1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM066.01A is suitable for the determination of difenoconazole, CGA205375, CGA142856 and CGA71019 (See Figure 1) in water. The limit of quantitation (LOQ) of the method has been established at a 0.10 µg/L (0.10 ppb).

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, US EPA Guidelines OCSPP 850.6100(2012) and EC Guidance Documents SANCO/3029/99 Rev 4(2000) and SANCO/825/00 Rev 8.1(2010).

1.2 Method Summary

Environmental water samples were analyzed directly upon treatment with acetonitrile using liquid chromatography tandem mass spectrometry (LC-MS/MS) technique when the instrument sensitivity allows. Alternatively, the water samples were concentrated for CGA71019 analysis using solid phase extraction (SPE) procedures prior to LC-MS/MS analysis. Thus, upon sample loading and SPE cartridge washing, the samples were eluted with 0.5% NH_4OH in methanol/water (90/10; v/v) from the SPE cartridges and collected. The collected NH_4OH containing methanol/water fractions were evaporated under a gentle stream of nitrogen in a water bath at approximately 40 °C and re-constituted with acetonitrile/H_2O (20:80; v/v) then subjected to LC-MS/MS analysis. The LOQ of the method is 0.10 µg/L (ppb) in 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 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

Individual primary stock solutions at the 100 µg/mL concentration level for difenoconazole, CGA205375, CGA142856 and CGA71019 are prepared by dissolving 10.0 mg of each compound into individual 100-mL glass volumetric flasks followed by dilution to the mark with 50:50 acetonitrile:water. The amounts weighed should be corrected for its respective % purity.

Alternatively, the appropriate volume of 50:50 acetonitrile: water (v/v; HPLC grade) is added 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 acetonitrile:water 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

A mixed standard solution at the 1.0 µg/mL concentration is prepared by combining 1.0 mL of each primary stock standard into a 100-mL volumetric flask and filling to the mark with 20:80 acetonitrile:water (v/v; HPLC grade). Serial dilutions of this mixed standard solution are prepared in 20:80 acetonitrile:water (v/v; HPLC grade) to create mixed fortification standards. 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.

2.3.3 Standards for LC-MS/MS

Calibration standard solutions should be prepared by serial dilution in acetonitrile/water (20/80; v/v; HPLC grade) from the stock solution or fortification solution. For example, transfer 10 mL of the 1.0 µg/mL fortification standard into a 100-mL volumetric flask and diluted with acetonitrile/water (20/80; v/v; HPLC grade) solution to the mark to yield a calibration standard solution at 0.10 µg/mL concentration.

A calibration curve should be generated for each analyte to quantify residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of the mixed standard in acetonitrile/water (20/80; v/v; HPLC grade) solution. A minimum of five levels of calibration standards should be used for calibration plot establishment. The following concentration levels of standards are prepared for difenoconazole, CGA205375, CGA142856 and CGA71019 calibration plots: 0.025 pg/µL, 0.05 pg/µL, 0.1 pg/µL, 0.20 pg/µL, 0.50 pg/µL, 1.0 pg/µL, 2.0 pg/µL 5.0 pg/µL and 10 pg/µL. Single point calibrations are not recommended for this method.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figures 7-11

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator 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 all compounds 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	Formic Acid	Ammonium Hydroxide
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-D,S	SHC-D, S
OES Short Term (mg/m^3)	105	310	N/A	24
OES Long Term (mg/m^3)	70	260	N/A	17

N/A not known

Syngenta Hazard Classification for difenoconazole has been assigned as SHC-B.

At present there are insufficient data available to assign a Syngenta Hazard Classification for CGA205375, CGA142856, and CGA71019. These compounds 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

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 a known amount of each analyte using the mixed fortification standard solution should be analyzed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired. Note that plastic containers should be avoided due to suspected difenoconazole and CGA205375 strong surface interactions with plastic materials.

A summary of the method is included in flow-chart as shown in Appendix 4.

3.1 Sample Preparation (Difenoconazole, CGA205375 and CGA142856)

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) Fortification sample preparation: Transfer 10 mL of water sample to be analyzed into a 20-mL glass scintillation vial. Fortify the recovery sample with the appropriate amount of each analyte using the mixed standard using no more than 0.1 mL. Cap the scintillation vial securely and shake gently to mix thoroughly. Proceed to Section 3.1. c.

c) Water samples (including recovery samples): Transfer 0.8 mL of water sample to a 2-mL injection vial containing 0.2 mL acetonitrile. Cap securely and mix sample using a vortex mixer. Samples can be further diluted in acetonitrile/water (20/80; v/v; HPLC grade) solution, if needed. Analyze sample using LC-MS/MS for residue determination.

d) If adequate sensitivity is not available for the direct injection of CGA71019, see Section 3.2 for a SPE/concentration procedure. The method development phase using the AB Sciex API 4000 triple quadrupole mass spectrometer did not allow for direct injection analysis of CGA71019.

During the Independent Laboratory Validation of this method, TK0180143, samples were analyzed using the AB Sciex Triple Quad™ 6500 LC-MS/MS System demonstrating that adequate sensitivity was achieved to allow for direct injection for the CGA71019 analysis, therefore, omitting the SPE/concentration step.

3.2 Solid Phase Extraction Procedure - Bond Elut Certify Cartridges – 300 mg, 3cc - (CGA71019)

a) Fortification samples: Transfer 10 mL of water sample to be analyzed into a 20-mL glass scintillation vial. Fortify the recovery sample using no more than 0.1 mL of the standard solution. Cap the vial securely and shake gently to mix thoroughly.

b) Condition Certify 300mg 3cc solid phase extraction cartridges as follows:

   a. Fill cartridges with methanol (MeOH), **twice**.
   b. 2 mL of 0.5 % ammonium hydroxide in 90:10 MeOH/H_2O, **twice**.
   c. 2 mL of HPLC H_2O, **twice**.
   d. 2 mL of 5% formic acid in MeOH, **twice**.
   e. 2 mL of 2% formic acid in H_2O, **once**.

c) For all samples: A 5 mL aliquot of each sample was loaded to their respective SPE cartridges. The sample load was allowed to drip through the cartridge under gravity and the load was discarded.

d) Each cartridge was then washed as follows:

   a. 2 mL of HPLC Grade water, **twice**.
   b. 1 mL of MeOH, **twice**. All washes were discarded.

e) Residues were eluted with three portions each of 2 mL of 0.5% ammonium hydroxide in 90:10 MeOH/ H_2O. All eluents were collected in a 15-mL glass centrifuge tube.

f) Eluents were evaporated under a nitrogen stream at 40 °C to approx. 0.5 mL.

g) Samples were reconstituted with 0.2 mL of acetonitrile and brought to a final volume of 1.0 mL with HPLC water.

h) Samples were sonicated to dissolve any residues and vialed for LC-MS/MS analysis.

3.3 Problems and Modifications

a) The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Bottled Optima grade ultra pure water 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.

c) To minimize possible carry-over issues, it is recommended that high level recoveries ($\geq$ 10 ppb), samples and standards with similar expected residues include blank injections of HPLC solvent blanks (acetonitrile/water; 20/80; v/v) following high level injections to observe possible carry-over greater than 10% of the LOQ. Difenoconazole has a strong tendency to adhere to the injection needle in some types of auto samplers not utilizing flow through needle cleaning. It is important to determine the best wash solutions for the needle washing to reduce the peak response in solvent blanks to <1%.

3.4 Time Required for Analysis

For direct injection, the methodology is normally performed with a batch of 20 samples. One skilled analyst can complete the analysis of 20 samples in 1 day (8 hour working period).

For water samples requiring concentration (CGA71019) by solid phase extraction (SPE) prior to LC-MS/MS analysis, a batch of 8-10 samples can be analyzed in 1 day (8 hour working period).

3.5 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

An integrated AB Sciex Triple Quad™ 6500 mass spectrometer was used to establish and validate the method. The system is controlled and data is processed by AB Sciex Analyst™ Software. Other instruments may also be used, however 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 instrument operation.

The following instrumentation and liquid chromatographic conditions are suitable for analysis of difenoconazole, CGA205375, CGA142856 and CGA71019.

For difenoconazole and CGA205375, final determination by LC-MS/MS using two transitions is considered to be highly specific; therefore no further confirmatory conditions are included.

For CGA142856 and CGA71019, final determination by LC-MS/MS is accomplished using one transition due to the low molecular weight of the parent compounds. Confirmatory procedures can be found for CGA142856 and CGA71019 in the following references:

a) For CGA142856 and CGA71019, alternative chromatographic conditions have been documented in Syngenta Report Number T013656-05 (Reference 3).
b) For CGA71019, alternative chromatographic conditions have been documented in Syngenta Report Number 444-00 (Reference 4).

4.1 Instrument Description/Operating Conditions for Difenoconazole, CGA205375, CGA142856 and CGA71019

Mobile phase composition

HPLC System:	**Agilent 1290 Infinity Series (UHPLC)**			
Flow Rate:	500 µL/min			
Column:	Agilent Zorbax SB-Aq 4.6 × 75 mm, 3.5 µm			
Column temperature:	40 °C			
Injection Volume:	20 µL			
Mobile Phase A:	0.3% Formic acid in Water			
Mobile Phase B:	Acetonitrile:methanol (70:30)			
Gradient Step Table:	Step	Total Time (min)	A (%)	B (%)
	0	0.0	95	5.0
	1	0.5	95	5.0
	2	1.5	5.0	95
	3	5.0	5.0	95
	4	5.1	95	5.0
	5	7.0	95	5.0

The retention time may vary depending upon chromatographic conditions and systems.

Retention times for each analyte under these conditions:
1) Difenonconazole: 3.53 min
2) CGA205375: 3.36 min
3) CGA142856: 2.11 min
4) CGA71019: 2.08 min

Mass Spectrometer Conditions:

Mass Spectrometer Detector:	ABSciex Triple Quad™ 6500 LC-MS/MS System
Polarity:	Positive
CUR:	35.0
IS	3500
CAD:	10.0
TEM:	550
GS1:	70.0
GS2:	70.0
EP:	10.0

MRM Operating Parameters:

MRM Conditions	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	DP	CE	CXP
Quantification Ions						
Difenoconazole	406.2	251.0	3.5	90.0	35.0	13.0
CGA205375	350.1[1]	69.9	3.3	70.0	55.0	8.0
CGA142856	128.1	70.0	2.3	50.0	25.0	8.0
CGA71019	70.0	43.1	2.1	140.0	28.0	7.0
Confirmation Ions						
Difenoconazole	406.2	187.9	3.5	90.0	60.0	10.0
CGA205375	352.1[2]	69.9	3.3	70.0	55.0	8.0

[1] CGA205375 with all ^{35}Cl

[2] CGA205375 with one ^{37}Cl

Optimization of an instrument can be conducted by infusing standard solutions of Difenoconazole, CGA205375, CGA142856 and CGA71019(0.1 µg/mL) in mobile phase directly into the mass spectrometer interface at a rate at of approximately 7 µL/min (Appendix 3).

Typical LC-MS/MS chromatograms from analysis of water samples are shown in Figure Section.

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

Residues 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 20 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 ms/ms transition. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

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).

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 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 for each analyte.

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

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

d) Calculate the 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.

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 110% 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

No significant matrix effects were observed in the water types tested during method development and non-matrix standards should generally be used for quantification.

7.2 Reagent and Solvent Interference

No interference has been observed from using of high purity solvents and reagents.

7.3 Labware Interference

All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

7.4 Potential Interferences

During the validation study (TK0180143), using AB Sciex Triple Quad™ 6500 system, a second peak with significant intensity was observed in standard and fortified-sample chromatograms of CGA71019 (*m/z* 69.9 → 43.1). This second peak has a retention time close to the expected retention time of CGA71019; however, it was not observed in previous development/validation work using AB Sciex API 4000 system. At the request of the study monitor, experiments were prepared to determine the source of the second peak (as per ILV

protocol Amendment 1). These experiments included the injection of single and mixed-analyte standards of CGA142856 and CGA71019, and also full product ion scans. These experiments demonstrate the second peak originates from possible in-source fragmentation of the parent ion of CGA142856 (mass 129) to the parent ion of CGA71019 (mass 69) and a fragment ion (mass 43) common to both CGA142856 and CGA71019. Product ion scan of a CGA142856 standard and extracted-ion chromatograms of CGA71019 and CGA142856 are presented in Appendix 5

8.0 METHOD VALIDATION

8.1 Recovery Data and Repeatability

Method validations have been carried out on the procedures described in Sections 3.0 and 4.0 of GRM066.01A. The method validation data are reported in TK0180143 report (Reference 5) and summaries are included in Tables 2-19.

8.2 Limit of Quantitation (LOQ)

The limit of quantitation of the method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and a mean recovery of 70-110% with a relative standard deviation of $\leq 20\%$ has been obtained. Generally, for accurate quantitation, the response for an analyte peak should be no lower than four times the mean amplitude of the background noise in an untreated sample at the corresponding retention time.

The limit of quantitation (LOQ) of the analytical method has been established at 0.10 µg/L (ppb), which is equivalent to 0.0016 ng on column.

8.3 Limit of Detection (LOD)

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as three times background noise. Note that the LOD may vary between runs and from instrument to instrument.

The limit of detection of the analytical method was estimated to be 0.025 µg/L (ppb), which is equivalent to 0.0005 ng on column with AB Sciex Triple Quad™ 6500 instrument.

8.4 Matrix Effects

No significant matrix effects were observed in the water types tested during method validation and non-matrix standards should generally be used for quantification. A summary of the matrix effects is included in Table 24.

8.5 Detector Linearity

For accurate quantitation of residue concentrations, analyses should be carried out within the linear range of the detector. For multi-point calibration, detector range and linearity will be demonstrated within each sample set.

The linearity of the LC-MS/MS detector response for difenoconazole, CGA205375, CGA142856 and CGA71019 was tested in the range from 0.025 µg/L to 10.0 µg/L concentrations injected (equivalent to 0.50 pg to 200 pg standards on-column when using a 20 µL injection volume) and was found to be linear during the validation using the AB Sciex 6500 triple quadrupole mass spectrometer.

If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantification is recommended.

Nine different standard concentration levels were injected and the response plotted against the standard concentration (i.e. analyte found) during method development for difenoconazole, CGA205375, CGA142856 and CGA71019 using Analyst ^{TM} software version 1.6.2 software. Representative plots of the detector responses versus the analyte concentration (pg found) for all calibration points from method testing are presented in Figures 2-6.

8.6 Final Extract Stability

Final water samples in acetonitrile/water (20/80; v/v; HPLC grade) solution retained in vials were found to be stable for at least 19 days upon storage at temperature of approximately 5° ± 2 °C during method validation. A summary of the stability data is presented in Table 20-23. It is not recommended to store for more than 7 days for accurate determination of residues. It is recommended to analyze the samples as soon as possible.

9.0 LIMITATIONS

The method has been tested on representative water types. It can reasonably be assumed that the method can be applied to other water matrices not tested in this method provided successful recovery tests at the relevant levels validate the suitability of the method.

10.0 CONCLUSIONS

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of residues of difenoconazole, CGA205375, CGA142856 and CGA71019 in environmental water. Only commercially available laboratory equipment and reagents are required. The direct analysis of 20 water samples can be completed by one skilled analyst in 1 day (8 working hour period). For water samples concentrated for CGA71019 analysis using solid phase extraction (SPE) procedures prior to LC-MS/MS analysis, a batch of 8-10 samples can be analyzed in 1 working day (8 working hour period).

Untreated and fortified samples should be analyzed with each set of samples to demonstrate absence of any interference and adequate recoveries, if possible. The LOQ of the method is 0.10 µg/L (ppb) for difenoconazole, CGA205375, CGA142856 and CGA71019 in water samples.

This method satisfies OECD Guidance Document ENV/JM/MONO(2007)17, US EPA Guidelines OCSPP 850.6100 (2012) and EC Guidance Documents SANCO/3029/99 rev 4 (2000) and SANCO/825/00 rev 8.1 (2010).

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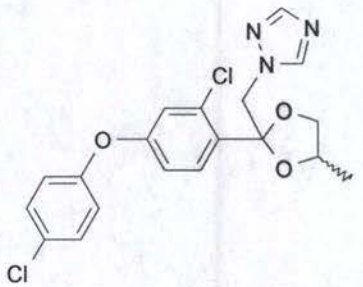
FIGURE 1 Chemical Structures

Compound Code Number	:	CGA169374 (Difenoconazole)
Alternative Compound Code Number	:	
CAS Number	:	119446-68-3
IUPAC Name	:	1-{2-[2-Chloro-4-(4-chloro-phenoxy)-phenyl]-4-methyl-[1,3]dioxolan-2-ylmethyl}-1H-[1,2,4]triazole
Molecular Formula	:	$C_{19}H_{17}Cl_2N_3O_3$
Molecular Weight	:	
Molecular Mass	:	



Compound Code Number	:	CGA205375
Alternative Compound Code Number	:	
CAS Number	:	117018-19-6
IUPAC Name	:	1-[2-Chloro-4-(4-chloro-phenoxy)-phenyl]-2-1,2,4-triazol-1-yl-ethanol
Molecular Formula	:	$C_{16}H_{13}Cl_2N_3O_2$
Molecular Weight	:	
Molecular Mass	:	

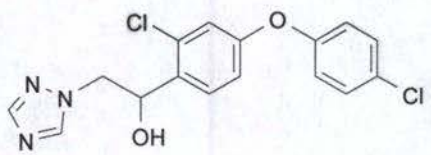
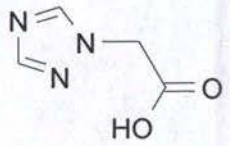
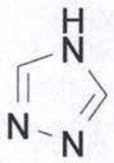


FIGURE 1 Chemical Structures (Continued)

Compound Code Number	:	CGA142856
Alternative Compound Code Number	:	
CAS Number	:	28711-29-7
IUPAC Name	:	1,2,4-Triazol-1-yl-acetic acid
Molecular Formula	:	$C_4 H_5 N_3 O_2$
Molecular Weight	:	
Molecular Mass	:	



Compound Code Number	:	CGA71019
Alternative Compound Code Number	:	
CAS Number	:	288-88-0
IUPAC Name	:	4H-1,2,4-Triazole
Molecular Formula	:	$C_2 H_3 N_3$
Molecular Weight	:	
Molecular Mass	:	



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
Sample processing station/Vacuum manifold	Waters Extraction Manifold	www.waters.com
Solid Phase Extraction cartridges	Bond Elut-C18; 100 mg, 3-mL	www.agilent.com
Column connectors	Suitable for various sizes of reservoirs	www.Biotage.com
Column reservoirs	Various sizes	www.Biotage.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
LC-MS/MS system Includes HPLC and autosampler units	AB Sciex API 4000 and AB Sciex Triple Quad ™ 6500 LC-MS/MS systems	www.absciex.com/
HPLC column	Agilent SB-AQ, 4.6 x 75 mm i.d., 3.5 µm particle size	www.agilent.com

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Ultra pure water	Optima grade	www.thermoscientific.com
Methanol	Optima grade	www.thermoscientific.com
Ultra pure water	HPLC grade	www.thermoscientific.com
Acetonitrile	HPLC grade	www.thermoscientific.com
Formic Acid	A.C.S. grade	www.thermoscientific.com
Ammonium Hydroxide	A.C.S. grade	www.thermoscientific.com
Difenoconazole analytical standard	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.
CGA205375 analytical standard	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.
CGA142856 analytical standard	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.
CGA71019 analytical standard	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) "0.3%" formic acid in water; prepared by mixing 3 mL of formic acid with 1,000 mL of Optima LC/MS grade water.

b) "Acetonitrile/Water (20/80; v/v; Optima LC/MS grade): prepared by mixing 200 mL of Optima LC/MS grade acetonitrile with 800 mL of Optima LC/MS grade water.

c) "0.5%" ammonium hydroxide in 90/10 MeOH/Water (HPLC grade): prepared by mixing 5.0 mL of ammonium hydroxide with 1,000 mL of 90/10 MeOH/Water (HPLC grade)

d) "2.0%" Formic Acid in H_2O; prepared by mixing 20 mL of formic acid with 980 mL of Optima LC/MS grade water.

e) "5.0%" Formic Acid in MeOH; prepared by mixing 50 mL of formic acid with 950 mL of HPLC grade MeOH.

f) Methanol/Water (90/10; v/v; HPLC grade): prepared by mixing 900 mL of HPLC grade MeOH with 100 mL of HPLC grade water.

g) Acetonitrile/Methanol (70/30; v/v; HPLC grade): prepared by mixing 700 mL of Optima LC/MS grade acetonitrile with 300 mL of Optima LC/MS grade MeOH.

APPENDIX 3 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

Infuse individual standard solutions (0.1 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 7µL/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* in positive ionization mode.

Compound	Parent ion signal m/z
Difenoconazole	406.2
CGA205375	350.1
CGA142856	128.1
CGA71019	69.9

Using the Analyst software quantitative optimization routine, tune the instrument, ensuring that the correct ions (See Section 4.0) are selected. If desired, manual tuning of the ion optics and collision energy 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 the infusion standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.

In positive mode, the molecular ion generated in the ion source is selected and subjected to further fragmentation by collisional activation. The most sensitive product ions are then selected and used for quantitative analysis.

Compound	Parent ion signal (m/z)	Product Ions
Difenoconazole	406.2	251.0, 187.9
CGA205375 [1]	350.1	69.9
CGA205375 [2]	352.1	69.9
CGA142856	128.1	69.9
CGA71019	69.9	43.1

[1] CGA205375 with all ^{35}Cl

[2] CGA205375 with one ^{37}Cl

For difenconazole and CGA205375, final determination by LC-MS/MS using two transitions is considered to be highly specific; therefore no further confirmatory conditions are included.

For CGA205375 and CGA142856, final determination by LC-MS/MS is accomplished using one transition due to the low molecular weight of the parent compounds. Confirmatory procedures can be found for CGA142856 and CGA71019 in the following references:

a) For CGA142856 and CGA71019, alternative chromatographic conditions have been documented in Syngenta Report Number T013656-05 (Reference 3).
b) For CGA71019, alternative chromatographic conditions have been documented in Syngenta Report Number 444-00 (Reference 4).

APPENDIX 4 Method Flow Chart

Transfer sample (10.0 mL) to a glass vial

↓

Fortify recovery sample, if needed

↓

Mix well by agitation (vortex)

Sample analysis by direct injection for (Difenoconazole, CGA205375, CGA142856 and CGA71019)	Sample analysis by SPE cleanup for CGA71019, if needed
	↓
	Bond Elut Certify (300 mg, 3cc)
↓	↓
Transfer 0.8 mL of sample into HPLC vial containing 0.2 mL of ACN	Condition the SPE cartridges
↓	↓
Analysis by LC-MS/MS	Load the water samples quantitatively
	↓
	Wash SPE cartridges
	↓
	Elute the SPE cartridges with basic 90/10 methanol/water and collect
	↓
	Evaporate to ~ 0.5 mL
	↓
	Re-constitute the sample by adding 0.2 mL ACN and bring up to 1.0 mL with water.
	↓
	Analyze by LC-MS/MS