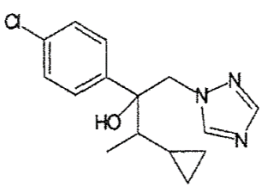
3.0 MATERIALS AND METHODS

3.1 Test Substance/Reference Substance

The test substance, Cyproconazole, was received on July 18, 2016 from Syngenta Crop Protection, Greensboro, North Carolina. The following information was provided:

Compound Structure	
Syngenta Code:	SAN619
Common Name:	Cyproconazole
CAS Name:	1H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-
CAS Number:	94361-06-5
IUPAC Name:	2-(4-Chloro-phenyl)-3-cyclopropyl-1-[1,2,4]triazol-1-yl-butan-2-ol
Batch Number:	WRS 1161/2
Molecular Weight:	291.8
Structural Formula	$C_{15}H_{18}ClN_3O$
Storage Conditions:	< 30ºC
Purity:	97.9%
Recertification Date:	End of April 2017

Upon receipt, the test substance (Cyproconazole) was stored at room temperature in the original container. Concentrations were adjusted for the purity of the test substance.

All solutions made from the test substance (analytical standard) were stored according to the method.

3.2 Test Systems

The test systems evaluated in this study were surface water and ground (drinking) water. These matrices were chosen because they are representative of the water the method is designed for.

Approximately 6L of Surface water was collected from Musconetcong River at Waterloo Road at Saxton Falls in New Jersey, United States on August 17, 2016. Upon arrival at Symbiotic Research on the same date, surface water was stored refrigerated. The following day, the surface water was removed from and returned to refrigerator following filtration with Whatman Qualitative Circles, Grade 1.

Groundwater (bottled natural spring water) was purchased from a local grocer in Chester, NJ on August 18, 2016, and stored room temperature in the laboratory following arrival at Symbiotic Research.

Refrigerator storage temperatures were monitored on a daily basis and were typically at *c.a.* 4.0^{o}C. Surface water was stored refrigerated, except for the periods during which the matrix was aliquoted for analysis. Groundwater was stored at room temperature for the entire duration of the study.

3.3 Equipment and Reagents/Supplies

The equipment and reagents/supplies used for the method validation were as outlined in the method. Identical or equivalent equipment and materials were used, as permitted by the method. The equivalent equipment and reagents used were as follows:

3.3.1 Equipment

Balance:	Mettler Toledo Microbalance, Model AT20
HPLC:	Hewlett Packard Series 1100 Modular HPLC System Agilent Technology, Wilmington, DE. Equipped with Degasser, Binary Pump, Autosampler, Column Oven, DAD.

3.3.2 Reagents

Acetonitrile:	HPLC Grade (GC Chemical)
Acetic Acid, glacial:	HPLC Grade (EMD Millipore)
Water (H_2O):	LC-MS Grade (Fluka via Sigma Aldrich)

3.4 Preparation of Standard Solutions

The preparation of Cyproconazole standard solutions used for this study is described below. The solutions were stored as recommended in the method when not in use (frozen, *c.a.* -20°C).

3.4.1 Stock Standard Solution

A small amount of Cyproconazole reference substance was added to a pre-tared small glass vial and the weight (4.95 milligrams) was recorded. The volume of acetonitrile (24.23 mL) needed to make a stock standard solution of Cyproconazole having a concentration of 200 µg/mL was calculated using the equation in section 2.3.1 of the method GRM033.04A. One (1) mL of acetonitrile was added to the vial and contents mixed by vortex and transferred to an appropriate sized larger vial. This step was repeated a total of four (4) times. The remaining volume of acetonitrile was added to the larger vial and contents mixed by vortex to give a final concentration of 200 µg/mL.

3.4.2 Fortification Solutions

Fortification Solutions

10-µg/mL:	0.5 mL of a 200-µg/mL Cyproconazole stock standard solution was transferred to a 15-mL polypropylene centrifuge tube. 9.5 mL acetonitrile was added and the solution was mixed well.
1.0-µg/mL:	1.0 mL of a 10-µg/mL Cyproconazole stock standard solution was transferred to a 15-mL polypropylene centrifuge tube. 9 mL acetonitrile was added and the solution was mixed well.
0.1-µg/mL:	1.0 mL of a 1.0-µg/mL fortification solution was transferred to a 15-mL polypropylene centrifuge tube. 9 mL acetonitrile was added and the solution was mixed well.
0.01-µg/mL:	1.0 mL of a 0.1-µg/mL fortification solution was transferred to a 15-mL polypropylene centrifuge tube. 9 mL acetonitrile was added and the solution was mixed well.
0.05-µg/mL:	5.0 mL of a 0.1-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.
0.025-µg/mL:	5.0 mL of a 0.05-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.
0.005-µg/mL:	5.0 mL of a 0.01-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.
0.002-µg/mL:	4.0 mL of a 0.005-µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed well.

3.4.3 HPLC (Calibration) Standard Solutions

Calibration standards were prepared from the fortification solutions and were stored frozen when not in use.

1 µg/L:	0.1 mL of a 0.1 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.
0.5 µg/L:	0.1 mL of a 0.05 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.
0.25 µg/L:	0.1 mL of a 0.025 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.
0.1 µg/L:	0.1 mL of a 0.01 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.
0.05 µg/L:	0.1 mL of a 0.005 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.
0.02 µg/L:	0.1 mL of a 0.002 µg/mL fortification solution was transferred to a 10-mL volumetric flask. The contents were brought to volume with ultra pure water and mixed well.

3.5 Analytical Method

Analytical method GRM033.04A was successfully independently validated in this study. See Appendix 1 for the complete text of the method. The following is a summary of that method:

> 10 mL of water samples were transferred into 15mL centrifuge tubes. Samples were fortified as appropriate, transferred into suitable autosampler vials, and submitted for LC-MS/MS analysis. The limit of quantitation (LOQ) is 0.05 µg/L (ppb) for Cyproconazole.

The analytical procedure was performed as written with the following exceptions. Surface water was filtered through Whatman Qualitative Circles, Grade 1, 110mm filter paper after collection. To increase accuracy, Calibration standards were prepared in 10 mL volumetric flasks instead of 15 mL polypropylene centrifuge tubes. A needle wash in acetonitrile was added to prevent carry over. Detector sensitivity at LOQ level was abundant, and for maintenance purposes, post column eluate was split to reduce material into the MS source.

Residue calculations were performed as specified in the analytical method and were conducted using Analyst (version 1.4.2) to prepare the calibration curve with 1/x weighting. The calculation worksheet can be found in Appendix 3.

3.5.1 Fortifications

Untreated surface and ground water samples were fortified using microliter amounts of the appropriate fortification standard for LOQ and 10X LOQ concentrations as per method. Fortifications used in this method validation are as follows:

Matrix	Fortification Volume (µL)	Fortification Conc. (µg/mL)	Final Volume (mL)	Final Conc. (µg/L)	Replicates
Surface Water	50	0.01	10	0.05 (LOQ)	5
Groundwater	50	0.01	10	0.05 (LOQ)	5
Surface Water	50	0.1	10	0.5 (10X LOQ)	5
Groundwater	50	0.1	10	0.5 (10X LOQ)	5

After fortification, the samples were mixed thoroughly before transferring to suitable HPLC vials.

3.6 Instrumentation Conditions

All samples were analyzed by LC-MS/MS detection. Typical conditions were as follows:

Chromatography Conditions

HPLC System	:	Hewlett Packard Series 1100 Modular HPLC System. Equipped with Degasser, Binary Pump, Autosampler, Column Oven, DAD.
Detector	:	Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software
Column	:	Kromasil 100 5µm C18, 3.0 x 50mm
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	5.0 min
Injection protocol	:	Analyze calibration standard after no more than 4 sample Injections
Mobile phase	:	Solvent A = 0.2% Acetic Acid in Ultra pure water Solvent B = Acetonitrile

Mobile Phase Composition

Time (min)	%A	%B	Flow Rate (mL/min)
0.00	70	30	1.0
3.00	20	80	1.0
3.50	20	80	1.0
3.60	70	30	1.0
5.00	70	30	1.0

Mass Spectrometer Conditions

Ion Source Parameters:

Ionization Mode	Positive (+)
Curtain Gas (CUR)	20
Collision Gas (CAD)	7
IonSpray Voltage (V)	5500
Temperature (TEM)	650
Ion Source Gas 1 (GS1)	35
Ion Source Gas 2 (GS2)	50
Declustering Potential (DP)	60
Entrance Potential (EP)	10

Note: The mass spectrometer tuning parameters shown here are for reference only. The analyst should always consult with instrument operation manual to obtain optimum conditions for all the analytes prior to residue analysis.

MRM Operating Parameters:

Cyproconazole	MS/MS Transition	Time (msec.)	CE (Volts)	CXP (Volts)
Quantification	292 → 70.2	200	40	12
Confirmation	292 → 125	200	40	12

3.7 Modifications, Interpretations, and Critical Steps

The analytical procedure was performed as written with the following exceptions. Surface water was filtered through Whatman Qualitative Circles, Grade 1, 110mm filter paper after collection. To increase accuracy, Calibration standards were prepared in 10 mL volumetric flasks instead of 15 mL polypropylene centrifuge tubes. A needle wash in acetonitrile was added to prevent carry over. Detector sensitivity at LOQ level was abundant, and for maintenance purposes, post column eluate was split to reduce material into the MS source.