

2.0 INTRODUCTION

This final report describes the independent laboratory validation (ILV) of Syngenta Analytical Method RAM 425/01 "Cyprodinil – Independent Laboratory Validation of Residue Method (RAM 425/01) for the Determination of Cyprodinil (CGA219417), CGA249287, CGA275535, and CGA321915 in Soil by LC-MS/MS" (Reference 1) as performed by ADPEN Laboratories, Inc. (ADPEN). The analytical method is presented in Appendix 2.

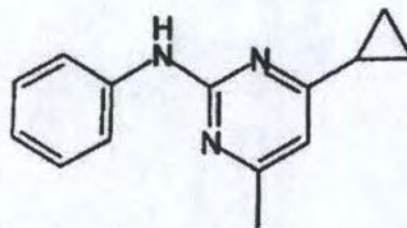
This study was designed to satisfy harmonized guideline requirements described in EPA Guideline OCSPP 850.6100 (2012) (Reference 2), EC SANCO/3029/99 Rev 4 (2000) (Reference 3), and EC SANCO/825/00 Rev 8.1 (2010) (Reference 4). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (Reference 5).

3.0 MATERIALS AND METHODS

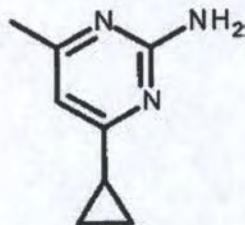
3.1 Test/Reference Substance

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

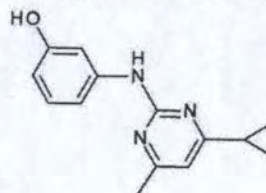
Common Name:	Cyprodinil
Code Name:	CGA219417
IUPAC Name:	(4-cyclopropyl-6-methyl-pyrimidin-2-yl)-phenyl-amine
CAS Number:	121552-61-2
Molecular Formula:	C ₁₄ H ₁₅ N ₃
Molecular Weight:	225.3 g/mol
Batch Identification:	AMS 452/3
Purity:	99.9%
Expiration Date:	End of July 2018
Storage Conditions:	< 30 °C
Source:	Syngenta Crop Protection, LLC



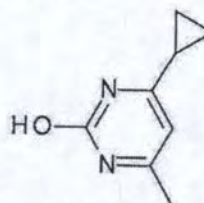
Common Name: None
Code Name: CGA249287
IUPAC Name: 4-cyclopropyl-6-methyl-pyrimidin-2-ylamine
CAS Number: 92238-61-4
Lot Number: GAN-XXXII-31-1
Purity: 99.4%
Expiration Date: May 31, 2016
Storage Conditions: Refrigerator
Source: Syngenta Crop Protection, LLC



Common Name: None
Code Name: CGA275535
IUPAC Name: N-(4-cyclopropyl-6-methyl-pyrimidin-2-yl)-3-hydroxy-aniline
Lot Number: DAH-XXVI-70
Purity: 99.7%
Expiration Date: October 31, 2014
Storage Conditions: Refrigerator
Source: Syngenta Crop Protection, LLC



Common Name: None
Code Name: CGA321915
IUPAC Name: 4-cyclopropyl-6-methyl-pyrimidin-2-ol
CAS Number: 1221553-48-8
Lot Number: GAN-XXXII-69
Purity: 99.8%
Expiration Date: October 31, 2014
Storage Conditions: Refrigerator
Source: Syngenta Crop Protection, LLC



The test/reference substances (analytical standards) used in this study were procured from the Sponsor and stored as directed. Characterization data for the test/reference standards 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 clay and sandy loam. The control samples used in this study were characterized by AGVISE Laboratories of Northwood, North Dakota and

reported to Syngenta Archive under Syngenta Study Number TK0002309. GLP characterization results are presented in Appendix 4 and summarized below.

Sample ID	Soil Type	Sample Description
RIMV00213-0001	Clay Loam	Underwood Farm, OH, 0-6"
RIMV00213-0002	Sandy Loam	San Luis Obispo Farm, CA, 0-6"

Control soil samples utilized for this study from Syngenta Study Number TK0165281 were sent from Syngenta to ADPEN on August 19, 2013 and received on August 20, 2013. Upon receipt, the samples were logged in and stored in freezer E-23, which had an average temperature during the course of this study of -17°C . Prior to analysis, the sample was sub-sampled and unique laboratory codes were assigned to each sub-sample and are cross-referenced on each page of the detailed residue reports in Appendix 6 to the Syngenta sample number. Sample extracts were stored in refrigerator E-20 while awaiting LC-MS/MS analysis. The average temperature during the course of this study for this refrigerator was 7°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 RAM 425/01.

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	HPLC grade	Fisher Scientific
Water	HPLC grade	EMD
Ammonium acetate	Analytical Reagent	EMD
Cyprodinil analytical standard	GLP certified	Syngenta
CGA249287 analytical standard	GLP certified	Syngenta
CGA275535 analytical standard	GLP certified	Syngenta
CGA321915 analytical standard	GLP certified	Syngenta

3.4.1 Preparation of Reagents

Reagents were prepared as described in the method.

3.4.2 Preparation of Stock Standard Solutions

Approximately 5 mg of each analytical standard was weighed into a 25-mL volumetric flask. The volume was brought up to the mark to prepare a 200 ng/ μ L stock solution of cyprodinil, CGA249287, CGA275535, and CGA321915. Stock solutions were stored in refrigerator E-109.

3.4.3 Preparation of Fortification Standard Solutions

Untreated control soil samples were fortified using 0.1 mL of the appropriate fortification standard at LOQ (0.01 ppm) and 10 \times LOQ (0.1 ppm) concentrations as per the method. Fortifications used in this method validation are as follows:

Matrix	Standard Concentration (ng/ μ L)	Aliquot Volume (mL)	Dilution Volume (mL)	Final Concentration (ng/ μ L)
Soil	10	2.0	20.0	1.0
	100	2.0	20.0	10.0

3.4.4 Preparation of Calibration Standard Solutions

Mixed-standard calibration solutions were prepared by volumetrically diluting intermediate standard solutions with (10:90 v/v) methanol:10mM ammonium acetate. The following table is a summary of the actual preparation of calibration solutions:

Parent Concentration (ng/ μ L)	Aliquot Volume (mL)	Dilution Volume (mL)	Final Concentration (ng/mL)
1.0	0.500	10.0	50.0
1.0	0.250	10.0	25.0
1.0	0.100	10.0	10.0
1.0	0.050	10.0	5.0
0.050	0.400	10.0	2.0
0.050	0.200	10.0	1.0
0.050	0.100	10.0	0.5
0.050	0.050	10.0	0.25

4.0 ANALYTICAL PROCEDURE

Each validation set included a reagent blank, two control soil samples, five control soil samples fortified at LOQ, and five control soil samples fortified at 10 \times LOQ.

4.1 Extraction

1. A 10-g aliquot of the control soil sample was weighed into a 250-mL round-bottomed flask.

2. Recovery samples were fortified with known amount of mix fortification standard solutions.
3. 100-mL of extraction solvent (80:20 methanol:water) was added to the sample.
4. The sample was placed on the heating mantle and heated under reflux for 1 hour.
5. Samples were cooled to room temperature and the soil extract was decanted into a 250-mL plastic centrifuge bottle.
6. Sample was centrifuged at 3500 rpm for 5 minutes.
7. Sample extract was decanted into a 100-mL graduated cylinder and the volume was adjusted with extraction solvent.
8. The sample extract was poured from the cylinder back into the centrifuge bottle and re-centrifuged at 3500 rpm for 5 minutes.
9. A 1-mL aliquot of the extract was transferred into a 10-mL graduated test tube and 10-mM ammonium acetate (0.700-mL) was added to the test tube.
10. The sample was evaporated to 1-mL under dry air in a heating block set to 40 °C.
11. The sample was transferred to a HPLC vial for analysis by LC-MS/MS.

4.2 Instrumentation/Operating Conditions

4.2.1 Chromatographic Conditions

HPLC Instrument:	Agilent 1290 UPLC			
Column:	Zorbax SB-AQ, 50 mm × 4.6 mm, 1.8 μm S/N USSQB01755			
Column temperature:	40°C			
Injection volume:	10 μL			
Flow rate:	1 mL/min			
Mobile phase A:	10 mM Ammonium Acetate in Optima Water			
Mobile phase B:	Methanol			
Gradient Step Table:	Step	Time (min)	A (%)	B (%)
	0	0	90	10
	1	2	5	95
	2	2.9	5	95
	3	3	90	10
	4	4	90	10

4.2.2 Mass Spectrometer Conditions

Mass Spectrometer:	Agilent 6490 Series QQQ
Ion Mode:	ESI+Agilent Jet Stream
Polarity:	Positive
Gas Temp (°C):	150 °C
Gas Flow (L/min):	14
Nebulizer (psi):	45
Sheath Gas Heater:	300
Sheath Gas Flow:	12
Capillary (V):	3000
V Charging:	1500
Scan type:	MRM

MRM Conditions	Q1 m/z	Q3 m/z	Retention Time (min)	Dwell time	Frag (V)	CE (V)	Cell Acc (V)
Quantification Ions							
Cyprodinil	226.1	93.0	2.5	100	380	36	7
CGA249287	150.1	66.9	2.0	100	380	40	7
CGA275535	242.1	93.0	2.3	20	380	40	7
CGA321915	151.1	93.0	1.6	10	380	28	7
Confirmation Ions							
Cyprodinil	226.1	77.0	2.5	100	380	56	7
CGA249287	150.1	118.0	2.0	100	380	24	7
CGA275535	242.1	64.9	2.3	20	380	52	7
CGA321915	151.1	108.0	1.6	10	380	16	7

4.3 Data Acquisition

Peak integration and peak area count quantitation were performed by MassHunter (version B.04.01) 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, relative standard deviations. Mean percent recoveries were calculated in LIMS (Laboratory Information System) and reported in Microsoft® Office Excel spreadsheets. Standard deviations and relative standard deviations were calculated and reported in Microsoft® Office Excel spreadsheets.

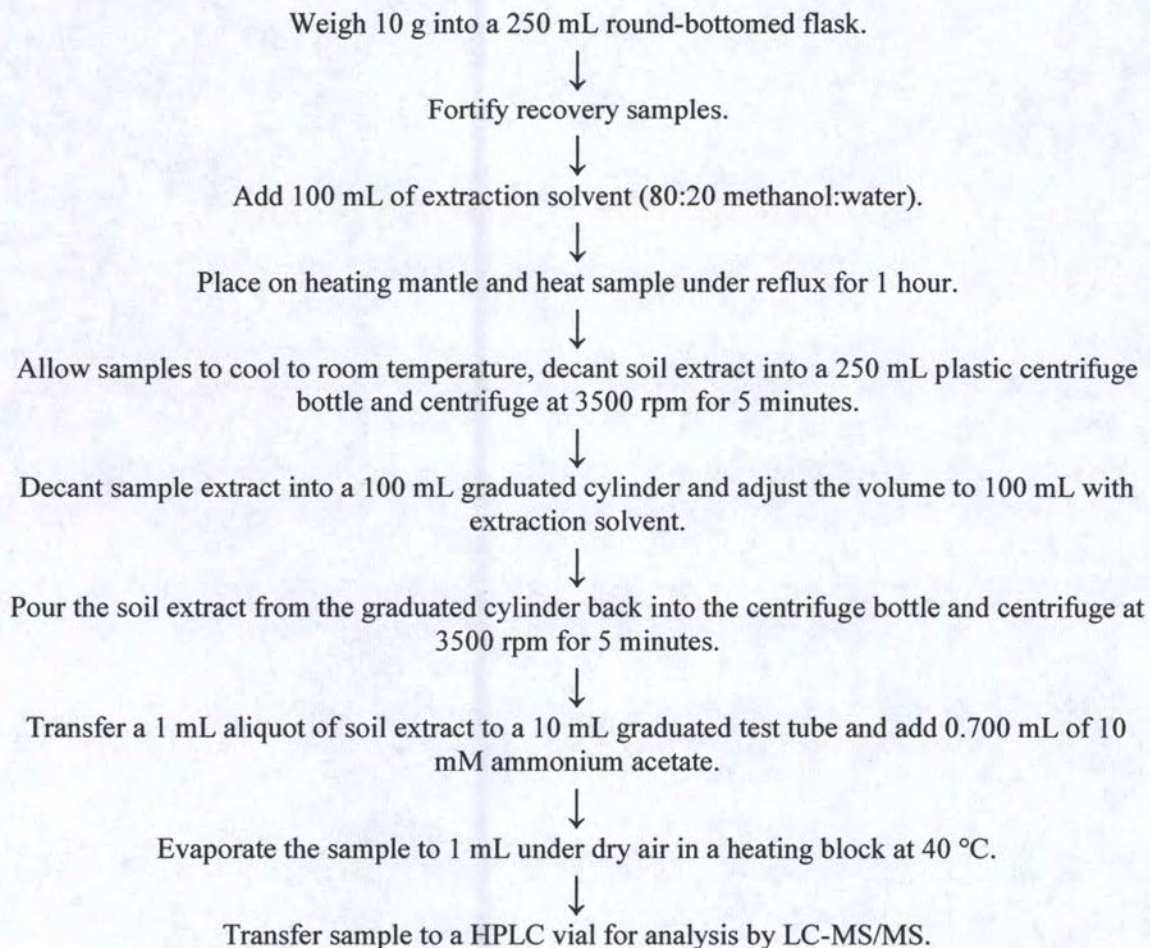
5.0

5.1 Method Establishment/Pre-Validation Evaluation

Initially, the mass spectrometer was optimized by infusing analyte standards to determine the optimum instrument operation parameters. Using these optimized instrument parameters, the retention times of the analytes, instrument detection limits and response linearity were established by injecting a series of calibration reference standards.

Prior to analysis of actual validation samples, a reagent blank and untreated control samples were analyzed to determine if interferences were present near the retention time of the analytes. The results of these evaluations indicated the selected control samples contained no detectable residues of cyprodinil and its metabolites CGA249287, CGA275535, and CGA321915 and had no peaks which might interfere with targeted analyte responses.

APPENDIX 1 Method Flow Chart



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 (MassHunter B.04.01). 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 for quantitation. LIMS was used to calculate the ppm and percent recovery and presented in Microsoft® Excel. The following equations were used for quantitation:

The following equations are used for residue calculations within MassHunter:

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 (mg) = $\frac{\text{Sample amt. (g)} \times \text{Inj. size } (\mu\text{L})}{\text{Final sample vol. (mL)}} \times \frac{1 \text{ mL}}{1000 \mu\text{L}} \times \frac{1000 \text{ mg}}{1 \text{ g}}$

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

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

As an example, calculations to obtain the percent recovery in control clay loam sample from WO-14062405 fortified with cyprodinil in lab code 14062405-Recovery1-1. The calculations are shown below:

a) Calibration curve: $y = (2896687.713711) * x - 1634.924959$

Solving for x : $x = \frac{21108 + 1634.924959}{2896687.713711} = 0.007851 \text{ ng}$

e) Amount of sample injected (mg) = $\frac{10.03 \text{ g} \times 10 \mu\text{L}}{100 \text{ mL}} \times \frac{1 \text{ mL}}{1000 \mu\text{L}} \times \frac{1000 \text{ mg}}{1 \text{ g}} = 1.003 \text{ mg}$

b) Analyte concentration (ppm) = $\frac{0.007851 \text{ ng}}{1.003 \text{ mg}} = 0.007828 \text{ ppm}$

Average residue found in the untreated sample (lab code: 140624001-001B and 140624001-001C) = 0.000000 ppm

c) Percent recovery = $\left(\frac{0.007828 \text{ ppm} - 0.000000 \text{ ppm}}{0.010 \text{ ppm}} \right) \times 100 = 78.3 \%$