1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM020.08B is suitable for the determination of CGA300405 (Figure 1) and CGA313458 (Figure 2) in water. The limit of quantitation (LOQ) of the method has been established at a 10.0 μg/L (10.0 ppb). Analytical method GRM020.08B supersedes method GRM020.08A. Version B has the addition of metabolite CGA313458 and improved chromatographic conditions.

This method satisfies US EPA guidelines EPA OCSPP 650.6100.

1.2 Method Summary

Rice paddy water samples are analyzed directly by LC-MS/MS after dilution with 30/70 acetonitrile/ultra pure water. Matrix match standards may be required for certain water types.

The LOQ of the method is 10.0 μg/L (10.0 ppb) for rice paddy 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 CGA300405 and CGA313458 by one of the following methods:

Weigh out accurately, using a five figure balance, sufficient CGA300405 and CGA313458 analytical standard into an amber "Class A" volumetric flask (100-mL). Dilute to the mark with methanol and mix well to give a 100 μg/mL stock solutions of CGA300405 and CGA313458. Standards should be prepared in amber bottles and stored under refrigeration.

Alternatively, the appropriate volume of methanol 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 methanol 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 CGA300405 and CGA313458 should be prepared by serial dilution in methanol 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. CGA300405 has been observed to have lower solubility in acetonitrile at higher concentrations. Mixed standards of CGA300405 and CGA313458 may be prepared if desired.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Due to possible photo degradation, standards should be prepared in amber bottles and stored in refrigeration away from light when not in use. If stability cannot be maintained, daily use standards and/or matrix match standards may be prepared from the 1.0 μg/mL, and 0.10 μg/mL fortification standard solutions. Serially dilute stock and fortification standards using 30/70 acetonitrile/ultra pure water if external standards are to be used. Using the LC-MS/MS instrumentation described within, the following concentration range of standards were prepared and used to construct calibration plots (0.10 ug/L -10 ug/L).

A calibration curve should be generated to quantify CGA300405 and CGA313458 residues. Standards over an appropriate concentration range should be prepared with a minimum of five levels.

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 30/70 acetonitrile/ultra pure water 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 CGA300405 and CGA313458 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	Methanol
Harmful Vapor	✓	✓
Highly Flammable	✓	✓
Harmful by Skin Absorption	✓	✓
Irritant to respiratory system and eyes	✓	✓
Causes severe burns	✗	✗
Syngenta Hazard Category (SHC)	C, S	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 CGA300405 and CGA313458. It 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 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 CGA300405 and CGA313458 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 30/70 acetonitrile/ultra pure water. Using the instrumentation described in section 4.0. The method LOQ (10 ppb) can be diluted 1 to 10 while maintaining acceptable signal/noise ratio at 1 ppb.

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 (10 ppb) when a final volume of 1 mL is maintained. Addition of any solvent (MeOH, 30/70 ACN/UPW) should remain the same for each standard and sample.

3.2 Problems and Modifications

Evaluation of alternate extraction procedures including sample cleanup by SPE were performed during the method development stage. Attempts to develop a SPE cleanup procedure for LC-MS/MS and GC/MSD were unsuccessful for the CGA300405 molecule. Any modifications should be evaluated using procedural recoveries on the intended matrix.

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 instruments. 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:	1.0mL/min
Column:	ACE 3 C18, 3.0 x 50 mm
Column Oven Temp:	40°C
Injection Vol.	50µL
Run Time:	3.0 minute
Detector:	Applied BioSystem API QTRAP 5500
Retention Time:	0.25 minutes
Mobile Phase A:	OPTIMA Grade MeOH/Water (50/50) in 10mM Ammonium Acetate
Mobile Phase B:	OPTIMA Grade Acetonitrile/Water (90/10) in 10mM Ammonium Acetate

Gradient:

Time	A%	B%
0.0	0	100
0.5	0	100
1.0	100	0
2.0	100	0
3.0	0	100

Under these conditions CGA300405 and CGA313458 co-elute at a retention time of 0.25 minutes. Due to the selectivity of LC-MS/MS, each analyte can be separated and quantitated based on molecular/product mass transitions.

4.2 Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	650 °C
Ionspray voltage	:	1500 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		CGA300405 primary transition	CGA300405 confirmatory transition	CGA313458 primary transition	CGA313458 confirmatory transition
Q1 *m/z*	:	203	203	241	241
Q3 *m/z*	:	69	157	83	69
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)	:	-26V	-12V	-30V	-35V
Collision cell exit potential (CXP)	:	-10V	-10V	-10V	-10V

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

4.3 Confirmatory Procedures for CGA300405 and CGA313458

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

Residues of CGA300405 and CGA313458 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. Quality Control standard solutions should be interspersed throughout the analysis to monitor any matrix effects.

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).

CGA300405 and CGA313458 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 CGA300405 and CGA313458 at an appropriate concentration either LC-MS/MS or GC/MSD operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for CGA300405 and CGA313458.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to CGA300405 and CGA313458.

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

d) Calculate the CGA300405 and CGA313458 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

Matrix effects were observed in some water types tested during method development and matrix-match standards can be used at the discretion of the study director.

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.

CHEMICAL STRUCTURES

FIGURE 1 CGA300405

Compound Code Number	:	CGA300405
CAS Name	:	1,2,3-Propanetricarboxylic acid, 2-ethyl ester
IUPAC Name	:	3-Ethoxycarbonyl-pentanedioic acid
Molecular Formula	:	$C_8H_{12}O_6$
Molecular Weight	:	204.17

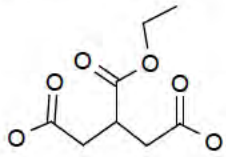
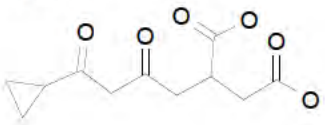


FIGURE 2 CGA313458

Compound Code Number	:	CGA313458
CAS Name	:	Butanedioic acid, (4-cyclopropyl-2,4-dioxobutyl)-
IUPAC Name	:	2-(4-Cyclopropyl-2,4-dioxo-butyl)-succinic acid
Molecular Formula	:	$C_{11}H_{14}O_6$
Molecular Weight	:	242.00



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
CGA300405/CGA313458 analytical standards	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) 90/10 Acetonitrile/Water (v/v) in 10mM Ammonium Acetate; prepared by adding 0.77g ammonium acetate and diluting 900 mL of acetonitrile to 1,000 mL using Optima/HPLC Grade water.

b) 50/50 Methanol/Water (v/v) in 10mM Ammonium Acetate; prepared by adding 0.77g ammonium acetate and diluting 500 mL of methanol to 1,000 mL using Optima/HPLC Grade water.

c) 30/70 Acetonitrile/Water (v/v); prepared by diluting 300mL 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 CGA300405 & CGA313458

Infuse a standard solution of CGA300405 and CGA313458 (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* 203 for CGA300405 in negative ionization mode and *m/z* 241 for CGA313458 in negative ionization mode.

Using the Analyst™ Software optimization routine, tune the instrument for CGA300405 and CGA313458, 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 CGA300405 and CGA313458 standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position and temperature, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas pressure for maximum sensitivity.

For CGA300405, in negative ionization mode, the de-protonated molecular ion generated in the ion source (*m/z* 203) is selected and subjected to further fragmentation by collision induced fragmentation. The product ion (*m/z* 69) is selected and used for quantitative analysis as the primary transition. Product ion *m/z* 157 is used for confirmatory purposes.

For CGA313458, in negative ionization mode, the de-protonated molecular ion generated in the ion source (*m/z* 241) is selected and subjected to further fragmentation by collision induced fragmentation. The product ion (*m/z* 83) is selected and used for quantitative analysis as the primary transition. Product ion *m/z* 69 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 15 mL poly tube

↓

Fortify recovery sample, if needed

↓

Dilute to 10 mL ACN/UPW (30/70)

↓

Mix well by agitation (vortex)

↓

If using a matrix match calibration, add pre-determined control matrix at equal concentrations (0.1 mL) to each calibration standard with a final volume of 1.0 mL to equal the same amount of matrix present in sample.

↓

Vial and analyze by LC-MS/MS