I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of propiconazole (Novartis code CGA-64250, Chemical Abstracts Registry (CAS) Number: 60207-90-1,1-[(2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl)methyl]-1H-1,2,4-triazole) and its degradates CGA-217495, CGA-91305, CGA-136735, CGA-118244, CGA-118245, and CGA-71019 in soil and water. The compounds are separated by high performance liquid chromatography (HPLC) and detected by mass spectrometry (LC/MS). A turboionspray atmospheric pressure ionization (API) interface is used to introduce the HPLC eluant into the mass spectrometer. The analytes are detected in the triple quadrupole mode (MS/MS) by passing the positive molecular ion through Q1, inducing fragmentation in Q2, and then monitoring a characteristic product ion in Q3. The chemical structures, chemical names, and Chemical Abstracts Registry numbers of the analytes are presented in Figure 1.

The limit of detection by LC/MS (smallest standard amount injected during the chromatographic run) is 0.25 ng for propiconazole, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735, and 0.375 ng for CGA-71019. The limit of determination (the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) for LC/MS analyses is 5 ppb for all analytes in soil and 0.1 ppb for all analytes in water.

B. Principle

A 20 gram subsample of soil is extracted with 100 mL of 70% (v/v) methanol/water under reflux conditions for one hour. The sample is centrifuged and filtered. A 50-mL aliquot is measured, made basic, and passed through a SAX SPE column to remove matrix interferences (the analytes are not retained). The methanol content is then removed by rotary evaporation, the sample is made acidic, and then passed through a ENV SPE column attached piggy-back style to a SCX SPE column.

(Propiconazole, CGA-217495, CGA-91305, CGA-136735, CGA-118244, and CGA-118245 are retained by the ENV SPE while CGA-71019 is retained by the SCX SPE.) The ENV analyltes are eluted with acetonitrile. The SCX analyte is eluted using 2.5% ammonium hydroxide in 70% methanol/water. The organic content is removed via rotary evaporation. The samples are adjusted to the desired final volume and organic content. LC/MS is used for analysis of the samples. A flow diagram for the soil method is presented in Figure 2.

The water method uses a sample volume of 100 mL. The sample is made acidic and then passed through a ENV SPE column attached piggy-back style to a SCX SPE column. (Propiconazole, CGA-217495, CGA-91305, CGA-136735, CGA-118244, and CGA-118245 are retained by the ENV SPE while CGA-71019 is retained by the SCX SPE.) The ENV analytes are eluted with acetonitrile. The SCX analyte is eluted using 2.5% ammonium hydroxide in 70% methanol/water. The organic content is removed via rotary evaporation. The samples are adjusted to the desired final volume and organic content. LC/MS is used for analysis of the samples. A flow diagram for the water method is presented in Figure 3.

II. MATERIALS AND METHODS

A. Apparatus

1.0 Balance, analytical (Sartorius R160P) or equivalent.

2.0 Beaker, glass, 250-ml (Fisher cat. #02-540K) or equivalent.

3.0 Bottle, amber Boston round, with Polyseal-lined cap (Fisher cat. #05-563-2E) or equivalent.

4.0 Bottle, polyallomer, wide-mouth (Fisher cat. #05-562-19A) or equivalent with cap (Fisher cat. #05-563-1D) or equivalent, appropriate size for centrifugation of soil samples.

5.0 Centrifuge, Sorvall Superspeed RC5-B (DuPont Instruments cat. #55228-9) or equivalent, with 6-place GSA rotor head (DuPont, Sorvall GSA cat. #08136) or equivalent.

6.0 Cylinder, graduated, 100-mL and 50-mL (Fisher cat. #08-551D and 08-551C) or equivalent.

7.0 Extraction column reservoir and adapter, (J. T. Baker #7120-03 and #7122-00, respectively) or equivalent.

8.0 Filter paper, 24-cm prepleated circles, Whatman 114V (Whatman cat. #1214 240) or equivalent.

9.0 Flasks, round bottom, 250-mL and 100-mL(Fisher cat. #10-067E and 10-067C) or equivalent.

10.0 Funnel, filter, 147-mm (Fisher cat. #10-373B) or equivalent.

11.0 Mechanical shaker, orbital (Fisher cat. #15-456-6) or equivalent.

12.0 Mixer, Vortex-Genie 2 (Fisher cat. #12-812) or equivalent.

13.0 Pasteur pipet (Fisher cat. #13-678-7C) or equivalent.

14.0 Pipets, glass, class A certified, assorted volumes. These pipets are used when an exact addition of liquid is required (i.e., final addition of solvent to samples).

15.0 Pipetters, Oxford BenchMate adjustable, 40-200 µL volume range (Fisher cat. #21-231), 200-1000 µL volume range (Fisher cat. #21-229) or equivalent. (Note: These adjustable pipetters may only be used for addition of liquid where an exact volume added is not critical, i.e., addition of acid or base.)

16.0 Rotary evaporator, Buchi (Fisher cat. #09-548-105F) or equivalent.

17.0 Solid phase extraction (SPE) columns: (1) Mega Bond Elut SAX, 1 gram/6 mL capacity/volume (Varian cat. #1225-6013), (2) Mega Bond Elut ENV, 1 gram/6 mL capacity/volume (Varian cat. #1225-5012), and (3) Mega Bond Elut SCX, 1 gram/6 mL capacity/volume (Varian cat. #1225-6011).

18.0 Tube, concentration, 50-mL, with 19/38 ground glass joint (Fisher cat. #05-538-40B) or equivalent, with 24/19 enlarging adapter (Fisher cat. #01-035D) or equivalent.

19.0 Vacuum manifold, (J. T. Baker #Spe-12G column processor) or equivalent.

20.0 Vials, clear or amber, 1.5-mL (Sun Brokers, Inc. cat. #200-002) or equivalent, with Teflon-lined, crimp-top seals (Sun Brokers, Inc. cat. #200-152) or equivalent.

B. Reagents and Analytical Standards

All reagents and polypropylene glycols are stored at room temperature. Solid analytical standards are stored in a freezer (temperature <-10°C).

1.0 Acetic acid, concentrated, HPLC grade (Fisher cat. #A35-500) or equivalent.

2.0 Acetic acid, 0.1%: mix 1.0 mL of conc. acetic acid with 999 ml of purified water.

3.0 Acetic acid, 0.2%: mix 2.0 mL of conc. acetic acid with 999 ml of purified water.

4.0 Acetonitrile, HPLC grade (Fisher cat. #A998-4) or equivalent.

5.0 Acetonitrile, 0.1% in acetic acid: mix 1.0 mL of conc. acetic acid with 999 mL of acetonitrile.

6.0 Ammonium acetate, HPLC grade (Fisher cat. #A639-500) or equivalent.

7.0 Ammonium formate, certified grade (Fisher cat. #A666-500) or equivalent.

8.0 Ammonium hydroxide, ACS grade, (Fisher cat. #A669-500) or equivalent.

9.0 Extraction solvent: 70% methanol in water. Mix 700 mL of methanol and 300 mL of purified water.

10.0 Methanol, HPLC grade (Fisher cat. #A452-4) or equivalent.

11.0 Polypropylene glycol, M.W. 425 (Aldrich cat. #20,230-4).

12.0 Polypropylene glycol, M.W. 1000 (Aldrich cat. #20,232-0).

13.0 Polypropylene glycol, M.W. 2000 (Aldrich cat. #20,233-9).

14.0 PPG tuning solution (for mass calibration of the LC/MS system). Dissolve 0.0014 g PPG 425, 0.0100 g PPG 1000, 0.0400 g PPG 2000, and 0.0126 g of ammonium formate in 50 ml of methanol, 50 ml water, and 0.1 ml of acetonitrile. Mix well. Store refrigerated in an amber bottle.

15.0 Sample diluent (ENV analytes): 30% acetonitrile in water. Mix 300 mL of acetonitrile with 700 mL of purified water.

16.0 Sample diluent (SCX analyte): 25% methanol in water. Mix 250 mL of methanol with 750 mL of purified water.

17.0 SAX SPE basic rinse: Add 2.0 ml of conc. ammonium hydroxide to 1000 mL of the extraction solvent (70% methanol in water).

18.0 SCX and ENV SPE acidic rinse: 0.2% acetic acid.

19.0 SCX eluting solution: 2.5% ammonium hydroxide in 70% methanol/water. Add 25 mL of conc. ammonium hydroxide to 975 mL of the extraction solvent (70% methanol/water).

20.0 Test analytes tuning solution, 2.5 ng/µL. Mix one volume of a 10 ng/µL mixed solution of analytes in acetonitrile with three volumes of 0.1% acetic acid. Store at refrigerated or frozen temperature.

21.0 Water, HPLC grade, purified in-house with a HYDRO™ purification system or equivalent.

22.0 Zorbax 300 SCX mobile phase 1: 25% MeOH/H2O, 0.1 % acetic acid. Mix 250 mL of methanol, 749 mL of water, and 1.0 mL of concentrated acetic acid.

23.0 Zorbax 300 SCX mobile phase 2: 25% MeOH/H_2O, 20 mM ammonium acetate. Mix 250 mL of methanol, 750 mL of water, and 1.54 g of ammonium acetate.

24.0 Propiconazole (CGA-64250), chemical purity: 90.0%; CGA-217495, chemical purity: 91.6%; CGA-91305, chemical purity: >99.9%, CGA-136735, chemical purity: 99.7%; CGA-118244, chemical purity: 98.5%; CGA-118245, chemical purity: >99.9%; and CGA-71019, chemical purity: >99.9%, Novartis Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

C. Safety and Health

Whereas most of the chemicals used and analyzed for in this method have not been completely characterized, general laboratory safety is advised (e.g., safety glasses, gloves, etc. should be used). The acetic acid and ammonium hydroxide used in this method are caustic and irritants and should be used in a well-ventilated area (i.e., a fume hood).

D. Analytical Procedure

Note: All glassware, including the polyallomer bottles for grass extraction, should be thoroughly cleaned and followed with a rinse of acetonitrile or methanol prior to use. The analysis system is very sensitive and may detect contamination from previous samples if all glassware and extraction bottles are not properly cleaned prior to each use.

1.0 Soil Moisture Determination

1.1 Label and record the actual weight of an appropriate-sized glass beaker or aluminum weighing pan that will be used to determine the soil moisture content.

1.2 Add approximately 10-20 g of soil sample to the beaker or pan. Record the weight of the container plus wet soil.

1.3 Place the sample in an oven set at 100-120°C and let it dry overnight, or 12-16 hours.

1.4 Remove the sample and allow it to cool to room temperature.

1.5 Record the weight of the container plus dry soil.

1.6 Calculate the moisture content using the equation:

$$m = \frac{W_{1.2} - W_{1.5}}{W_{1.2} - W_{1.1}}$$

where m is the moisture content expressed in decimal form (i.e., 0.1 = 10%), $W_{1.1}$ is the weight of the container (from Step 1.1), $W_{1.2}$ is the weight of wet soil plus container (from Step 1.2), and $W_{1.5}$ is the weight of the dry soil plus container (from Step 1.5).

2.0 Soil

(Note: Samples must be homogenized prior to analysis using suitable sample preparation techniques.)

Soil characterization data for the soil used in these validation experiments is presented in Table I.

2.1 Weigh and record 20 ± 0.1 g of soil sample into a 250-mL round bottom flask.

2.2 Sample fortification, if required for this particular sample, is to be done at this time (refer to Section II.K.2.0).

2.3 Add 100 mL of 70% methanol in water. Briefly swirl the contents. Attach to a reflux condenser, chilled with cold water, and heat under reflux for one hour using an electrical heating mantle.

2.4 Remove the heating mantle from the round bottom flask and permit the sample to cool before disconnecting from the reflux condenser. Transfer the sample to a centrifugable polyallomer bottle. Centrifuge the sample at approximately 9,000 RPM for 10 minutes, or at an alternate speed and time if the results are considered satisfactory.

2.5 Decant the sample extract through filter paper into a 50-mL graduated cylinder. Collect 50 mL for subsequent cleanup.

2.6 Precondition a SAX SPE column with approximately 5 mL of methanol, followed by 5 mL of the SAX SPE basic rinse solvent. (Note: Do not allow the SPE column to dry out between rinses.) Discard the eluate.

2.7 Add 100 µl of concentrated ammonium hydroxide to each sample. Place a stopper on the cylinder and mix the contents well.

2.8 Pass the sample at a fast drip rate through the SAX SPE column via the reservoir, collecting the eluate in a 100-mL round bottom flask, or alternatively in a beaker and then transferring the eluate to a 100-mL round bottom flask. (Note: The analytes are non-retained.)

2.9 Rinse the graduated cylinder with approximately 5 mL of the SAX SPE basic rinse solvent, making sure to rinse the walls of the reservoir. Pass through the SPE column. Collect this eluate in the 100-ml round bottom flask.

2.10 Remove the methanol solvent from the samples via rotary evaporation using a water bath temperature of approximately 40°C.

2.11 Add 100 µL of concentrated acetic acid to the sample. Mix. Add purified water, if necessary, to the sample so that the sample volume is approximately 15 mL.

2.12 Pass the sample through a Varian ENV SPE column attached piggy-back style to a Varian SCX SPE. Discard the eluate. (The SPE columns are preconditioned by passing 5 mL of methanol and then 5 mL of 0.2% acetic acid through each column. Add 2-3 mL of 0.2% acetic acid to the lower SCX column just prior to adding the sample to the top ENV column. Do not permit the SCX column to go dry while the top ENV column still contains sample. Add 0.2% acetic acid to lower SCX column, as needed, to prevent it from going dry.)

2.13 Add approximately 5.0 mL of 0.2% acetic acid to the round bottom flask from Step 2.11 and swirl to dissolve any residues still on the glass. Pass through both SPE columns. Discard the eluate.

2.14 Rinse the SPE columns (columns are still attached) with approximately 5 mL of purified water.

2.15 Disconnect the two SPE columns. Rinse the SCX column with approximately 5 mL of 70% methanol/water. Discard the eluate.

ENV Analytes

2.16 Place a precalibrated 50-mL concentration tube containing approximately 2 mL of purified water beneath the ENV SPE column. Elute the analytes (CGA-64250, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735) with 15 mL of acetonitrile. (Note: The concentration tube is calibrated to the desired volume by pipetting, via an accurate pipette, the desired final volume and then marking the meniscus line on the tube using a marker pen.)

2.17 Remove the organic solvent via rotary evaporation with a water bath temperature of approximately 40°C until less than 3 mL of aqueous remains. Add 1.5 mL of acetonitrile and dilute to the 5.0 mL calibration mark using purified water. The sample may be further diluted using 30% acetonitrile/water, if needed. Store the samples in a refrigerator (<5°C)until the time of analysis.

2.18 Analyze the ENV analytes (CGA-64250, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735) by LC/MS

with the reversed phase HPLC system as detailed in Table II.

SCX Analyte (CGA-71019)

2.19 Place a precalibrated 50-mL concentration tube beneath the SCX SPE column. Elute CGA-71019 with 10 mL of 2.5% ammonium hydroxide in 70% methanol/water. (Note: The concentration tube is calibrated to the desired volume by pipetting, via an accurate pipette, the desired final volume and then marking the meniscus line on the tube using a marker pen.)

2.20 Remove the organic solvent via rotary evaporation with a water bath temperature of approximately 40°C until less than 3 mL of aqueous remains. Add approximately 1.25 mL of methanol and dilute to the 5.0 mL calibration mark using purified water. The sample may be further diluted using 25% methanol/water, if needed. Store the samples in a refrigerator (<5°C)until the time of analysis.

2.21 Analyze the CGA-71019 by LC/MS with the cation exchange (SCX) HPLC system as detailed in Table III.

3.0 Water

3.1 Measure 100 mL of a water sample, using a 100-mL graduated cylinder.

3.2 Sample fortification, if required for this particular sample, is to be done at this time (refer to Section II.K.2.0).

3.3 If suspended solids are present in the sample, centrifuge the sample in a centrifugable polyallomer bottle at approximately 9,000 RPM for 10

minutes, or at an alternate speed and time if the results are considered satisfactory. Pass the sample through filter paper and collect in a 100-mL graduated cylinder. Record the volume of the sample. (Note: It is more convenient to centrifuge and filter >100 mL of water samples that do not require fortification. The analyst then measures 100 mL of the filtered sample for sample preparation.)

3.4 Add 200 µL of concentrated acetic acid. Mix with the sample.

3.5 The remainder of the cleanup procedure follows Steps 2.12 through 2.21 of the soil procedure (the SAX SPE cleanup step to remove soil matrix is not used). The final volume of the water samples may need to be reduced to approximately 2.0 mL, depending on instrument sensitivity. The volume of organic solvent added to the final sample should be adjusted to maintain the same ratio of organic/aqueous as in the soil samples.

E. Instrumentation

1.0 Description and Operating Conditions: HPLC

See Table II for a description of the reversed phase HPLC system and chromatographic conditions used for the analysis of the ENV SPE analytes. See Table III for a description of the cation exchange HPLC system and chromatographic conditions used for the analysis of CGA-71019.

2.0 Description and Operating Conditions: LC/MS

Propiconazole, CGA-217495, CGA-91305, CGA-136735, CGA-118244, CGA-118245, and CGA-71019 are monitored as positive ions. Triple quadrupole analysis (MS/MS) of the

unique precurser/product ion pair is suggested, although single quadrupole analysis (MS) utilizing the molecular ion may be performed provided that no interferences are present in the sample matrix. See Table IV for a description of the mass spectrometer instrumentation and operating conditions.

3.0 Description and Operating Conditions: LC/MS Turboionspray Interface

The optimized values for the turboionspray interface may vary with time and may need to be periodically re-optimized. See Table IV for a description of typical turboionspray operating conditions used with the analytes in Analytical Method AG-677.

4.0 Calibration and Standardization: LC/MS

4.1 Calibrate and tune the mass spectrometer prior to analyzing samples. Check the calibration and tune by infusing a standard solution of polypropylene glycol (PPG) into the mass spectrometer using the ionspray interface while monitoring positive ions. Weekly calibrations and tunes with the PPG solution are considered sufficient provided that instrument mass calibration stability is demonstrated for that time interval.

4.2 Determine the specific ion to monitor for each analyte by infusion of an analyte test solution (approx. 2.5 ng/µL in 50% acetonitrile/water, 0.1% acetic acid) while scanning the Q1 quadrupole mass analyzer to find the optimum ion. Determine the specific product ion fragment to monitor for each analyte in the MS/MS mode by passing the characteristic precurser ion through Q1, fragmenting the ion in Q2, and scanning the resulting ion fragments in Q3. The selected

product ion chosen to monitor will depend on the intensity of the ion fragment along with the possibility that an interference also has the same fragment ion. Table IV lists the precurser ion and monitored product ion for each analyte. Typical ionspray MS/MS mass fragmentation spectra are presented in Figure 4 for the analytes.

4.3 Determine the retention time of the analytes by injecting a standard solution into the HPLC. During a series of analyses, the analyte retention time should vary no more than 2% from its mean value, on a daily basis.

4.4 Calibrate the instrument by constructing a calibration curve from detector response (chromatographic peak height or area) and the amount of analyte injected, encompassing a range from 2.5 to 5 ng (100 µl injections), for the ENV analytes, and from 0.375 to 7.5 ng (150 µl) for CGA-71019. The response curve can be constructed manually or, preferably, by generation of a linear regression equation by use of a computer or appropriate calculator. Typical chromatograms of analytical standards are presented in Figures 5-6.

F. Interferences

1.0 There are no known interferences originating from the sample cleanup procedure. However, interferences can originate from impure chemicals, solvents, contaminated glassware, and the HPLC water supply.

G. Confirmatory Techniques

1.0 No confirmatory analysis procedure is included in this method. This method employs highly specific LC/MS or LC/MS/MS for the detection mode, coupled with the

characteristic retention time observed for the analyte on the appropriate HPLC column.

H. Time Required

1.0 A set of twelve water samples, or eight soil samples, can be processed in an eight-hour working day by a chemist familiar with this method.

2.0 Each HPLC analysis requires approximately 25 minutes with the reversed phase column and 8 minutes with the cation exchange column.

I. Modifications and Potential Problems

1.0 Contaminants from chemicals, solvents, glassware, and the HPLC water supply can interfere with the analysis. It is recommended that a reagent blank be run with an analysis set to verify that no interferences are originating from the chemicals and reagents used in this procedure. MS techniques are very sensitive. All glassware should be solvent rinsed before use to prevent inadvertent contamination of control or low level samples.

2.0 Analytical Method AG-677 was validated only for the soil type listed in the final method. Other soil samples from different locations may exhibit binding or interference problems which were not observed with these samples.

3.0 "Bumping" is sometimes observed for soil samples during the solvent removal steps via rotary evaporation. Periodic venting of the vacuum and the use of solvent traps helps minimize inadvertent losses during these steps.

4.0 Ion suppression due to soil matrix has been seen while using LC/MS detection. This effect is minimized by the SAX SPE cleanup procedure which removes the majority of the interfering matrix.

5.0 Analytical standards for CGA-118244, CGA-118245, and CGA-136735 contain up to four stereoisomers for each analyte. The stereoisomers of the three hydroxy degradates can not all be chromatographically resolved from one another. Therefore, the peak area for all of these isomers is summed for quantitative calculations. This represents the "total hydroxy residue". MS/MS can not distinguish between these three positional isomers. Figure 7 shows the distribution, and retention, of the various stereoisomers for each of the hydroxy degradates.

6.0 Two stereoisomers are observed for propiconazole when a C8 or C18 column is used for analysis, as seen by a split peak.

7.0 No analyte stability or solubility problems have been observed when solutions have been prepared and stored as detailed in Section II.J.

8.0 Long-term optimization of the LC/MS signal by infusion of a test mixture of analytes into the system will result in lingering high backgrounds for the molecular ions. While the background signals will decrease with time or cleaning of the orifice plate, it may be severe enough to affect the ability to achieve desired signal to noise ratios for lowest standards. For this reason it is highly recommended that optimizing/calibrating with analytical standards be done with dilute solutions and the optimizing/calibrating time be minimized. It is also recommended after calibrating/optimizing with test analytes, to turn the power off to the electronics, remove the ionspray interface, and thoroughly wipe clean the orifice plate using a lint-free tissue wetted with methanol. Repeat several times.

9.0 This method will work on the PE Sciex API-300, API-365, and API-III+ LC/MS systems using the turboionspray interface.

Additionally, single quadrupole MS detection may be achieved using the PE Sciex single quadrupole systems with the turboionspray interface. The ability of LC/MS systems from other manufacturers to satisfactorily perform the analysis is unknown.

10.0 The ratio of mobile phase A and B for use with the cation exchange (SCX) HPLC column may need to be varied on a column-to-column basis, or if an SCX column of a different manufacturer is used. Increasing the composition of the mobile phase containing ammonium acetate will decrease the retention time of CGA-71019.

11.0 The cation exchange HPLC column may be cleaned of bound components by passing 50% A/B through the column for approximately 20 minutes. The column should then be equilibrated for approximately 30 minutes with the mobile phase composition used for analysis prior to starting a new sample run sequence. It is recommended to perform this cleaning procedure on a frequent basis.

12.0 If poor recoveries of CGA-71019 are observed, it may be due to the SCX SPE having increased retention compared to the cartridges used in this validation. If this is observed, it may be necessary to either increase the volume of SCX eluting solvent used or to increase the percentage of ammonium hydroxide used in the eluting solution.

13.0 Reversed phase columns from other manufacturers may be substituted for the column used in this study provided that the analyst demonstrates acceptable peak shape and sensitivity with the substituted column. The mobile phase gradient may need to be altered if a different column is used.

14.0 CGA-71019 is volatile. Adequate water content must be maintained in the sample during rotary evaporation steps to avoid significant losses of the analyte.

J. Preparation of Standard Solutions

All stock are stored in amber bottles in a freezer (< -10°C) when not in use. Mixed standards may be stored in a freezer or refrigerated (< 5°C). No analyte stability or solubility problems have been observed in the standard solutions used in this study. The mixed standards are used for fortifications and as HPLC standards.

1.0 Prepare individual 100 ng/µl stock solutions for propiconazole, CGA-217495, CGA-91305, CGA-71019, CGA-118244, CGA-118245, and CGA-136735. Weigh approximately 10.0 mg of analyte. Determine the appropriate volume of acetonitrile to add using the equation presented below. The concentration of the analytical standard is corrected for its chemical purity.

$$V\,(\text{ml}) = \frac{w(\text{mg}) \times P}{C\,(\text{ng/ul})} \times 10^3$$

Where V is the volume of acetonitrile needed; W is the weight, in mg, of the solid analytical standard; P is the purity, in decimal form, of the analytical standard; C is the desired concentration of the final solution, in ng/µl; and 10^3 is a conversion factor.

For example:

The volume of solvent required to dilute 9.9 mg of an analyte, of 98.0% purity, to a final concentration of 200 ng/µl is:

$$V\,(\text{ml}) = \frac{9.9\ \text{mg} \times 0.98}{200\ \text{ng/ul}} \times 10^3 = 48.5\ \text{ml}$$

2.0 Fortification standards are prepared by combination of the analyte stock solutions and dilution with acetonitrile and water. Prepare a 10 ng/µL mixed solution by pipetting 5.0 mL of each analyte 100 ng/µL stock solution into a 50-mL volumetric flask and then diluting to the calibration mark

with water. Subsequent dilutions of this solution with 30% acetonitrile/water will depend upon the desired fortification level(s). Fortification standards should be prepared such that no more than 1.0 ml of the fortification solution is added to a sample. (Example: For a 100-ml water sample, the addition of 1.0 ml of a 0.01 ng/µl fortification solution will result in a fortification level of 0.1 ppb.)

3.0 Prepare a 1 ng/µL mixed standard for generating external calibration curves for the ENV analytes (CGA-64250, CGA-217495, CGA-91305, CGA-118244, CGA-118245, CGA-136735)on the LC/MS system. Pipette 1.0 mL from the 100 ng/µL stock solutions for CGA-64250, CGA-217495, CGA-91305, CGA-118244, CGA-118245, and CGA-136735 into a 100-mL volumetric flask and dilute to the calibration mark using 30% acetonitrile/ water. Subsequent dilutions in 30% acetonitrile/water are made to prepare a series of calibration standards.

4.0 Prepare a 1 ng/µL mixed standard for generating external calibration curves for CGA-71019 for use with the LC/MS system. Pipette 1.0 mL from the 100 ng/µL stock solutions into a 100-mL volumetric flask and dilute to the calibration mark using 25% methanol/water. Subsequent dilutions in 25% methanol/water are made to prepare a series of calibration standards.

K. Methods of Calculation

1.0 Determination of Residues in Samples

1.1 Inject the sample solution from Step II.D.2.18 or II.D.2.21 into the analysis system. The sample solution may be diluted if the analyte response exceeds the range of the calibration curve. The amount of analyte injected (ng) is determined by entering the value of the chromatographic peak height, or area,

in the calibration response curve (Step II.E.4.4) and calculating (by computer, calculator, or manual means) the corresponding value of nanograms injected. Typical chromatograms for fortified water and soil samples are presented in Figures 8-11.

2.0 Determination of Residues in Fortified Samples

Validate the method for each set of samples analyzed by including a control sample and one or more control samples fortified prior to the extraction procedure with 0.1 ppb or more of each analyte in water and with 5 ppb or more of each analyte in soil.

2.1 Add an appropriate volume of a fortification solution (from Step II.J.2.0) to the sample prior to any of the cleanup steps. The total volume of the added fortification solution should not exceed 1.0 ml.

2.2 Proceed with the sample cleanup procedure (Step II.D.2.3 for soil and Step II.D.3.3 for water).

3.0 Calculations

Calculations may be performed by computer program or manually as follows (soil concentrations are based on their wet weight):

3.1 Calculate the analyte concentration (in ppb) for field samples from equation (1):

$$(1)\ \text{ppb analyte} = \frac{\text{ng analyte found}}{\text{g sample injected}} \times \frac{1}{R}$$

where R is the recovery factor expressed in decimal form (i.e., 0.8 = 80%) and is calculated from equation (4), and the chemical purity

of the analytical standard has been accounted for in the preparation of the standard solutions. The use of the recovery correction factor "1/R" is left to the discretion of the study director.

The grams of sample injected for soil and water are calculated from equations (2) and (3), respectively.

The grams of sample injected for soil is calculated from equation (2).

$$(2)\ \text{g soil injected} = \frac{g}{V_e + V_{std} + (m \times g)} \times \frac{V_a}{V_f} \quad V_i$$

where, g is the grams of soil (wet weight) used, V_a is the aliquot volume of extracted sample used for analysis, V_e is the volume of extract solvent used, V_{std} is the volume (mL) of fortification standard added (if any), V_i is the volume (mL) injected onto the HPLC column, m is the percent moisture in the sample, expressed in decimal form (ex. 0.1 = 10%), and V_f is the final volume (mL) of the cleaned-up sample (from Step II.D.2.18 or II.D.2.21). (Note: the term "(m x g)" is a dilution correction factor due to the moisture in the soil, where 1.0 g = 1.0 mL.

$$(3)\ \text{g sample injected (water)} = \frac{g \times V_i}{V_f}$$

where g is the grams of sample (Step II.D.3.1 or II.D.3.3) used (for water, 100.0 ml = 100.0 g), V_i is the volume (ml) of sample injected onto the HPLC, and V_f is the final volume (ml) of the sample (from Step II.D.2.18 or II.D.2.21).

The recovery factor, expressed as a percentage (R%), is calculated from fortification experiments and is presented in equation (4).

$$(4)\ R\% = \frac{\text{ppb analyte found - ppb analyte (control)}}{\text{ppb analyte added}} \times 100\%$$

The amount (ppb) of analyte found is calculated from equation (5).

$$(5)\ \text{ppb analyte found} = \frac{\text{ng analyte found}}{\text{g sample injected}}$$

Residues of degradates found in test samples may also be expressed as parent equivalents by multiplying the amount found by the ratio of the molecular weight of propiconazole to that of the degradate (equation (6)).

$$(6)\ \text{ppb propiconazole equiv.} = \text{ppb metabolite} \times \frac{MW\,(p)}{MW\,(m)}$$

where MW(p) is the average molecular weight of propiconazole (342.2) and MW(m) is the average molecular weight of the metabolite, 344.2 for CGA-217495, 258.11 for CGA-91305, 69.07 for CGA-71019, and 358.2 for CGA-136735, CGA-118244, and CGA-118245.

3.2 The accuracy of the method is determined by the average recovery of the analytes fortified into the test substrate. The precision is estimated by the relative standard deviation of the determined concentration.

TABLE II. HPLC SYSTEM AND OPERATING CONDITIONS (ENV ANALYTES)

This system is used for the analysis of CGA-64250, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735

Instrumentation:

Perkin-Elmer Series 200 Gradient Pump
Perkin-Elmer Series 200 Autosampler
Eppendorf Model CH-30 Column Heater

Operating Conditions:

Column Heater: 30°C
Injection Volume: 100 µl
Mobile Phase Flow Rate: 1.5 ml/min
Column: Intersil 5 C8 (MetaChem Technologies, Inc., #0296-150X046, 15 cm x 4.6 mm, dp = 5 µm, equipped with an Upchurch (#A-318) pre-column filter (0.5 µm)
Mobile Phase A: 0.1% (v/v) acetic acid in acetonitrile
Mobile Phase B: 0.1% (v/v) acetic acid in purified water

Mobile Phase Gradient Program:

Time (min.)	% A	% B	Curve
0.0	30	70	-
10.0	75	25	1
0.5	100	0	1
3.0	100	0	-
0.5	30	70	1
6.0	30	70	-

Total Run Time: 20.0 min.

Analyte Retention Times:

CGA-91305	5.1 min
CGA-118245*	5.7
CGA-118244*	5.9
CGA-136735*	7.0 min
CGA-217495	8.4 min
Propiconazole*	10.0 min

* Multiple peaks are observed for these analytes due to stereoisomerism.

TABLE III. HPLC SYSTEM AND OPERATING CONDITIONS (CGA-71019)

Instrumentation:

Perkin-Elmer Series 200 Gradient Pump
Perkin-Elmer Series 200 Autosampler
Eppendorf Model CH-30 Column Heater

Operating Conditions:

Column Heater: 30°C
Injection Volume: 150 µl
Mobile Phase Flow Rate: 1.0 ml/min
Column: Zorbax 300 SCX (Mac-Mod Analytical, Inc., #883952-704, 15 cm x 4.6 mm, dp = 7 µm equipped with an Upchurch (#A-318) pre-column filter (0.5 µm)
Mobile Phase A: 25% methanol/water, 0.1% acetic acid
Mobile Phase B: 25% methanol/water, 20 mM in ammonium acetate
Mobile Phase Composition: 95% A/B

Total Run Time: 8 min.

Analyte Retention Time:

CGA-71019	3.5	min

TABLE IV. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS: LC/MS

Instrumentation:

PE Sciex API 300 Triple Quadrupole Mass Spectrometer
TurboIonSpray Liquid Introduction Interface
Instrument Control and Data Collection: Apple MacIntosh
Power PC Computer, Model 8500/180

Software:

Apple System 7.5

PE Sciex Software:

LC2Tune v. 1.0
Sample Control v.1.2
MacDAD v. 1.2
MacQuan v. 1.4
Multiview v. 1.2

Data Acquisition:

ENV Analytes

Scan type: MRM
Polarity: Positive
Acquisition mode: Profile
Pause time: 2 ms

Q1/Q3 masses	Dwell (ms)
258/70	200
344/256	200
358/256	200
342/159	200

CGA-71019

Scan Type: MRM
Polarity: Positive
Acquisition mode: Profile
Pause time: 2 ms

Q1/Q3 masses	Dwell (ms)
70/70	500

TABLE IV. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS: LC/MS (CONT.)

Typical State File Values

CGA-71019	
Positive Ions	
Gases	
NEB	14
CUR	12
CAD	3
Controls	
IS	5200
NC	0
TEM	425
OR	47
RNG	300
Q0	-7.5
IQ1	-9
ST	-15
RO1	-10
IQ2	-13.5
RO2	-18
IQ3	-24.5
RO3	-25
DF	-200
CEM	2000
Q1 Resolution	
Mass	**Offset**
30	0.033
100	0.045
1000	0.145
2000	0.255
Q3 Resolution	
Mass	**Offset**
30	0.025
100	0.020
1000	0.045
2000	0.070

ENV Analytes	
Positive Ions	
Gases	
NEB	14
CUR	12
CAD	3
Controls	
IS	5000
NC	0
TEM	425
OR	35
RNG	380
Q0	-7.5
IQ1	-9.5
ST	-14
RO1	-10
IQ2	-18
RO2	-32
IQ3	-48
RO3	-37
DF	-200
CEM	2000
Q1 Resolution	
Mass	**Offset**
30	0.033
100	0.045
1000	0.145
2000	0.255
Q3 Resolution	
Mass	**Offset**
30	0.025
100	0.020
1000	0.045
2000	0.070

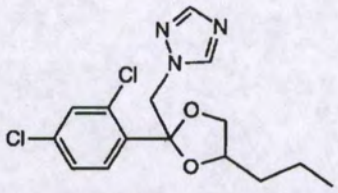
* Note: State file values will vary slightly from instrument to instrument. The values often will be changed slightly on a daily basis during instrument optimization procedures.

TABLE V. TYPICAL ANALYTE MONITORING IONS: LC/MS

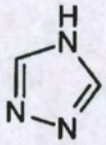
Analyte	Exact Molecular Weight	Q1 Molecular Ion	Q3 Product Ion
Propiconazole	341.07	342.1	159.0
CGA-217495	343.01	344.0	256.0
CGA-91305	257.01	258.0	70.0
CGA-71019	69.03	70.0	70.0
CGA-136735	357.06	358.1	256.0
CGA-118244	357.06	358.1	256.0
CGA-118245	357.06	358.1	256.0

Note: CGA-71019 does not form a product ion of any significant abundance in the MS/MS mode. The signal-to-noise ratio is enhanced, however, due to reduction of Q1 solvent noise at mass 70.

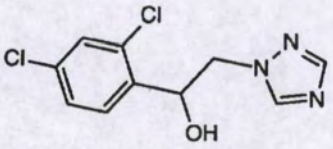
FIGURE 1. CHEMICAL NAMES AND STRUCTURES



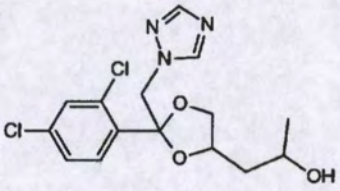
Propiconazole (CGA-64250)
CAS Number: 60207-90-1
1H-1,2,4-Triazole, 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-



CGA-71019
CAS Number: 288-88-0
1H-1,2,4-Triazole

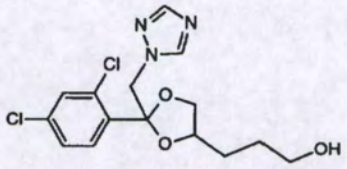


CGA-91305
CAS Number: 58905-18-3
1H-1,2,4-Triazole-1-ethanol, .alpha.-(2,4-dichlorophenyl)-

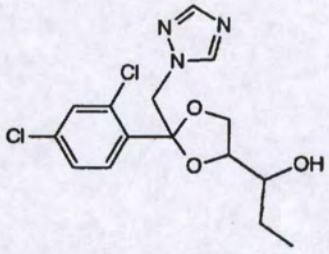


CGA-118244
CAS Number: 104390-57-0
1,3-Dioxolane-4-ethanol, 2-(2,4-dichlorophenyl)-.alpha.-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-

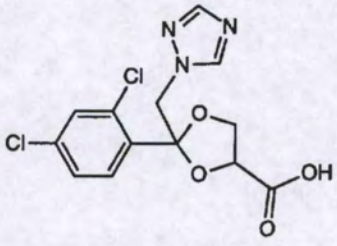
FIGURE 1. CHEMICAL NAMES AND STRUCTURES (CONT.)



CGA-118245
CAS Number: 104390-58-1
1,3-Dioxolane-4-propanol, 2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-



CGA-136735
CAS Number: 119725-85-8
1,3-Dioxolane-4-methanol, 2-(2,4-dichlorophenyl)-.alpha.-ethyl-2-(1H-1,2,4-triazol-1-ylmethyl)-



CGA-217495
CAS Number: 119725-91-6
1,3-Dioxolane-4-carboxylic acid, 2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-

FIGURE 2. AG-677 FLOW DIAGRAM FOR SOIL

Weigh 20 g sample of soil.
(Fortify, if necessary)
Extract under reflux for 1 hr with 100 mL of 70% methanol/water.
Centrifuge/filter.
Measure 50 mL aliquot of sample.

↓

Basify with ammonium hydroxide.
Pass through Varian SAX SPE, collect non-retained eluate
(analytes are not retained).
Remove organic via rotary evaporation.
Acidify with acetic acid.

↓

Pass sample through a preconditioned Varian ENV SPE connected piggyback style to a Varian SCX SPE column.
Rinse SPE columns with acidified water followed by purified water.
Disconnect SPE columns

↓

Elute ENV SPE with acetonitrile, collect in a concentration tube.
Add water to serve as an analyte trap.
Remove organic via rotary evaporation until only aqueous remains.
Dilute to precalibrated mark using acetonitrile and water.
Analyze by LC/MS using reversed phase HPLC.
(Note: This fraction contains CGA-64250, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735.)

↓

Rinse SCX SPE with 70% methanol/water.
Elute with 2.5% ammonium hydroxide in 70% methanol/water, collect in concentration tube.
Remove organic via rotary evaporation until only aqueous remains.
Dilute to precalibrated mark using methanol and water.
Analyze by LC/MS using cation exchange HPLC.

FIGURE 3. AG-677 FLOW DIAGRAM FOR WATER

Measure 100-ml aliquot of water.
(Fortify, if necessary.)
(Centrifuge/filter, if necessary)
Acidify with acetic acid.

↓

Pass sample through a preconditioned Varian ENV SPE connected piggyback style to a Varian SCX SPE column.
Rinse SPE columns with acidified water followed by purified water.
Disconnect SPE columns

↓

Elute ENV SPE with acetonitrile, collect in a concentration tube.
Add water to serve as an analyte trap.
Remove organic via rotary evaporation until only aqueous remains.
Dilute to precalibrated mark using acetonitrile and water.
Analyze by LC/MS using reversed phase HPLC.
(Note: This fraction contains CGA-64250, CGA-91305, CGA-217495, CGA-118244, CGA-118245, and CGA-136735.)

↓

Rinse SCX SPE with 70% methanol/water.
Elute with 2.5% ammonium hydroxide in 70% methanol/water, collect in concentration tube.
Remove organic via rotary evaporation until only aqueous remains.
Dilute to precalibrated mark using methanol and water.
Analyze by LC/MS using cation exchange HPLC.