2.0 INTRODUCTION

This report describes the independent laboratory validation (ILV) of Syngenta Analytical Method GRM033.01A [1] as performed by North Coast Laboratories, Ltd. (NCL) for the determination of the cyproconazole, 1,2,4-triazole, and triazole acetic acid in soil using liquid chromatography-electrospray ionization tandem mass spectrometry (LC/MS/MS).

This study was conducted to satisfy guideline requirements described in the harmonized guidelines from the OPPTS, "Public Draft" - Data Reporting for Environmental Chemistry Methods, OPPTS 850.7100 [2]. In addition, this report also satisfies the requirements outlined in the US EPA FIFRA Pesticide Assessment Guidelines for Subdivisions N, E, and K, and addenda for Data Reporting Guideline for Environmental Methods [3].

3.0 MATERIALS AND METHODS

3.1 Test / Reference Substance

The test/reference substances were shipped from Syngenta Crop Protection, Inc., Greensboro, North Carolina to NCL. The 1,2,4-triazole and triazole acetic acid were received on September 25, 2008; the cyproconazole was received on September 29, 2008. The test/reference substances that were used for the validation are described as follows:

Common name:	Cyproconazole
CAS name:	1H-1,2,4-triazole-1-ethanol,alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-
CAS numbers.:	94361-06-5 & 94361-07-6
Batch identification:	410441
Stated purity:	96.2%
Expiration date:	September 30, 2009
Storage conditions:	2°C to 6°C

Product name:	CGA-71019
Chemical name:	1H-1,2,4-Triazole
CAS number:	288-88-0
Batch identification:	WFH-IV-5
Stated purity:	99.9%
Expiration date:	April 30, 2009
Storage conditions:	2°C to 6°C

Product name:	CGA-142856
Chemical name:	1H-1,2,4-Triazole-1-acetic acid
CAS number:	28711-29-7
Batch identification:	DAH-XXX-93
Stated purity:	99.7%
Expiration date:	October 31, 2009
Storage conditions:	2°C to 6°C

The Analytical and Product Chemistry Department of Syngenta Crop Protection, Inc., Greensboro, North Carolina maintains the characterization and stability data for the test/reference substance.

Note: The Certificate of Analysis that accompanied the cyproconazole neat reference substance indicated a storage temperature of < 30°C. The neat reference substance was stored refrigerated at 2°C to 6°C, and the sponsor confirmed that refrigerated storage was appropriate.

Stock standard solutions were prepared from the neat test/reference substances for use in the preparation of fortification solutions and instrument calibration solutions. All standard solutions were prepared as per the method. These stock standard solutions were stored refrigerated when not in use. Section **3.5.4** describes of the preparation of single analyte stock solutions, and section **4.7.2** provides example calculations.

3.2 Equipment and Reagents

3.2.1 Solvents and Reagents

Water, Fisher HPLC grade
Methanol, Fisher HPLC grade
Ammonium Hydroxide, Fisher A.C.S., Plus
Fisher ACS Certified 88% Formic Acid

3.2.2 Solutions

Methanol/Water, 5/95 (v/v); 5 mL methanol; bring the final volume up to (q.s.) 100 mL with water
Methanol/Water, 50/50 (v/v); 100 mL water; q.s. to 200 mL with methanol
Methanol/Water, 80/20 (v/v); 200 mL water; q.s. to 1 L with methanol
Methanol/Water, 90/10 (v/v); 20 mL water; q.s. to 200 mL with methanol
0.5% Ammonium Hydroxide in 90/10 Methanol/Water; 1.0 mL ammonium hydroxide into 100 mL 90/10 (v/v) methanol/water
1% Ammonium Hydroxide in Methanol; 1.0 mL ammonium hydroxide into 100 mL methanol
1% Ammonium Hydroxide in Water; 1.0 mL ammonium hydroxide into 100 mL water
5% Formic Acid in Methanol; 5 mL formic acid added to 95 mL methanol
2% Formic Acid in Methanol; 4 mL formic acid into 196 mL methanol

2% Formic Acid in Water; 2 mL formic acid into 98 mL water
0.1% Formic Acid in Acetonitrile (Mobile Phase "B"); 1.0 mL formic acid; q.s. to 1 L with ACN
0.1% Formic Acid in Methanol (Mobile Phase "B"); 1.0 mL formic acid; q.s. to 1 L with methanol
0.1% Formic Acid in Water (Mobile Phase "A"); 1.0 mL formic acid; q.s. to 1 L with water

3.2.3 Apparatus

A list of apparatus used in the method validation trial is shown below. Similar equipment from other suppliers may also be used.

Mettler AB204-2 Analytical Balance
Mettler Top Loading Balance
Büchner funnels, 7 cm or 9 cm
Centrifuge (DuPont RC-5B Refrigerated Superspeed or IEC Centra-8)
Centrifuge Tubes, polypropylene, graduated, screw-capped, 15-mL and 50-mL capacity
VWR mini vortexer
Organomation Associates N-EVAP™112 nitrogen evaporator
Pipettors, Automatic - capable of accurately dispensing volumes of 1.0 µL to 50 mL
Pipettes, Graduated or Volumetric suitable of accurately delivering 0.5 to 10 mL
Pipettes, Pasteur, disposable
Platform shaker, reciprocal, Eberbach
Brinkmann Buchi 121 Rotary Vacuum Evaporators
Supelco Visiprep 24™ DL SPE Vacuum Manifold
Vacuum flasks, 250 mL or 500 mL
SPE cartidges, Varian Bond Elut Certify, 300 mg/3-mL, Catalog No. 12102081
SPE cartridges, Waters Oasis MAX, 150 mg/6-mL, 60µm, Catalog No. 186000370
Kimbal Screw Thread Amber 12-mL glass vials with Teflon-lined screw-caps
CRS 1.8-mL clear screw top standard mouth glass autosampler vials with caps
Whatman 2V filter paper

3.2.4 LC/MS/MS Instrumentation

Analysis was performed using a high pressure liquid chromatograph with a tandem mass spectrometer (LC/MS-MS). The following equipment was used:

(2) Shimadzu LC-10ADvp pumps and Shimadzu SCL-10Avp pump system controller
Applied Biosystems/MDS Sciex Turbo V™ Ion Source for the API 4000 mass spectrometer, operated in positive ion mode for cyproconazole and 1,2,4-triazole and in negative ion mode for triazole acetic acid
Applied Biosystems/MDS Sciex API 4000 LC/MS-MS triple quadrapole mass spectrometer
Agilent ZORBAX SB-Aq Rapid Resolution 4.6 x 75mm 3.5-µm HPLC column equipped with a Phenomenex MAX-RP "Security Guard" cartridge type guard column
Perkin Elmer (PE) ISS 200 autosampler
Applied Biosystems/MDS Sciex Analyst software version 1.4.2

3.3 Safety and Health

This method was performed by trained personnel who acted in accord with the cyproconazole material safety data sheet that documents the hazards associated with the use of this chemical.

3.4 Test System and Sample Storage

The matrix, bulk control soil, used for the validation was provided by the sponsor and arrived in good frozen condition on dry ice on September 24, 2008. The soil sample had a unique Syngenta number and received a unique North Coast Laboratories, Ltd. (NCL) sample number on receipt at NCL. The sample was stored frozen (less than or equal to -10 °C) in a limited access freezer, except when in use. Table 2 presents the dates of events for each use of this sample.

The control soil sample used in this ILV was collected from Greene County, Iowa for Syngenta Study T002340-06 (labeled as "Bulk Soil (control)" with Sample Numeric ID of RIEN00707-0002). The bulk soil sample was processed by Syngenta Crop Protection in Greensboro, NC, and a sub-sample of this untreated soil was provided to NCL. This control soil sample was checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in the validated Syngenta Method GRM033.01A.

Soil for the study site was characterized by Agvise Laboratories of Northwood, North Dakota and reported to Syngenta for Syngenta Study T002340-06 and the original raw data for the soil characterization will be stored in Syngenta Archive under the Syngenta Study Number T002340-06. The characterization results of the control soil are summarized below.

USDA Texural Class	Percent Sand	Percent Silt	Percent Clay	Percent Organic Matter	pH	Cation Exchange Capacity (meq/100g)	Bulk Density Disturbed (g/cc	Percent Moisture at 1/3 bar Disturbed	Percent Moisture at 15 bar Disturbed
Sandy Loam	54	26	20	2.6	7.3	17.3	1.23	18.4	10.4

3.5 Analytical Method and Method Establishment

3.5.1 Principle of the Method

Soil samples (10 g) were extracted two times by shaking with solvent at room temperature for 20 minutes. The extracts were combined upon centrifugation and filtration to separate the suspended solids. The volatile organics of the combined extracts were removed under vacuum with a rotary evaporator at a bath temperature of approximately 35°C. The resulting concentrated aqueous extracts were quantitatively transferred to a plastic centrifuge tube and

were diluted with deionized water to a final volume of 10 mL. Aliquots of this final extract were taken for solid phase extraction (SPE) workup. Cleanup for cyproconazole and 1,2,4-triazole were accomplished by a mixed mode cation exchange SPE, while triazole acetic acid was process by a mixed mode anion exchange SPE. Upon SPE cleanup and subsequent concentration, an aliquot of the final sample extract was transferred to an autosampler vial and subjected to LC/MS/MS analysis. Two LC/MS/MS analyses were performed: ESI positive mode for cyproconazole and triazole; and ESI negative mode for triazole acetic acid. Residue quantification was carried out using external standard calibrations.

3.5.2 Limits of Quantitation

The limit of quantitation (LOQ) for each analyte was 1 ng/g (ppb).

3.5.3 Validation Sample Set

One method trial was conducted and the set consisted of the following samples:

Positive mode:
Instrument calibration working standards
One reagent blank
Two unfortified control samples
Five samples fortified with cyproconazole and 1,2,4-triazole at 1.0 ng/g (ppb; LOQ)
Five samples fortified with cyproconazole and 1,2,4-triazole at 10 ng/g (ppb; 10xLOQ)

Negative mode:
Instrument calibration working standards
One reagent blank
Two unfortified control samples
Five samples fortified with triazole acetic acid at 1.0 ng/g (ppb; LOQ)
Five samples fortified with triazole acetic acid at 10 ng/g (ppb; 10xLOQ)

3.5.4 Preparation of Single Analyte Stock Standard Solutions

Section **4.7.2** provides example calculations describing the preparation of the stock standard solutions.

An aliquot of 0.0105 g of cyproconazole was weighed out into an amber glass vial. The appropriate amount of 5/95 methanol/water (v/v) was added to the vial to yield a 100 µg/mL standard solution. The 1,2,4-triazole and the triazole acetic acid were prepared similarly. The single analyte standard solutions were stored refrigerated at 2 to 6 °C.

3.5.5 Preparation of Mixed Analyte Fortification and Calibration Standard Solutions

A 1.0 µg/mL mixed standard solution was prepared by combining 1.0 mL each of the 100 µg/mL primary stock standards into a 100-mL volumetric flask and bringing the solution

up to the 100-mL final volume with 5/95 methanol/water (v/v; HPLC grade). A 1:10 dilution of the mixed standard solution produced a 0.10 µg/mL mixed standard solution.

Preparation of Mixed Analyte Instrument Calibration Working Standard Solutions

Six levels of mixed analyte instrument calibration working standards were prepared (0.5x, 1x, 2x, 5x, 10x and 20xLOQ) and named with respect to the concentration in the fortified samples (see the table below and the example calculations presented in section **4.7.4**). The standards described in the table below were brought up to a final 10-mL volume with 5/95 methanol/water (v/v).

Mixed Analyte Instrument Calibration Working Standard Solutions				
Analyte Concentration Relative to the Sample	Concentration of Mixed or Single Analyte Stock Solution (ng/µL)	Volume of Mixed or Single Analyte Stock Solution (µL)	Final Volume (mL)	In-solution Analyte Concentration (ng/mL)
0.5xLOQ = 0.5 ng/g	1.0	5	10	0.50
1xLOQ = 1 ng/g	1.0	10	10	1.0
2xLOQ = 2 ng/g	1.0	20	10	2.0
5xLOQ = 5 ng/g	1.0	50	10	5.0
10xLOQ = 10 ng/g	1.0	100	10	10
20xLOQ = 20 ng/g	1.0	200	10	20

3.5.7 Preparation of Samples and Weighing

The soil sample was prepared at Syngenta Crop Protection, Inc. and shipped frozen on dry ice to NCL, *via* FedEx priority overnight service. Immediately prior to the first extraction, the sample was thoroughly mixed. Sub-samples (10 ± 0.1 g) were weighed into 50-mL disposable plastic centrifuge tubes.

3.5.8 Preparation of Fortification Samples

A 100-µL aliquot of the 0.10 ng/µL, ppm (µg/mL) mixed standard solution was applied to each replicate LOQ fortification. A 100-µL aliquot of the 1.0 µg/mL (ng/µL) mixed standard solution was applied to each replicate 10xLOQ fortification. The fortified samples were allowed to sit for 5 minutes before proceeding with the extraction. Section **4.7.3** presents the calculations used to prepare the fortified samples.

3.5.9 Extraction Procedure

The extraction procedure was performed exactly as written in the method. The method is incorporated into the Study Protocol which is presented in Appendix 4; pages 21 through 23 of the Protocol/sections 3.2 and 3.3 of the method describe the extraction procedures that were followed at NCL.

3.5.10 LC/MS/MS Operating Parameters

3.5.10.1 Conditions

Column:	Agilent ZORBAX SB-Aq Rapid Resolution 4.6 x 75mm 3.5-µm HPLC column
Mobile phases:	Water, 0.1% formic acid (Mobile Phase A)
	Methanol, 0.1% formic acid (Mobile Phase B)
Flow rate:	0.500 mL/min.
Injection volume:	60 µL

Cyproconazole and 1,2,4-Triazole

Scan type:	MRM	
Polarity:	Positive	
Expected Retention time:	Cyproconazole	4.47 min.
	1,2,4-Triazole	3.00 min

Gradient Program:

Model: SCL-10Avp
Power: On
Event 1: On
Event 2: Off
Event 3: On
Event 4: Off
Time Program

Time	Module	Events	Parameter
1.80	System Controller	Event	3
2.00	Pumps	Pump B Conc.	2
2.50	Pumps	Pump B Conc.	90
6.00	System Controller	Event	1
8.00	Pumps	Pump B Conc.	90
8.01	Pumps	Total Flow	0.5
8.11	Pumps	Pump B Conc.	2
8.20	Pumps	Total Flow	1
10.00	Pumps	Total Flow	1
10.10	Pumps	Total Flow	0.5
11.00	System Controller	Stop	

Triazole Acetic Acid

Scan type:	MRM
Polarity:	Negative
Expected Retention time:	2.56 min.

Gradient program:

Model: SCL-10Avp
Power: On
Event 1: On
Event 2: Off
Event 3: Off
Event 4: Off
Time Program

Time	Module	Events	Parameter
1.70	System Controller	Event	3
3.50	System Controller	Event	1
3.51	Pumps	Pump B Conc.	2
3.60	Pumps	Pump B Conc.	98
6.60	Pumps	Pump B Conc.	0.0
6.70	Pumps	Pump B Conc.	2
6.71	Pumps	Total Flow	1
9.00	Pumps	Total Flow	0.5
11.00	System Controller	Stop	

3.5.10.2 Mass Spectrometer Mass Calibration, Optimization and Operation

The sample was introduced into the mass spectrometer via liquid chromatography by atmospheric pressure ionization (API) pneumatically assisted electrospray (Turbo V™ Ion Source) operated in the positive ion mode for the analysis of cyproconazole and 1,2,4-triazole, and in the negative ion mode for the analysis of triazole acetic acid.

3.5.10.3 Monitored Transitions

The primary transition was used for quantitation of each analyte. Copies of example chromatograms are included in the Figures Section. The transitions used were as follows:

Cyproconazole:	292.0/125.1 amu
1,2,4-triazole:	70.1 / 43.1 amu
Triazole acetic acid:	125.8/ 82.2 amu

3.5.10.4 Calibration Procedures

Instrument calibration working standard solutions were prepared as described in section **4.7.4**. Six instrument calibration working standards were positioned within the analytical batch sequence, bracketing no more than 3 samples between standards. The standard concentrations were 0.5x, 1x, 2x, 5x, 10x and 20xLOQ for each analyte (0.5, 1, 2, 5, 10 and 20 ng/g, ppb, respectively). The Applied Biosystems/MDS Sciex Analyst software (version 1.4.2) generated a quadratic calibration curve and the associated correlation coefficient (r) for each analyte by plotting the analyte peak area count versus analyte concentration (1/x weighting). The correlation coefficient (r) for each analyte was required to be greater than or equal to 0.995. Analyst also calculated the sample residue results printed on each chromatogram (see Figures section). The equation generated by analyst was verified by software from a second source (TotalChrom Version 6.3.1, © PerkinElmer, Inc. 2006).