1.0 Introduction and Summary

1.1 Scope and Chemical Structures

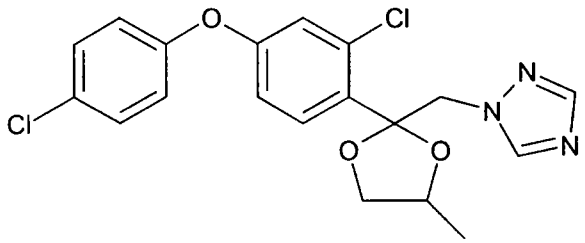
The method described herein is suitable for the determination of residues of difenoconazole and its metabolites, CGA-205375, CGA-142856 and CGA-71019 in soil. This method combines and modifies the existing Syngenta Methods RAM 435/01 and RAM 448/01 (References 1 and 2). The method LOQ has been established at 1.0 ppb (ng/g). Chemical structures and CAS information for the method analytes are shown below.

Name/Synonym: Difenoconazole

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

CAS Number: 119446-68-3

Structure:

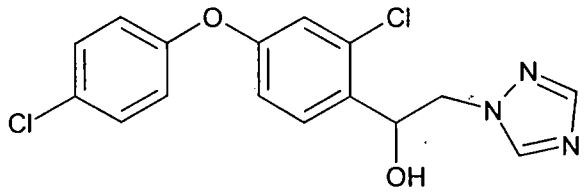


Name/Synonym: CGA-205375

IUPAC Name: 1-[2-chloro-4-(4-chloro-phenoxy)-phenyl]-2-[1,2,4]triazaol-1-yl-ethanol

CAS Number: 117018-19-6

Structure:

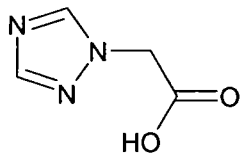


Name/Synonym: CGA-142856

IUPAC Name: Benzoic acid, 4-(methylsulfonyl)-2-nitro-

CAS Number: 110964-79-9

Structure:

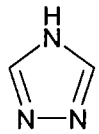


Name/Synonym: CGA-71019

IUPAC Name: 1,2,4 triazole

CAS Number: 288-88-0

Structure:



1.2 Method Summary

A 10.0 g sub sample of soil is extracted by reflux with acetonitrile/0.3% formic solution (70:30, v/v) for one hour. For difenoconazole and CGA-205375 analysis, an aliquot of the centrifuged soil extract is taken and diluted with water. It is analyzed by high performance liquid chromatography with triple quadrupole mass spectrometry detection (LC/MS/MS) using reverse phase.

For CGA-71019 analysis, a second aliquot of the centrifuged extract is taken and reacted with dansyl chloride to form the dansyl triazole derivative. The dansyl triazole (DT) is partitioned into dichloromethane, the dichloromethane evaporated to dryness and the sample re-dissolved in acetonitrile/water solution. It is analyzed by reverse phase LC/MS/MS.

For CGA-142856, a third aliquot of the extract is subjected to a cation exchange resin concentration procedure. The column eluate containing CGA-142856 is rotary evaporated to dryness and re-dissolved in acetonitrile/0.3% formic acid solution. It is analyzed using normal phase LC/MS/MS.

The limit of quantification (LOQ) of the method is 1.00 ng/g (ppb).

2.0 Materials and Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.1 Reagents and Analytical Standards

All solvents and other 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. See Appendix 2 for a list of reagents, solutions and analytical standards for the method.

2.2 Preparation of Analytical Stock Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear approved eye protection, gloves and a laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area(s) immediately.

These are suggested concentrations and preparation procedures. Different schemes may be used to prepare different and/or additional standard solutions.

Accurately weigh with a five-figure balance approximately 10.0 mg each of difenoconazole, CGA-205375, CGA-71019 and CGA-142856 analytical standard into 10.0 mL volumetric flasks. Fill the volumetric to the calibration mark with acetonitrile/water solution (1:1, v/v). Mix. This gives four stock standards each of a nominal 1000 ppm. Calculate the exact concentration of the stocks using the following formula.

$$C\,(ppm) = \frac{Wt.\,(mg) \times P \times 1000}{V\,(mL)}$$

Where "V" is the volume of diluting solvent; "Wt." is the weight, in mg, of the solid analytical standard; "P" is the purity, in decimal form, of the analytical standard; and 1000 is conversion factor from ppm to ppb.

Prepare an exact 10.0 ppm mixed fortification solution containing the 4 analytes. Use the following formula to determine the required delivery volume of each stock standard.

$$V\,(mL) = \frac{10.0\,ppm \times 10.0\,mL}{C_{stock}\,(ppm)}$$

Use an adjustable 250 uL or 100 uL Microman pipette and deliver the appropriate volumes to a 10.0 mL volumetric flask. Fill to the calibration mark with acetonitrile. Mix.

Prepare the following solutions from the mixed fortification solution with acetonitrile/water solution (1:1, v/v).

1.00 µg/mL(ppm) - 1.0 mL of the 10.0 ppm fortification solution is diluted with 9.0 mL acetonitrile/water solution (1:1, v/v) to give a 1.00 ppm fortification solution.

100 ng/mL(ppb) - 1.0 mL of the 1.00 ppm fortification solution is diluted with 9.0 mL acetonitrile/water solution (1:1, v/v) to give a 100 ppb fortification solution.

10 ng/mL(ppb) - 1.0 mL of the 100 ppb fortification solution is diluted with 9.0 mL acetonitrile/water solution (1:1, v/v) to give a 10 ppb fortification solution.

When not in use, always store the standard solutions in a refrigerator at about 5°C to prevent decomposition and/or concentration of the standard. An expiration date of one year is recommended for the stock and fortification standards unless additional data is generated that shows a longer storage period.

2.3 Preparation of Cation Resin and Cation Exchange Columns for CGA-142856 Analysis

Cation Resin Preparation Procedure:

Add 500 mL high purity water to approximately 200 g of Bio-Rad AG 50W-X4 (200-400 mesh size) resin in a 1 L conical flask and swirl gently to mix. Allow the resin to settle and decant the water. Add a further 500 mL ultra pure water and 1.0 mL of concentrated formic acid and swirl gently to mix. Cover or stopper the flask and leave to equilibrate overnight.

Cation Exchange Column Preparation Procedure:

Place a 10 mL (16mm ID x 80 mm) polypropylene column onto a suitable vacuum manifold (e.g. Supelco Visiprep®). Tightly pack the bottom of the column with a double layer formed by two 21 mm glass fiber filter papers (Whatman 934AH). It is helpful to have a 14 mm piston that fits within the column to help fit the papers tightly and securely. Keep the manifold tap closed and use a large bore 5 mL pipette to transfer small volumes of the resin slurry to the column. Attain a resin bed height of 2.5 cm (5 mL). Allow the resin to settle and adjust the height as required. Open the manifold tap and allow the water from the slurry to drain **under gravity** until the level is approximately 1-2 mm above the resin bed surface. Keep the flow rate at approximately 2 mL min^{-1}. Close the manifold tap. Do not allow the resin bed to go dry. Add 10 mL water to the column. Drain through the resin bed **under gravity** discarding the wash. Keep the flow rate at approximately 2 mL min^{-1}. Stop when the level is approximately 1-2 mm above the resin bed surface. Add 10 mL acetonitrile/2%

formic acid in water 70:30 (v/v) solution and drain through the resin bed **under gravity**. Keep the flow rate at approximately 2 mL min^{-1}. Discard the wash. Stop when the level is approximately 1-2 mm above the resin bed. Close the manifold taps. The cation exchange column is now ready to use.

2.4 Safety Precautions and Hazards

Whereas most of the chemicals in this method have not been completely characterized, general laboratory safety precautions are advised (e.g., safety glasses, gloves, etc.). The user(s) should consult the relevant MSDS for commonly used reagents and materials.

Solvent Hazards

	Acetonitrile	Acetic Acid	Dichloro-methane	Acetone	Formic acid
Harmful Vapour	✓	✓	✓	✓	✓
Highly Flammable	✓	×	×	✓	×
Harmful by Skin Absorption	✓	✓	✓	×	✓
OES Short Term (mg m^{-3})	**105**	**37**	**870 (MEL)**	**3560**	-

In all cases avoid breathing vapors. Avoid contact with eyes and skin.

Difenoconazole and CGA-71019 have been assigned a Syngenta toxicity classification of 4. At present there is insufficient data available to assign a Syngenta toxicity classification for CGA-205375. It should be treated as a class 3 compound until further information indicates otherwise. The toxicity classification scale rates highly toxic chemicals as class 1 and non toxic chemicals as class 5.

Dansyl chloride has been assigned a Syngenta toxicity classification of 3. It causes burns therefore suitable eye and skin protection should be used.

3.0 Analytical Procedure

Note: Due to the low detection limit of the method it is important that precautions be taken to avoid cross contamination in the laboratory.
Specifically:

- Where possible disposable glassware/plastic-ware has been specified, clean glassware/plastic-ware should be used for each batch of samples.
- High purity distilled-in-glass pesticide grade solvents should be used.

- Existing glassware should be washed and solvent (acetone or methanol) rinsed, before use in the method and between batches of samples

3.1 Sample Preparation

It is important that a homogeneous soil sample be available for analysis. All samples should be prepared using an approved method of preparation for residue analysis prior to analysis. (References 3, 4 and 5).

3.2 Extraction

a) Weigh a representative amount of soil (10.0 g) into a round-bottomed flask (250 mL size). At least one untreated control and two control samples fortified with known amounts of difenoconazole, CGA-205375, CGA-71019 and CGA-142856 in acetonitrile/water solution 1:1 (v/v) should be analyzed alongside each batch of samples to demonstrate acceptable performance of the method.

b) Add 100 mL acetonitrile/0.3% formic acid in water 70:30 (v/v) to the sample and place the round-bottomed flask on a heating mantle and heat the sample under reflux for 1 hour. Allow the sample to cool to room temperature. Pour 45 mL of the sample into a 50 mL polypropylene centrifuge tube. Discard the remainder. This solution will be cloudy and so it is necessary to centrifuge it. Centrifuge the sample at a speed that separates the particulate matter from the liquid e.g. 3500 rpm for five minutes. Store cool.

3.3 Difenoconazole and CGA-205375 Analysis

a) Transfer an aliquot of the soil extract from Section 3.2 (b) equivalent to 0.050 g soil (0.50 mL) into an autosampler vial. Add water (0.50 mL) to the sample, cap the vial and vortex for a few seconds to mix the sample. The sample is ready for LC/MS/MS analysis.

3.4 CGA-71019 Analysis

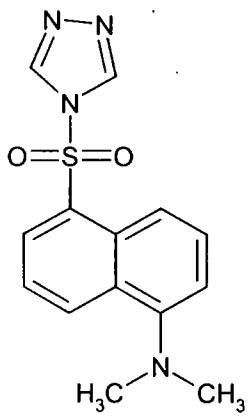
a) Transfer an aliquot of the soil extract from Section 3.2 (b) equivalent to 0.1 g soil (1.0 mL) into a screw capped glass test tube (15 mL size). Add 1 mL of 0.1 M sodium bicarbonate solution, 20 µL of 10% ammonium hydroxide, 100 µL of 10% EDTA and 100 µL of 50 mM dansyl chloride in acetone solution to the sample. Cap the tube and vortex for a few seconds to mix.
Note: The dansyl chloride solution should be prepared weekly and stored in an amber bottle in a refrigerator when not in use. Dansyl chloride neat material is prone to degradation and is moisture sensitive. It is to be kept cold and in a dessicator.

b) Place the vial in a heating block at 40°C for 30 minutes. Do not expose samples to direct light during this procedure. Cover the samples with aluminum foil during the derivatization process.

c) After 30 minutes, remove the samples from the heating block and cool for 10 minutes. Add 2 mL of dichloromethane to the sample, cap the vial and vortex for 30 seconds. Add 5 mL of water. Centrifuge at 1000 ppm for 1 minute to cleanly separate the phases. Carefully pipette the lower dichloromethane layer containing the dansyl triazole into a test tube (4 mL size). Evaporate the dichloromethane to dryness under a stream of clean, dry nitrogen.

d) Re-dissolve the sample in 1.0 mL pH 11 water/acetonitrile 60:40 (v/v) and ultrasonicate. Transfer the sample to an autosampler vial ready for LC/MS/MS analysis.

Dansyl Triazole Derivative



Note: Due to the limited stability of the dansyl triazole derivative, samples need to be kept stored deep-frozen if not immediately analyzed. Stored samples should be removed from the freezer just prior to analysis, thawed and well mixed. The LC/MS/MS should have an autosampler equipped with a chiller. Maintain the sample tray between 3 to 7°C.

3.5 CGA-142856 Analysis

a) Transfer an aliquot of the soil extract from Section 3.2 (b) equivalent to 2.0 g soil (20 mL) into a disposable 40 mL screw capped test tube and acidify with 400 µL concentrated formic acid. Cap the tube and invert it several times to mix it thoroughly.

b) Transfer the sample to a cation exchange column (Section 2.3) attached to a vacuum manifold and allow it to drain through the bed under gravity. Adjust the flow to approximately 2 mL min^{-1}. Discard the column eluate.

Stop when the level is approximately 1-2 mm above the resin bed surface. Close the manifold taps.

c) Rinse the tube with 5 mL water and add to the cation exchange column. Allow the water to drain through the bed under gravity. Adjust the flow to approximately 2 mL min^{-1}. Discard the wash. Stop when the level is approximately 1-2 mm above the resin bed surface.

d) Remove the cartridge from the vacuum manifold and insert it into the neck of a 125 mL round bottom flask with a 24/40 ground glass joint. Add 20 mL methanol/conc. ammonia 75:25 (v/v) to the column. Allow the solvent to drain through the bed under gravity eluting the CGA-142856. Remove the cation exchange column from the neck of the 125 mL flask.

e) Rotary evaporate the sample to dryness under reduced pressure with a water bath temperature of 35-40°C.

f) Re-dissolve the sample in 4.0 mL acetonitrile/0.3% formic acid in water 50:50 (v/v). Ultrasonicate.

g) Transfer 1.0 mL of sample to an autosampler vial ready for final determination by LC/MS/MS. Store cool.

3.6 Time Required for Analysis

The methodology is normally performed with a batch of 15 samples. A method flow diagram is shown in Figure 1. One person can complete the reflux of 15 samples in 1 hour. Preparation of the difenoconazole and CGA-205375 extracts takes about 1 hour. Preparation of the dansyl derivative extracts of CGA-71019 takes about 4 hours. Preparation of the CGA-142856 extracts takes about 5 hours. The total time required for preparation of 15 samples is 11 working hours. Instrumental analysis requires three separate runs. A single set of extracts can be run in about 4 hours. Total run time is about 12 hours.

3.7 Method Stopping Points

The analytical procedure can be stopped after the soil extraction step (section 3.2). The preparation of the cleaned up extracts of each analyte should be completed entirely without stopping. (Section 3.3, 3.4 or 3.5). The raw soil extract of Section 3.2 should be stored in sealed container and refrigerated. Final extracts suitable for analysis can be stored indefinitely as long as they kept deep-frozen (< -10° C). Acceptable recoveries (70-120%) of procedural fortification(s) prepared together with an analytical set validate the extract storage.

3.8 Preparation of Calibration Standards for LC/MS/MS

A minimum of five standard levels is recommended for generation of the external calibration curves. Solutions of analytical standards are interspersed with the samples to form a sequence of analyses. The first and the last injections used in an analytical set must be standards. The smallest standard within a set will determine the limit of detection (LOD) for the set. The smallest standard generally corresponds to about 50% of the limit of quantitation (LOQ) of the analytical method.

The MS/MS response of the samples should fall within the limits of the standard curve. One exception would be for control samples containing residues less than the method LOQ. Any samples with residues less than the method LOQ are typically reported as <1.0 ppb. See section 5.0 of this report for details regarding calculation of results.

The calibration standards should be stored in glass and refrigerated. An expiration date of six months is recommended unless additional data are generated that show a longer expiration date. The expiration date may be extended to a maximum of one year.

3.8.1 Difenoconazole and CGA-205375

LC/MS/MS calibration standards should be prepared at suitable concentrations in acetonitrile/water solution (1:1, v/v). For example, to prepare a 10.0 ppb calibration standard, transfer 1.0 mL of a 100 ppb difenoconazole and CGA-205375 mixed standard to a volumetric flask (10.0 mL) and dilute to 10.0 mL volume with acetonitrile/water solution 1:1 (v/v).

3.8.2 CGA-71019

A 100 ppb stock dansyl triazole LC/MS/MS calibration standard should be derivatized alongside each analysis batch. Prepare the 100 ppb dansyl triazole calibration standard by transferring 100 µL of a 1.00 ppm CGA-71019 standard to a 15 mL screw capped tube. Add 1.0 mL of acetonitrile/0.3% formic (70:30, v/v) and derivatize according to the procedure of Section 3.4 (b to d). Prepare suitable calibration standards from the derivatized stock by serial dilutions in pH 11 water/acetonitrile 60:40 (v/v).

3.8.3 CGA-142856

Standards suitable for external calibration should be prepared in acetonitrile/0.3% formic acid in water 50:50 (v/v). For example, to prepare a 10.0 ppb calibration standard, transfer 1.0 mL of a 100 ppb of mixed standard to a volumetric flask (10.0 mL) and dilute to 10.0 mL volume with acetonitrile/0.3% formic acid in water 50:50 (v/v).

4.0 Final Determination by LC/MS/MS

Three different LC systems are recommended for the analysis of difenoconazole, CGA-205375, CGA-71019 and CGA-142856. Difenoconazole and CGA-205375 are analyzed together. The metabolites CGA-71019 (triazole) and CGA-205375 (triazole acetic acid) are analyzed using separate systems.

The difenoconazole and CGA-205375 utilize a reverse phase silica based LC column. A formic acid mobile phase is required to achieve good sensitivity.

The dansyl derivative of CGA-71019 also utilizes a reverse phase silica based LC column. An acetic acid mobile phase is required. Substitution of the acetic mobile phase with a formic mobile phase significantly reduces the dansyl derivative sensitivity.

CGA-142856 is analyzed by normal phase chromatography on a pentafluorophenyl phase with a propyl spacer (pPFP). The column is highly retentive for basic analytes. CGA-42856 elution can be readily increased or decreased by small changes (~ 1%) of the aqueous percentage. The mobile phase linear gradient needs to be adjusted to give suitable retention and peak shape on the pPFP column. An aqueous composition somewhere in the range of 10% is generally suitable for elution of the analyte.

Difenoconazole, CGA-205375 and the dansyl derivative of CGA-71019 analytes are determined by positive ion electrospray MS/MS. CGA-142856 (triazole acetic acid) is determined by negative ion electrospray MS/MS.

The following instrumentation and conditions have been found suitable for this analysis. Other instrumentation can be used, however optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should be consulted to ensure safe and optimum use.

4.1 LC/MS/MS System Description and Operating Conditions: System 1

4.1.1 System 1: Difenoconazole and CGA-205375

LC: Instrumentation: CTC HTS PAL Autosampler
Perkin Elmer Series 200 Micropumps

Column	:	Aquasil C18 3.0 x 150 mm,3 um
Column Oven Temperature	:	40°C
Flow rate	:	0.500 mL min^{-1}
Injection volume	:	25 µL
Stop Time	:	10 minutes
Mobile phase	:	Solvent A = 0.2% formic acid in water (v/v) Solvent B = Acetonitrile

Mobile Phase Program (linear)

Time (min.)	% A	% B
0	50	50
1.0	50	50
4.0	10	90
7.0	10	90
7.1	50	50
10	50	50

Typical Analyte LC Retention Times:

Analyte	Approx. Retention time, min.
Difenoconazole	6.0 (doublet)
CGA-205375	4.9

Note: To help minimize ion source contamination, it is recommended that a timed event controlled switching valve be used to divert the LC stream to waste during periods of no data collection (e.g., from injection to 4.0 minutes and 7.0 minutes to run completion).

Typical chromatograms are shown in Figures 3, 4, 5 and 6.

4.1.2 Mass Spectrometer System Description and Operating Conditions

Applied Biosystems Sciex API 4000 LC/MS/MS triple quadrupole mass spectrometer.
Sciex Turbo Ion Spray (TIS) sample introduction unit for the API 4000 mass spectrometer.
Computer: Dell Computer Precision Workstation 360 x86- based PC
Software: OS Microsoft Windows 2000 Professional Version 5.0.2195
Applied Biosystems Analyst 4.1

General Operating Conditions

Interface	:	Turbo Spray	
Polarity	:	Positive	
GS1 (NEB)	:	Air set at 60 (arbitrary units)	
GS2 (AUX)	:	Air set at 60 (arbitrary units)	
Curtain gas (CUR)	:	Nitrogen set at 10 (arbitrary units)	
Temperature (TEM)	:	600°C	
Ionspray voltage	:	5500 V	
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)	
Scan type	:	MRM	
		Difenoconazole	CGA-205375
Q1 mass	:	406.0	350.1
Q3 mass	:	251.1	69.9
Dwell time	:	100 ms	100ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	116 V	81 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	37 V	45 V
Collision cell exit potential (CXP)	:	8 V	6 V
Electron multiplier setting (CEM)	:	2300 V	2300 V

4.2 LC/MS/MS System Description and Operating Conditions: System 2

4.2.1 System 2: CGA-71019 (triazole) as Dansyl Derivative

LC: Instrumentation: CTC HTS PAL Autosampler
Perkin Elmer Series 200 Micropumps

Column	:	Aquasil C18 3.0 x 150 mm,3 um
Column Oven Temperature	:	40°C
Flow rate	:	0.500 mL min^{-1}
Injection volume	:	50 µL
Stop Time	:	10 minutes
Mobile phase	:	Solvent A = 0.2% acetic acid in water (v/v) Solvent B = Acetonitrile

Mobile Phase Program (linear)

Time (min.)	% A	% B
0	60	40
1.0	60	40
4.0	10	90
8.0	10	90
8.1	60	40
10	60	40

Typical Analyte LC Retention Times:

Analyte	Approx. Retention time, min.
Dansyl triazole	5.3

Note: To help minimize ion source contamination, it is recommended that a timed event controlled switching valve be used to divert the LC stream to waste during periods of no data collection (e.g., from injection to 4.0 minutes and 8.0 minutes to run completion).

Typical chromatograms are shown in Figures 7 and 8.

4.2.2 Mass Spectrometer System Description and Operating Conditions

Applied Biosystems Sciex API 4000 LC/MS/MS triple quadrupole mass spectrometer.
Sciex Turbo Ion Spray (TIS) sample introduction unit for the API 4000 mass spectrometer.
Computer: Dell Computer Precision Workstation 360 x86- based PC
Software: OS Microsoft Windows 2000 Professional Version 5.0.2195
Applied Biosystems Analyst 4.1

General Operating Conditions

Interface	:	Turbo Spray
Polarity	:	Positive
GS1 (NEB)	:	Air set at 60 (arbitrary units)
GS2 (AUX)	:	Air set at 60 (arbitrary units)
Curtain gas (CUR)	:	Nitrogen set at 6 (arbitrary units)
Temperature (TEM)	:	600°C
Ionspray voltage	:	5500 V
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)
Scan type	:	MRM
		Dansyl triazole
Q1 mass	:	303.0
Q3 mass	:	181.00
Dwell time	:	250 ms
Resolution Q1	:	Unit
Resolution Q3	:	Unit
Declustering potential (DP)	:	76 V
Entrance potential (EP)	:	10 V
Collision energy (CE)	:	39 V
Collision cell exit potential (CXP)	:	12 V
Electron multiplier setting (CEM)	:	2300 V

4.3 LC/MS/MS System Description and Operating Conditions: System 3

4.3.1 System 3: CGA-142856 (triazole acetic acid)

LC: Instrumentation: CTC HTS PAL Autosampler,
Perkin Elmer Series 200 Micropumps

Column	:	Allure PFP Propyl 3.2 x 250, 5um
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	10 minutes
Mobile phase	:	Solvent A = 20% 5 mM AmAc at pH 4.5 in ACN Solvent B = ACN

Mobile Phase Program (linear)

Time (min.)	% A	% B	Flow (mL min^{-1})
0	0	100	1.0
2.0	0	100	1.0
6.0	100	0	0.8
8.0	100	0	0.8
10.0	0	100	1.0

Typical Analyte LC Retention Times:

Analyte	Approx. Retention time, min.
CGA-142856	4.2

It is recommended to equilibrate the Allure pPFP column prior to use. Condition with 100% acetonitrile at 1.0 mL min^{-1} for 1 hour, followed by 20% 5 mM AmAc at pH 4.5 in acetonitrile at 0.5 mL min^{-1} for 1 hour, followed by 5% AmAc at pH 4.5 in acetonitrile at 1.0 mL min^{-1} for 1 hour. Monitor the column pressure.

The column backpressure may increase significantly after 80-100 runs. Reverse the column and rinse with 50:50 Acetonitrile water for 2 to 4 hours to remove salts that can precipitate on the head of the column. Flushing will restore the column function.

The retention time of CGA-142856 will vary between 3.5 to 6 minutes on the column depending on the aqueous percentage. Optimize the chromatography to obtain the best possible peak shape.

Note: To help minimize ion source contamination, it is recommended that a timed event controlled switching valve be used to divert the LC stream to waste during periods of no data collection (e.g., from injection to 1.0 minutes and 6.0 minutes to run completion).

Typical chromatograms are shown in Figure 9 and 10.

4.3.2 Mass Spectrometer System Description and Operating Conditions

Applied Biosystems Sciex API 4000 LC/MS/MS triple quadrupole mass spectrometer.
Sciex Turbo Ion Spray (TIS) sample introduction unit for the API 4000 mass spectrometer.
Computer: Dell Computer Precision Workstation 360 x86- based PC
Software: OS Microsoft Windows 2000 Professional Version 5.0.2195
Applied Biosystems Analyst 4.1

General Operating Conditions

Interface	:	Turbo Spray
Polarity	:	Negative
GS1 (NEB)	:	Air set at 60 (arbitrary units)
GS2 (AUX)	:	Air set at 60 (arbitrary units)
Curtain gas (CUR)	:	Nitrogen set at 10 (arbitrary units)
Temperature (TEM)	:	600°C
Ionspray voltage	:	-3800 V
Collision gas setting (CAD)	:	Nitrogen set at 9 (arbitrary units)
Scan type	:	MRM
		CGA-142856
Q1 mass	:	125.80
Q3 mass	:	81.9
Dwell time	:	1000 ms
Resolution Q1	:	Unit
Resolution Q3	:	Unit
Declustering potential (DP)	:	-46 V
Entrance potential (EP)	:	-10 V
Collision energy (CE)	:	-14 V
Collision cell exit potential (CXP)	:	-3 V
Electron multiplier setting (CEM)	:	2200 V

4.3.3 MS/MS Ion transitions

Representative MS/MS product ion scans for difenoconazole and its metabolites CGA-205375, CGA-71019 and CGA-142856 are shown in Figure 2. For difenoconazole, the most intense MS/MS transition is from the positive parent ion at m/z 406 → 251. For CGA-205375 the monitored transition is 350 → 70 and for CGA-71019 as the dansyl derivative the monitored transition is 303 → 181. For CGA-142856 the most intense MS/MS transition is from the negative parent ion at m/z 126 → 82.

5.0 Calculation of Results

5.1 Determination of Residues in Samples:

Inject the sample extract from 3.3(a), 3.4 (d) and 3.5 (g) into its analysis system. The sample solution must be diluted if the analyte response exceeds the linear range of the calibration curve. Quantitation is achieved using a linear least squares curve fit to the external standards. Acceptable calibration curve fits include linear, linear forced through zero, or linear weighted 1/x, as appropriate.

5.2 Determination of Residues in Fortified Samples:

Verify the method performance for each set of samples analyzed by including a control sample and two or more control samples fortified with known amounts of difenoconazole, CGA-205375, CGA-71019 and CGA-142856 prior to the extraction procedure. One fortification should be performed at the LOQ and the second fortification level should approximate the expected residue levels in the study samples.

Recovery data are generally considered acceptable when the mean values are between 70% and 120% with a relative standard deviation of ≤20%.

Calculations:

Calculations may be performed with a computer program (preferred) or manually as shown below.

Calculate the analyte concentration (in ppb) for field-incurred residues using the equation:

$$RES(ppb) = \frac{\text{Analyte found (ng)}}{\text{SWI (g)}}$$

where RES is the residue value in ppb (ng/g), analyte found (ng) is calculated from a standard calibration curve, and SWI is the sample weight injected (g).

The amount, in milligrams, of sample weight injected (SWI) can be calculated using the equation:

$$SWI(g) = \frac{FW(g) \times IV(\mu L)}{FV(mL)} \times 1000$$

where FW = final sample weight (g), IV = LC injection volume (µL) and FV = final volume in which sample is dissolved (mL).

The final sample weight (FW) is calculated by the equation:

$$FW(g) = \left[\frac{SWE(g) \times A1(mL)}{EV(mL) + \{SWE(g) \times M(\%)/100\}}\right] \times \left[\frac{A2(mL)}{INV(mL)}\right]$$

where FW = final weight (g), SWE = sample weight extracted (g), A1 = aliquot 1 volume (mL), EV = total extraction solvent volume (mL), M = sample moisture in percent, A2 = aliquot 2 volume (mL), if needed, INV = interim volume (mL) is the total volume from which the 2nd aliquot is taken.

NOTES: Either actual or nominal sample weights may be used in the calculations. All of the calculations performed in this report used the 10.0 g nominal sample weight. For method performance (recovery) samples, the M% (moisture) value is set to zero since

the fortifications are based upon their wet weights. If no sample dilutions are performed, the second term in the equation (i.e., A2/INV) is equal to one.

The recovery factor, expressed as a percentage (R%), is calculated using the following equation.

$$R\% = \frac{\text{RES fortified (ppb)} - \text{RES control (ppb)}}{\text{ppb analyte added}} \times 100$$

To correct a residue value to its dry weight value, the following equation may be used:

$$\text{SDW(ppb)} = \frac{\text{CR (ppb)}}{\frac{(100 - \text{M(\%)})}{100}}$$

where SDW = soil dry weight residue (ppb), CR = corrected soil residue (ppb), and M = soil moisture (%). For study samples, soil moistures should be determined following the appropriate SOP.

6.0 Interferences and Confirmation

Final determination by LC/MS/MS is considered to be highly specific; therefore no confirmatory conditions are included. It is recommended that batches of solvent and/or reagents be checked for potential contamination if residue levels are detected in the method blanks. This method uses disposable lab ware, where possible. All reusable glassware should be detergent washed then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

7.0 Modifications and Potential Problems

7.1 Modifications

This method is a combination of two existing methods of analysis, Syngenta Method RAM 435/01 for the determination of difenoconazole and its metabolites CGA-205375 and CGA-71019 in soil [1] and Syngenta Method RAM 448/01 for the determination of residues of CGA-142856 in soil [2]. The major modifications to the Syngenta methods are:

1. The use of a single extraction of all residues with an acetonitrile/0.3% formic solution (70:30, v/v) reflux in place of two separate extractions, by reflux in RAM 435/01 and by accelerated solvent extraction (ASE) in RAM 448/01.
2. The derivatization conditions for the dansyl triazole in RAM 435/01 were altered to improve the ruggedness of the procedure. Modification to the derivatization procedure include:
   1) Increase of the concentration of the dansyl chloride derivatizing reagent from 5 mM to 50 mM.

2) Addition of a small amount of ammonium hydroxide to the derivatization extract prior to heating to increase its pH.
3) Addition of EDTA to the derivatization extract prior to heating to chelate any divalent cations present. Cations will reduce the reaction efficiency.

3. Simplification of the cleanup methodology for CGA-142856 in RAM 448/01. The Oasis SPE cartridge cleanup step following the cation exchange column was removed.
4. Improvement in the ruggedness of cation exchange procedure. The bed volume was increased from 1 mL to 5 mL (2.5 cm bed in a 16 x 80 mm column). The volume and strength of the elution solvent was increased to 20 mL of 75:25 methanol/conc. ammonia (v/v).
5. The alteration of the chromatography conditions for CGA-142856 in RAM 448/01. The cleaned extracts from the cation exchange column were chromatographed by normal phase chromatography on an Allure pPFP column.

7.2 Potential Problems

1. The preparation of the dansyl triazole derivative can be problematic. Alternate lot numbers of the neat dansyl chloride material may need to be obtained from the supplier if the reaction fails. The neat dansyl chloride is a hygroscopic, light sensitive material and is prone to degradation. It should be stored frozen in the dark in a dessicator. Fresh derivatization solution in acetone should be prepared only as required and discarded within 7 days.
2. CGA-71019 (triazole) is a ubiquitous compound and may be present at significant concentrations in soil, even soils of non-agricultural origin. Soil chosen for controls may need to be analyzed and evaluated for residues prior to use.
3. The capacity of the cation exchange cartridge may need to be increased if the recoveries of CGA-142856 are low or erratic. Increase the length of the resin bed from 2.5 cm (5 mL) to 3 cm (6 mL). Additional elution solvent may be necessary to completely remove the CGA-142856 from the resin.
4. Ensure that the cation exchange column is prepared so that no resin can escape from the bed into the extract. Contaminating resin will appear as an oily film coating the round bottom used for evaporation.
5. Poor sensitivity of the LC/MS/MS system for CGA-142856 may require that the final volume of the CGA-142856 extract be reduced from 4.0 mL (section 3.5 (f)). For instance, if the LC/MS/MS system cannot be optimized to detect a 0.250 ppb calibration standard (Section 8.4) the extract residue from Section 3.5 (f) may be reconstituted in 1.0 mL of acetonitrile/0.3% formic acid in water 50:50 (v/v). For this scenario optimization of a 1.0 ppb LOD standard would be sufficient.
6. The chromatography on the Allure pPFP column may need to be optimized by altering the aqueous percentage of the mobile phase. Reducing the aqueous by 1-2% will retain the CGA-142856 longer. Increasing the aqueous will decrease the retention time.

FIGURES

Figure 1. Method Flow Diagram

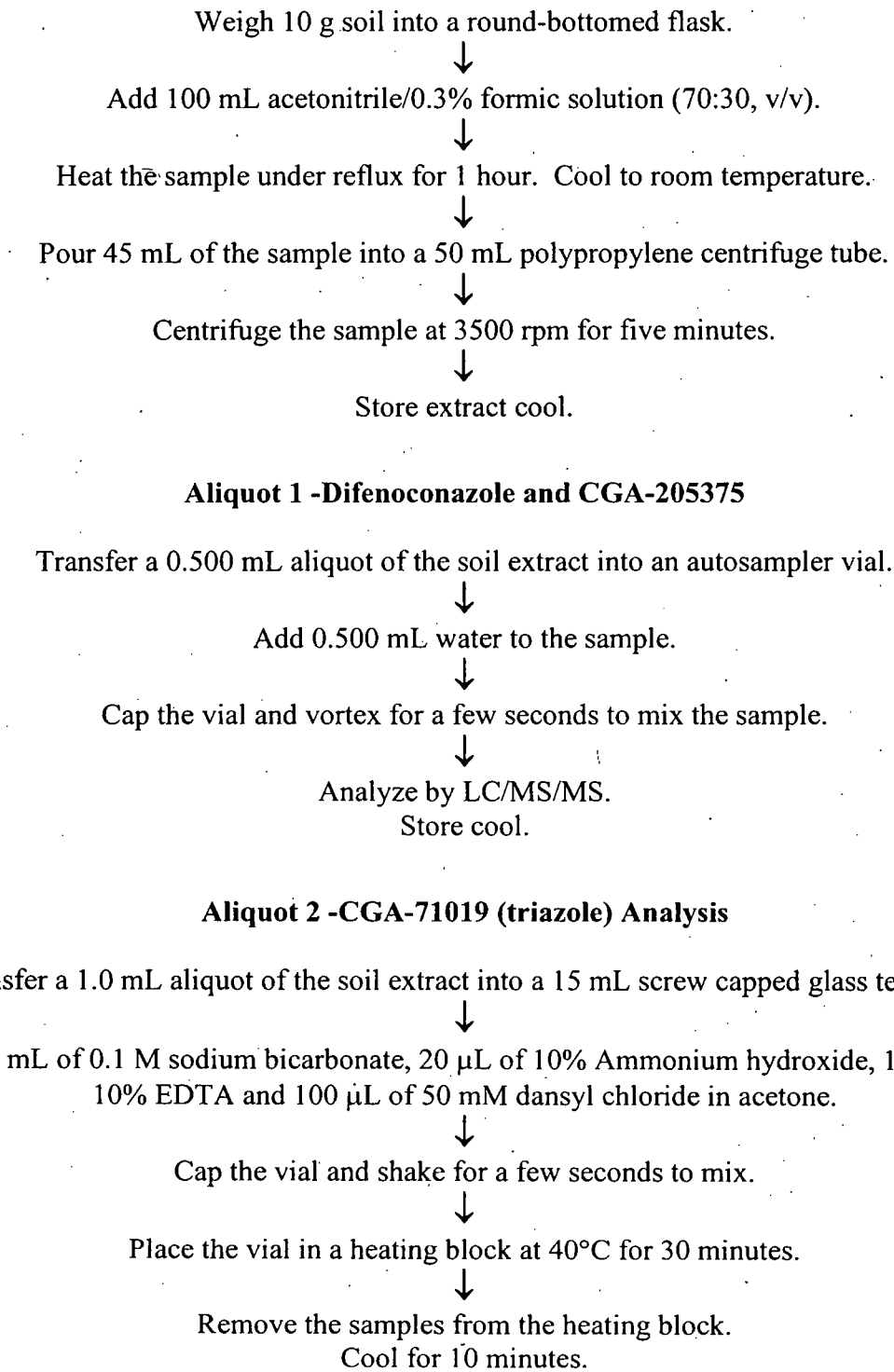


Figure 1. Method Flow Diagram (Continued)

↓

Add 2 mL dichloromethane to the sample, cap and vortex for 30 seconds.

↓

Add 5 mL of water and centrifuge at 1000 rpm for 1 minute or until the phases cleanly separate.

↓

Carefully pipette the lower layer into a 4 mL test tube.

↓

Evaporate the dichloromethane to dryness under a stream of clean, dry nitrogen.

↓

Re-dissolve the sample with 1.0 mL acetonitrile/water (60:40) at pH 11 and ultrasonicate.

↓

Transfer to an autosampler vial ready for LC/MS/MS analysis.
Store deep-frozen.

Aliquot 3 -CGA-142856 (triazole acetic acid) Analysis

Transfer a 20 mL aliquot of the soil extract into a disposable 40 mL screw capped test tube and acidify with 400 µL concentrated formic acid.

↓

Cap the tubes and invert several times to mix.
Transfer the sample to a cation resin exchange column

↓

Draw through under gravity at a rate of approximately 2 mL min^{-1}.

↓

Stop when the level is approximately 1-2 mm above the resin bed surface.
Discard the column eluate.

↓

Rinse the tube with 5 mL water and it to the column.

↓

Draw through the resin bed at a flow rate of approximately 2 mL min^{-1}.

↓

Stop when the level is approximately 1-2 mm above the resin bed surface.
Discard the wash.

↓

Remove the SPE column from the vacuum manifold and place directly into a 125 mL round bottom flask.

↓

Add 20 mL methanol/ concentrated ammonia solution (75:25, v/v) to the column.

Figure 1. Method Flow Diagram (Continued)

↓

Allow to drip by gravity to elute the CGA-142856.

↓

Rotary evaporate the sample to dryness under reduced pressure with water bath temperature at 35-40 °C.

↓

Re-dissolve the sample in 4.00 mL acetonitrile/0.3% formic (50:50). Ultrasonicate.

↓

Transfer 1.0 mL of sample to an autosampler vial for analysis by LC/MS/MS. Store cool.

APPENDICES

Appendix 1. Apparatus

General laboratory glassware (beakers, graduated cylinders, disposable pipettes, pipette bulbs, etc.) available from a general laboratory supply company.

Balance, analytical (Mettler model AG245) or equivalent. Electronic display of 0.01 mg, for weighing neat standard materials.

Balance, laboratory (Mettler model PB4002-S or PJ6000), or equivalent. Electronic display of 0.01 g, for weighing soil samples.

Micropipettes, Microman® 25, 50, 100, 250 and 1000 µL, Gilson, (models M25, M50, M100 and M1000). (These pipettes should be used for sample fortification and standard solution preparations).

Suitable Microman® plastic tips to match, Gilson (Cat. No. CP25, CP50, CP100 and CP1000).

Pipette, Socorex, 0.5 - 5 mL, (model 831).

Suitable Socorex 5.0 mL plastic tips to match.

Class A Volumetric Flasks 10 mL, Kimble Glass Inc., (Cat. No. 28014).

Round-bottom flasks, 250 mL and 125 mL with 24/40 ground glass necks, Pyrex (Cat. No. 4100).

1.0 L Erlenmeyer flask, Pyrex (Cat. No. 5100).

Reflux condensors 24/40, Pyrex, (Cat. No. 2480-300).

Heating Mantle 6 position, Barnstead Electrothermal.

pH meter Fisher Scientific. Accumet Research. AR-15.

Glass fiber filter papers, Whatman 934AH, (Cat. No. 1827-150).

Mixer, Vortex-Genie 2, (Fisher Scientific Cat. No. 12-812) or equivalent.

Centrifuge, Centrific Model 225, (Fisher Scientific Cat. No. 04-978-50), or equivalent.

N-Evap Model 111 nitrogen evaporator (Organomation Associates, Berlin, MA), or equivalent.

Tubes, disposable centrifuge, polypropylene, 50-mL, VWR, Cat. No. 21008-240) or equivalent.

Ultrasonic bath, VWR-Scientific Products, 550-T Aquasonic, or equivalent.

Autosampler vials, amber, 1.5 mL, National Scientific, Inc. (Cat. No. C4013), with caps (Cat No. C4013-A).

Autosampler vials, clear, National Scientific Inc. (Cat no. C4013-1) with caps (Cat No. C4013-A).

10 mL disposable glass test tubes, Fisher Scientific (Cat No. 14-961-29).

15 mL screw capped borosilicate glass tubes with Teflon lined solid phenolic closures, available from Kimble Glass Inc. (Cat. No. 45066A-16125).

40 mL screw capped borosilicate glass tubes with Teflon lined solid phenolic closures, available from Kimble Glass Inc. (Kimax. Cat. No. 45066A-25150).

Poly-prep chromatography columns, 10 mL size (16 mm ID x 80 mm), available from Reservoir Analytical, International. (Cat. No. D6).

Vacuum manifold Supelco Visiprep® 12 position.

Vacuum rotary evaporator (Heidolph, Laborota-4000 coupled with Buchi Vac. (Cat. No. V-500) equipped with thermostatically controlled water bath, from IKA

API 3000 LC/MS/MS system equipped with a Turbo Ion Spray source, available from Applied Biosystems, 850 Lincoln Center, Foster City, CA 94404-1128, USA.

API 4000 LC/MS/MS system equipped with a Turbo Ion Spray source, available from Applied Biosystems, 850 Lincoln Center, Foster City, CA 94404-1128, USA.

CTC HTS PAL autosampler, available from LEAP Technologies Inc., P.O. Box 969, Carrboro, NC 27510 USA.

Perkin-Elmer Series 200 Autosampler, available from Perkin-Elmer Corp., 761 Main Ave. Norwalk, CT 06859-0012, USA.

Perkin-Elmer Series 200 HPLC Micro Pump system, available from Perkin-Elmer Corp. 761 Main Ave. Norwalk, CT 06859-0012, USA.

HPLC column Allure PFP Propyl 5 um 250x3.2 mm available from Restek 110 Benner Circle Bellefonte PA 16823, USA

HPLC column, Aquasil C18 3 um 150 x 3.0 mm available from Thermo Electron Corp. Penn Eagle Industrial Park 320 Rolling Ridge Drive Bellefonte PA 16823, USA.

Appendix 2. Reagents, Solution Preparation and Analytical Standards

Reagents:
All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. All reagents except dansyl chloride are stored at room temperature.

Acetic acid, glacial, EMD Chemical Inc. AnalaR®. (Cat. No. B1001-78), or equivalent.

Acetone, HPLC grade, EMD Chemical Inc. OmniSolv®. (Cat. No. AX0116-1), or equivalent.

Acetonitrile, HPLC grade, EMD Chemical Inc. OmniSolv®. (Cat. No. AX0142-1), or equivalent.

Ammonium acetate, BDH Inc., (Cat. No. B10013), or equivalent.

Ammonium hydroxide, (0.88 specific gravity) Fisher Scientific. (Cat. No. A669-212), or equivalent.

Dansyl chloride, 98% Aldrich (Cat. No. D14,335-9), or equivalent.

Dichloromethane, HPLC grade, EMD Chemical Inc. OmniSolv®. (Cat. No. DX0831-1), or equivalent.

EDTA (ethylenediamine-tetra acetic acid tetrasodium salt dihydrate) certified ACS (EM Science. Cat. No. B10093-34.), or equivalent.

Formic acid, 90%, certified ACS, Fisher Chemicals (Cat. No. A118P-100), or equivalent.

Mass Spectrometer Standard Kit (PPGs) PE Applied Biosystems, Part No. 410936

Methanol, HPLC grade, EMD Chemical Inc. OmniSolv®. (Cat. No. MX0488-1.), or equivalent.

Sodium Bicarbonate, certified ACS (Fisher Chemicals. Cat. No. BP328-500.), or equivalent.

Strong cation exchange resin AG 50W-X4, 200-400 mesh size, Bio-Rad Laboratories, (Cat. No. 142-1351).

Water, ultra pure or HPLC grade (EMD Chemical Inc. OmniSolv®. Cat. No. WX0004-1.) or purified in-house with a Millipore® purification system or equivalent.

Preparation of Solutions:

A. Acetonitrile/0.3% formic acid in water (70:30 v/v) - Add 3.6 mL of formic acid and 1200 mL of de-ionized water to 2800 mL of acetonitrile. Mix well.

B. 0.1M sodium bicarbonate – Stir and dissolve 8.4 g of sodium bicarbonate in 1000 mL of de-ionized water.

C. 50 mM dansyl chloride in acetone - Stir and dissolve 133 mg of dansyl chloride in 10.0 mL acetone. PREPARE WEEKLY/EXPIRES WEEKLY.

D. 10% NH_4OH - SLOWLY stir 10 mL of conc. Ammonium hydroxide into 90 mL of de-ionized water.

E. 10% EDTA – Stir and dissolve 90 g of ethylenediamine-tetra acetic acid tetrasodium salt dihydrate (EDTA) in 900 mL of de-ionized water.

F. pH 11 water/acetonitrile 60:40 (v/v) - Mix 400 mL of acetonitrile with 600 mL of de-ionized water. Add conc. ammonium hydroxide (approx. 5 mL), adjusting the pH to 11. Verify with pH meter.

G. Acetonitrile/2% formic acid in water 70:30 (v/v) - Add 24 mL of formic acid and 1176 mL of bottled water to 2800 mL of acetonitrile. Mix well.

H. Methanol/conc. ammonia 75:25 (v/v) - SLOWLY stir 750 mL of conc. Ammonium hydroxide into 2250 mL of methanol

I. Acetonitrile/0.3% formic acid in water 50:50 (v/v) - Add 3.0 mL of formic acid and 1000 mL of de-ionized water to 1000 mL of acetonitrile. Mix well.

J. Cation exchange resin - Add 500 mL of de-ionized water to approx. 200 g of Bio-Rad AG 500W-X4 (200-400 mesh size) resin in a 1 L conical flask and swirl gently. Allow the resin to settle and decant the water. Add another 500 mL of bottled water followed by 1 mL of conc. formic acid. Swirl gently to mix. Cover and equilibrate overnight. Expires in 1 week.

K. 1000 mM Ammonium acetate (AmAc) - Stir and dissolve 77.1 g ammonium acetate into 1000 mL of HPLC grade water.

L. 5 mM AmAc at pH 4.5 - Add 5 mL of 1000 mM AmAc to 1000 mL HPLC grade water. Mix. Stir and adjust to pH 4.5 with ammonium hydroxide.

M. 5% ammonium hydroxide in methanol - Stir 50 mL of conc. ammonium hydroxide into 1000 mL of methanol. Store in a squirt bottle.

Preparation of Mobile Phases:

A. 0.2 % formic acid in water (v/v) - Add 2.0 mL formic acid (90%) to 1000 mL HPLC grade water. Mix. Degas.

B. 0.2% acetic acid in water (v/v) - Add 2.0 mL glacial acetic acid to 1000 mL HPLC grade water. Degas.

C. 10% 5mM AmAc at pH 4.5 in acetonitrile (v/v) - Add 100 mL of 5 mM AmAc at pH 4.5 to 900 mL acetonitrile. Mix. Degas.

D. 20% 5mM AmAc at pH 4.5 in acetonitrile (v/v) - Add 200 mL of 5 mM AmAc at pH 4.5 to 800 mL acetonitrile. Mix. Degas.

Analytical Standards:

Solid analytical standards are stored in a freezer (temperature < -10°C) unless specified otherwise on the sample shipment paperwork.

Difenoconazole, obtained from Syngenta Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

CGA-205375 obtained from Syngenta Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

CGA-71019, obtained from Syngenta Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

CGA-142856 obtained from Syngenta Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.