363431, CGA359009, CGA300407, CGA255548, CGA363430 and SYN510306. Prepare fresh matrix-matched calibration standards per Section 2.2.3 using basified control sample and inject them along with samples for analysis of CGA215944, CGA215525, CGA363431, CGA359009, CGA300407, CGA255548, CGA363430 and SYN510306. See Sections 4.4 and 4.5 for LC-MS/MS analysis. **Note: For analysis of this section, recovery samples and standards should be prepared freshly and injected immediately after bench work is completed.**

3.4 Experimental Precautions

a) Bottled Optima Grade ultrapure water and MeOH are 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 (>1 ppb) and samples with expected residues greater than 1 ppb should be diluted so that the final analyte concentration does not exceed 1 ng/mL. 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.

c) Some of analytes are not stable in aqueous solutions. Inject samples and calibration standards on the LC-MS/MS system immediately after bench work is completed.

d) Instrument warm-up should be performed by injecting at least 5 warm-up samples.

3.5 Time Required for Analysis

The methodology is normally performed with a batch of 24 samples. One person can complete the analysis of 24 samples in 1 day (8 hour working period).

3.6 Method Stopping Points

The analytical procedure should be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an AB Sciex 5500 Qtrap instrument. 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

Pump	:	Waters Aquity UPLC I class with a sample manager (autosampler) and a column manager
Detector	:	AB Sciex 5500 Qtrap

4.2 LC Conditions for CGA294849 and CGA371075

Column	:	ACE 5 C18 PFP 100 x 3.0 mm i.d., 5 µm particle size
Column Oven Temperature	:	Ambient
Injection volume	:	50-75 µL
Stop Time	:	12 mins
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	100	0	0.4
1	100	0	0.4
5.5	50	50	0.4
7.0	5	95	0.4
8.5	5	95	0.4
8.6	100	0	0.4
12	100	0	0.4

Notes : The column eluate is diverted to waste for the first 1 minute to prevent ionic material from the sample contaminating the mass spectrometer front plate.

Typical retention times

Analyte	Retention time (mins)	Analyte	Retention time (mins)
CGA294849	3.6	CGA371075	5.3

4.3 Mass Spectrometer Conditions for CGA294849 and CGA371075

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	600°C
Ionspray voltage	:	-4200 V
Collision gas setting (CAD)	:	Nitrogen set at medium
Gas 1 (GS1)	:	Air set at 55 (arbitrary units)
Gas 2 (GS2)	:	Air set at 45 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		CGA294849 primary transition	CGA294849 confirmatory transition	CGA371075 primary transition	CGA371075 confirmatory transition
Q1 *m/z*	:	141.0	141.0	140.0	140.0
Q3 *m/z*	:	42.0	124.9	42.0	68.0
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)	:	-32 V	-32 V	-44 V	-44 V
Entrance potential (EP)	:	-7 V	-8 V	-7 V	-9 V
Collision energy (CE)	:	-34 V	-17 V	-35 V	-21 V
Collision cell exit potential (CXP)	:	-10 V	-20 V	-10 V	-9 V

4.4 LC Conditions for CGA215944, CGA215525, CGA300407, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306

Column	:	Agilent Zorbax SB-Aq 50 x 4.5 mm i.d., 3.5 µm particle size
Column Oven Temperature	:	Ambient
Injection volume	:	50-75 µL
Stop Time	:	25 mins
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	solvent 1: 0.003 - 0.006% NH_4OH in ultrapure water solvent 2: 0.003 - 0.006% NH_4OH in MeOH

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	100	0	0.4
1	100	0	0.4
5.5	50	50	0.4
7.0	5	95	0.4
15.0	5	95	0.4
15.1	100	0	0.4
25	100	0	0.4

Note: The column eluate is diverted to waste for the first 1 minute to prevent ionic material from the sample contaminating the mass spectrometer front plate.

Typical retention times

Analyte	Retention time (mins)	Analyte	Retention time (mins)
CGA215944	7.4	CGA300407	6.4
CGA215525	4.1	SYN510306	6.3
CGA255548	4.6	CGA363430	5.4
CGA363431	5.5	CGA359009	6.9

4.5 Mass Spectrometer Conditions for CGA215944, CGA215525, CGA300407, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 20 (arbitrary units)
Temperature (TEM)	:	620°C
Ionspray voltage	:	5200V
Collision gas setting (CAD)	:	Nitrogen set at medium
Gas 1 (GS1)	:	Air set at 50 (arbitrary units)
Gas 2 (GS2)	:	Air set at 45 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**CGA215944 primary transition**	**CGA215944 confirmatory transition**	**CGA363431 primary transition**	**CGA363431 confirmatory transition**
Q1 *m/z*	:	217.8	217.8	250.1	250.1
Q3 *m/z*	:	105.0	78.0	121.1	103.1
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)	:	62 V	62 V	95 V	95 V
Entrance potential (EP)	:	10 V	10 V	10 V	8 V
Collision energy (CE)	:	27 V	60 V	26 V	55 V
Collision cell exit potential (CXP)	:	9 V	10 V	15 V	15 V

MRM Conditions		CGA215525 primary transition	CGA215525 confirmatory transition	CGA300407 primary transition	CGA300407 confirmatory transition
Q1 *m/z*	:	129.0	129.0	108.0	108.0
Q3 *m/z*	:	44.9	129.0 (88.1)	80.2	53.0
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)	:	60 V	45 V (60)	165 V	165 V
Entrance potential (EP)	:	10 V	10V (10)	8 V	8 V
Collision energy (CE)	:	17 V	17 V (13)	24 V	33 V
Collision cell exit potential (CXP)	:	7 V	10 V (7)	10 V	12V

MRM Conditions		CGA359009 primary transition	CGA359009 confirmatory transition	CGA363430 primary transition	CGA363430 confirmatory transition
Q1 *m/z*	:	234.0	234.0	248.0	248.0
Q3 *m/z*	:	105.2	92.1	121.1	103.1
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)	:	50 V	50 V	80 V	80 V
Entrance potential (EP)	:	10 V	10 V	10 V	10 V
Collision energy (CE)	:	29 V	41 V	23 V	45 V
Collision cell exit potential (CXP)	:	10 V	10 V	18 V	15 V

MRM Conditions		SYN510306 primary transition	SYN510306 confirmatory transition	CGA255548 primary transition	CGA255548 confirmatory transition
Q1 *m/z*	:	234.0	234.0	124.0	124.0
Q3 *m/z*	:	121.3	103.1	106.1	78.1
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)	:	55 V	55 V	58 V	58 V
Entrance potential (EP)	:	10 V	5 V	10 V	10 V
Collision energy (CE)	:	29 V	52 V	23 V	32 V
Collision cell exit potential (CXP)	:	8 V	8 V	9 V	10 V

Typical chromatograms are shown in the Figures Section.

4.6 LC Conditions for Analysis of CGA249257

Column	:	ACE Excel 3 C18 AR 150 x 3.0 mm i.d., 5 µm particle size
Column Oven Temperature	:	40 °C
Injection volume	:	50 - 75 µL
Stop Time	:	14 mins
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	solvent 1: 0.05% acetic acid in ultrapure water solvent 2: MeOH

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	95	5	0.4
1	95	5	0.4
5.5	50	50	0.4
7.0	5	95	0.4
10	5	95	0.4
10.1	95	5	0.4
14	95	5	0.4

Notes : The column eluate is diverted to waste for the first 1 minute to prevent ionic material from the sample contaminating the mass spectrometer front plate.

Typical retention times

Analyte	Retention time (mins)
CGA249257	3.6

4.7 Optional LC Conditions for Analysis of CGA249257

Column	:	Agilent Pursuit XRs 3 Diphenyl 100 x 4.6 mm, i.d.
Column Oven Temperature	:	40 °C
Injection volume	:	50 - 75 µL
Stop Time	:	14 mins
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	solvent 1: 5 mM ammonium acetate in ultrapure water solvent 2: MeOH

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	95	5	0.4
1	95	5	0.4
5.5	50	50	0.4
7.0	5	95	0.4
10	5	95	0.4
10.1	95	5	0.4
14	95	5	0.4

Notes : The column eluate is diverted to waste for the first 1 minute to prevent ionic material from the sample contaminating the mass spectrometer front plate.

Typical retention times

Analyte	Retention time (mins)
CGA249257	6.4

4.8 Mass Spectrometer Conditions for CGA249257

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 20 (arbitrary units)
Temperature (TEM)	:	550°C
Ionspray voltage	:	2000 V
Collision gas setting (CAD)	:	Nitrogen set at medium
Gas 1 (GS1)	:	Air set at 60 (arbitrary units)
Gas 2 (GS2)	:	Air set at 60 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**CGA249257 primary transition**	**CGA249257 confirmatory transition**
Q1 *m/z*	:	113.8	113.8
Q3 *m/z*	:	72.9	70.8
Dwell time	:	100 ms	100 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	60 V	60 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	17.3 V	18.7
Collision cell exit potential (CXP)	:	12.5 V	12.5 V

Typical chromatograms are shown in the Figures Section.

4.9 Confirmatory Procedures

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

CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 residues may be calculated in ppb or µg/L 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).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306.

c) Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

d) Generate calibration curve parameters using an appropriate regression package.

e) 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 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

$$x = \frac{y - c}{m}$$

f) Calculate the CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 residues in the sample, expressed as ppb (µg/L) as follows:

$$\text{Residue (ppb or } \mu g/L) = \frac{\text{Analyte found (pg)}}{\text{Sample injected on column (uL)}}$$

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

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

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (ppb or } \mu\text{g/L)}$$

5.2 Single-Point Calibration Procedure

CGA215944 CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 residues may be calculated in mg/kg 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 CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for CGA215944, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306.

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

d) Calculate the CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 residues in the sample, expressed as ppb (µg/L) using a mean standard response from each of the injections bracketing the sample as follows.

$$\text{Residue (ppb or } \mu\text{g/L)} = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard injected (pg)}}{\text{Sample Volume Injected on Column}}$$

Peak response for sample

Average peak response for bracketing standards

Standard injected on column (pg)

Sample volumes injected on column (µL).

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

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (ppb or } \mu g/L)$$

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).

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.

At least two recovery samples (control samples accurately fortified with known amounts of CGA215944, CGA215525, CGA300407, CGA294849, CGA371075, CGA363431, CGA359009, CGA363430, CGA255548, CGA249257 and SYN510306 should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels 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%.

Where 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.

APPENDIX 3 Method Flow Chart

Measure 40 mL water sample into 50 mL polypropylene tube

↓

Prepare fortification standards and fortify sample as needed

↓	↓	↓
Take aliquot (1 mL) into injection vial for analysis of CGA294849 and CGA371075	Take 20 mL and add 10 µL of NH_4OH to sample and take aliquot (1 mL) into injection vial for analysis of CGA215944, CGA215525, CGA300407, CGA363431, CGA359009, CGA363430, CGA255548 and SYN510306	Take 10 mL and mix with 10 mL of MeOH; Evaporate to <1 mL and bring back to 1 mL with water; vial up for analysis of, CGA249257