2.0 INTRODUCTION

This report describes the independent laboratory validation (ILV) of Syngenta Analytical Method GRM066.01A "Difenoconazole - Residue Method for the Determination of Difenoconazole, CGA205375, CGA142856 and CGA71019 in Water" (Reference 1) as performed by ADPEN Laboratories, Inc. The analytical method is presented in Appendix 2.

This study was designed to satisfy harmonized guideline requirements described in OECD Guidance Document ENV/JM/MONO(2007)17 (Reference 2), EPA Guideline OCSPP 850.6100 (Reference 3), EC Guidance Document SANCO/3029/99 Rev.4 (2000) (Reference 4), and EC Guidance Document SANCO/825/00 Rev.8.1 (2010) (Reference 5). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (Reference 6).

The residue analytical method is suitable for the determination of Difenoconazole, CGA205375, CGA142856 and CGA71019 in surface and ground water LOQ level of 0.10 ppb.

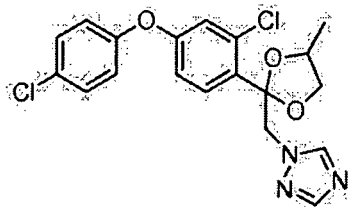
Environmental water samples were analyzed directly upon treatment with acetonitrile using liquid chromatography tandem mass spectrometry (LC-MS/MS) technique when the instrument sensitivity allows. Alternatively, the water samples were concentrated for CGA71019 analysis using solid phase extraction (SPE) 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 (90:10) methanol/water from the SPE cartridges and collected. The collected 0.5% NH_4OH in (90:10) methanol/water fractions were evaporated under a gentle stream of nitrogen in a water bath at approximately 40 °C and re-constituted with acetonitrile/H2O (20:80; v/v) then subjected to LC-MS/MS analysis. The LOQ of the method is 0.10 µg/L (ppb) for the environmental water samples.

3.0 MATERIALS AND METHODS

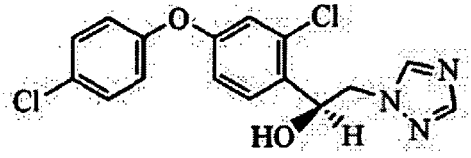
3.1 Test/Reference Substance

The test/reference substances were obtained from Syngenta Crop Protection, LLC. The following test/reference substances were used:

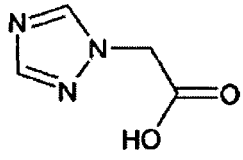
Compound Code Number :	**CGA169374 (Difenoconazole)**
CAS Number:	119446-68-3
IUPAC Name:	cis,trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether
Lot Number:	WRS 1324/1
Molecular Formula:	$C_{19}H_{17}Cl_2N_3O_3$
Molecular Weight:	406.3
Purity:	96.3%
Expiration Date:	February 2019
Storage Conditions:	< 30 °C



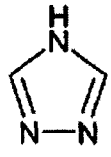
Compound Code Number : **CGA205375**
CAS Number: 117018-19-6
IUPAC Name: 1-[2-chloro-4-(4-chloro-phenoxy)-phenyl]-2-[1,2,4]-triazaol-1-yl-ethanol
Lot Number: DAH-XXXI-17
Molecular Formula $C_{16}H_{13}Cl_2N_3O_2$
Purity: 99.3%
Expiration Date: August 31, 2015
Storage Conditions: refrigerator



Compound Code Number : **CGA142856**
CAS Number: 28711-29-7
IUPAC Name: [1,2,4]-Triazol-1-yl-acetic acid
Lot Number: DAH-XXX-93
Molecular Formula $C_4H_5N_3O_2$
Purity: 98.7%
Expiration Date: December 31, 2014
Storage Conditions: refrigerator



Compound Code Number : **CGA71019**
CAS Number: 288-88-0
IUPAC Name: 1,2,4-Triazole
Lot Number: DAH-XXX-87
Molecular Formula $C_2H_3N_3$
Purity: 96.7%
Expiration Date: December 31, 2014
Storage Conditions: freezer



The test/reference substance (analytical standard) used in this study was procured from the Sponsor and stored as directed. Characterization data for the test/reference standard are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificates of Analysis are included in Appendix 3.

3.2 Test System

The test systems evaluated in this study were surface water and ground water. The control sample used in this study was characterized by AGVISE Laboratories of Northwood, North Dakota and reported to Syngenta Archive under Syngenta Study Number TK0048240. GLP characterization results are presented in Appendix 4 and summarized below.

Sample ID	Water Type	pH	Calcium (ppm)	Magnesium (ppm)
RIMV00312-0001	Surface Water	7.3	6.0	2.9
RIMV00312-0002	Ground Water	7.5	16	4.5

Control water samples were sent from Syngenta on 23 September 2013 and received at ADPEN Laboratories, Inc. on 24 September 2013. Upon receipt, samples were logged in and stored in freezer E 23, then allowed to defrost prior to analysis in refrigerator E 57. Temperature ranges during the course of this study were −15±2 °C. Prior to analysis, the samples were sub-sampled and unique laboratory codes were assigned to each sub-sample and are cross-referenced on each page of the detailed residue reports to the Syngenta sample number. Sample extracts were stored in refrigerator E 109 while awaiting LC-MS/MS analysis. The temperature range during the course of this study for this refrigerator was 5±2°C.

The control samples were checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in Syngenta Method GRM066.01A.

3.3 Apparatus

The equipment and apparatus used for the method validation were as outlined in the method. Identical or equivalent equipment was used, as permitted by the method.

3.4 Reagents

Reagent	Description	Supplier
Methanol	Optima grade	Fisher Scientific
Ultra-pure water	HPLC grade	EMD
Acetonitrile	HPLC grade	EMD
Formic Acid	A.C.S. grade	EMD
Ammonium Hydroxide	A.C.S. grade	EMD
Difenoconazole analytical standard	GLP certified	SYNGENTA
CGA205375 analytical standard	GLP certified	SYNGENTA
CGA142856 analytical standard	GLP certified	SYNGENTA
CGA71019 analytical standard	GLP certified	SYNGENTA

3.4.1 Preparation of Reagents

Reagents prepared as described in the method.

3.5 Preparation of Standard Solutions

All standard solutions were prepared and stored as recommended in the method.

3.6 Analytical Procedures and Modifications

Analytical Method GRM066.01A was independently validated as written.

3.6.1 Modifications

No modifications to the method were made.

3.6.2 Fortifications

Untreated control water samples were fortified using 0.1 mL of the appropriate fortification standard at LOQ (0.1 ppb) and 10×LOQ (1.0 ppb) concentrations as per the method. Fortifications used in this method validation are as follows:

Matrix	Standard Concentration (ng/mL)	Aliquot Volume (mL)	Sample Size (mL)	Final Concentration (ppb)	Replicates
Water	10	0.1	10	0.1	5
	100	0.1	10	1.0	5

4.0 ANALYTICAL PROCEDURE

Each validation set included a reagent blank, two control water samples, five control water samples fortified at LOQ, and five control samples fortified at 10× LOQ. Plastic containers were 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 1.

4.1 Sample Preparation (Difenoconazole, CGA205375, CGA142856, and CGA71019)

1. Water samples were allowed to defrost completely at room temperature. Defrosted samples were shaken thoroughly to ensure sample homogeneity prior to subsequent aliquot for further treatment or analysis.

2. Fortification sample preparation: Water sample (10 mL) was transferred into a 20-mL glass scintillation vial. The recovery samples were fortified with the appropriate amount of each analyte using the spiking solution (0.1 mL). The scintillation vial was capped securely and shaken gently to mix thoroughly.

3. Water samples (including recovery samples): Water sample (0.8 mL) was transferred to a 2-mL injection vial containing 0.2 mL acetonitrile. The vial was capped securely and sample was mixed using a vortex mixer.

4. Samples were analyzed using LC-MS/MS for residue determination.

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

1. Fortification samples: Water samples (10 mL) were fortified previously in section 4.1 above.

2. SPE cartridges were conditioned 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**.

3. 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.

4. 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.

5. 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.

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

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

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

4.3 Instrumentation/Operating Conditions

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

Mass Spectrometer Detector:	**ABSciex 6500 LC-MS/MS System**
Polarity:	Positive
CUR:	35.0
CAD:	10.0
TEM:	550
GS1:	70.0
GS2:	70.0
EP:	10.0

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	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	69.9	3.3	70.0	55.0	8.0

4.4 Data Acquisition

Peak integration and peak area count quantitation were performed by Analyst® (version 1.6.2) data handling software. A best-fit, linear regression equation was derived and used in conjunction with the analyte response in each sample to calculate the concentration of the

analyte. The square of correlation coefficients (R^2) for the calibration curves for each analytical set was greater than 0.99. Recovery results were computed for each sample.

A statistical treatment of the data includes the calculation of averages, standard deviations, and relative standard deviations. Mean percent recoveries, standard deviations, and relative standard deviations were calculated in LIMS and reported in Microsoft® Office Excel spreadsheets.

5.3 Limit of detection

The limit of detection of the analytical method was defined as 0.025 µg/L (ppb), which is equivalent to 0.0005 ng on column.

5.4 Limit of quantitation

The limit of quantitation (LOQ) of the analytical method has been established at 0.10 µg/L (ppb), which is equivalent to 0.002 ng on column.

5.5 Potential Interferences

During this study, using AB Sciex 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 4000 system. At the request of the study monitor, experiments were prepared to determine the source of the second peak (as per 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 8.

Minimal matrix effects were observed for all analytes in the surface and ground water samples, with the exception of CGA142856, which showed suppression of approximately 26% (Surface Water). A summary of the matrix effects results is presented in Table 24.

5.6 Communications

All pertinent communications during the study are provided in Appendix 7.

5.7 Time Required for Analysis

A single analyst completed a set of 13 samples in less than one working day with LC-MS/MS analysis performed overnight.

5.8 Deviations

No protocol, SOP, or method deviations were generated during this validation.

5.9 Circumstances Affecting Data

The configuration of the LC component of the instrument hardware was modified with the installation of a 100 µL loop. This change in configuration caused retention times for the analytes of interest to shift as shown in the supporting data.

6.0 CONCLUSIONS

In summary, ADPEN Laboratories, Inc. successfully independently validated on the first trial Syngenta Analytical Method GRM066.01A entitled "Difenoconazole - Residue Method for the Determination of Difenoconazole, CGA205375, CGA142856 and CGA71019 in Water".

The analytical method was demonstrated to be suitable for the determination of difenoconazole, CGA205375, CGA142856, and CGA71019 in surface and ground water at an LOQ of 0.1 ppb and at 10×LOQ. Additionally, the direct injection method was demonstrated to be suitable for the determination of CGA71019 in surface and ground water using an AB Sciex 6500 mass spectrometer. The method is well-written and contains sufficient information to guide the analyst through the procedure for the first time.

7.0 REFERENCES

1. Manuli, M., Huang, S. "Difenoconazole - Residue Method for the Determination of Difenoconazole, CGA205375, CGA142856 and CGA71019 in Water". Syngenta Crop Protection. Draft Analytical Method.

2. Organization for Economic Co-Operation and Development (OECD). Guidance Document on Pesticide Residue Analytical Methods, ENV/JM/MONO(2007)17.

3. United States Environmental Protection Agency, Office of Prevention, Pesticide and Toxic Substances, Ecological Effects Test Guidelines EPA Documents EPA 712-C-001 (OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation, Jan, 2012. Internet address: http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm

4. European Commission, Directorate General Health and Consumer Protection, (SANCO/3029/99 Rev.4) Residues: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414; November 07, 2000. Internet Address: http://ec.europa.eu/food/plant/plant_protection_products/guidance_documents/docs/wrkdoc12_en.pdf

5. European Commission, Directorate General Health and Consumer Protection, (SANCO/825/00 Rev.8.1) Guidance Document on Pesticide Residue Analytical Methods; November 16, 2010. Internet Address: http://ec.europa.eu/food/plant/plant_protection_products/guidance_documents/docs/guide_doc_825-00_rev8_en.pdf

6. FIFRA Good Laboratory Practice Standards. *Code of Federal Regulations*, Section 160, Title 40, 1982; *Fed. Regist.* **1983,** *48,* 53946 ff.

APPENDIX 5. Example Calculations

Residue results are calculated by comparison to the standard curves obtained from a linear regression analysis of the data found by the data system. The equation for the fit of the standard curve was used to calculate intercept and slope of the linear regression curve. The intercept and the slope were used in the equation used for quantitation. LIMS was used to calculate the ppb and percent recovery and presented in Microsoft® Excel. The following equations were used for quantitation:

The following equations are used for residue calculations within Analyst:

a) Calibration curve $y = mx + b$: Solving for x: $x = \frac{y - b}{m}$

Where, m = Slope
b = y-intercept
x = Amount found (ng)
y = Peak area

b) Amount of sample injected (mL) = $\frac{\text{Sample amt. (mL)} \times \text{Inj. size (mL)}}{\text{Final sample vol. (mL)}}$

c) Analyte concentration (ppb) = $\frac{\text{Amount found (ng)}}{\text{Amount of sample injected (mL)}}$

d) Percent recovery = $\left(\frac{\text{ppb in sample} - \text{ppb in control}}{\text{ppb added}}\right) \times 100$

As an example, calculations to obtain the percent recovery in a control water sample from WO-13093005 fortified with difenoconazole in lab code 13093005-Recovery1-1. The calculations are shown below:

a) Calibration curve: $y = (2.79e{+}008)x - 4.85e{+}003$

Solving for x: $x = \frac{437166 + 4.85e{+}003}{2.79e{+}008} = 0.00159$ ng

b) Amount of sample injected (mL) = $\frac{10.0 \text{ mL} \times 0.02 \text{ mL}}{12.5 \text{ mL}} = 0.016$ mL

c) Analyte concentration (ppb) = $\frac{0.00159 \text{ ng}}{0.016 \text{ mL}} = 0.09938$ ppb

Average residue found in the untreated sample (lab code: 130924001-001A and 130924001-001 B) = 0.00156 ppb

d) Percent recovery = $\left(\frac{0.09938 \text{ ppb} - 0.00156 \text{ ppb}}{0.1 \text{ ppb}}\right) \times 100 = 97.8\%$