I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of CGA-279202 including its stereoisomer metabolites (CGA-357261, CGA-357262, and CGA-331409) and its acidic metabolites (CGA-321113 and CGA-373466) in soil. The compounds are separated by high performance liquid chromatography (HPLC) and detected by UV absorption detection. The structures, chemical names, and Chemical Abstracts Registry numbers of the analytes (if available) are presented in Figure 1. Analytical Method AG-654 is being reissued as AG-654A to include the change of eluting solvent (step. 2.15) for the mixed phase cation extraction disc to improve the recovery of acid metabolites and replace the silica extraction disc (item II.B.19.0) to 0.5-g silica cartridge (Varian) to improve the method precision, particularly for CGA-331409.

The limit of detection (smallest standard amount injected during the chromatographic run) is 2.5 ng for all analytes. The limit of determination (the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) is 10 ppb in soil.

B. Principle

Soil samples (20 g) are extracted twice with 10% water/methanol using mechanical shaking at room temperature. The samples are centrifuged and filtered. Methanol is removed via rotary evaporation until only a small amount of aqueous remains. The aqueous is acidified and then passed through a mixed phase cation extraction disc cartridge. The analytes are then eluted from the cartridge using 65% ethyl acetate/hexane. The eluate is evaporated to near dryness and dissolved in 1/2 (v/v) methanol/0.1% ammonium hydroxide. The solution is passed through an Aminopropyl (NH_2) extraction disc cartridge (the analytes are not retained). The eluate (methanol aqueous solution) is partitioned twice with hexane under basic condition. The hexane extract is evaporated to dryness and dissolved in hexane before being loaded

onto a silica gel cartridge. CGA-279202 and its stereoisomers are retained in the cartridge and are eluted with 40% (v/v) methyl-tert butyl ether/hexane. The solvent is evaporated and the analytes are dissolved with aqueous acetonitrile for HPLC analysis. The acid metabolites (CGA-321113 and CGA-373466) are isolated from the methanol aqueous solution by partitioning with hexane under acidic condition. Hexane is evaporated and the analytes are dissolved in aqueous acetonitrile for HPLC analysis. A flow diagram for the method is presented in Figure 2.

II. MATERIALS AND METHODS

A. Apparatus

1.0 Balance, analytical (Sartorius R160P) or equivalent; top loading (Mettler BB1300) or equivalent.

2.0 Beaker, glass, 150-mL (Fisher cat. #02-540J) or equivalent.

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

4.0 Bottle, polypropylene, (Fisher cat. #05-562-23) or equivalent with cap. Appropriate size for soil extraction.

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 Concentration tube, 50-mL (Fisher cat. #05-538-40B) with Teflon® stopper or equivalent.

7.0 Cylinder, graduated, 50-mL, 100-mL, and 1000-mL (Fisher cat. #08-556C, #08-556D, #08-556G), or equivalent.

8.0 Filter, paper, for filtering soil extracts prior to rotary evaporation, 24-cm prepleated circles, Whatman 2V (Fisher cat. #09-832D) or equivalent.

9.0 Flasks, round bottom, 500-mL (Fisher cat. #10-067E) or equivalent.

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

11.0 Mixer, vortex (Fisher cat. #12-810-10) or equivalent.

12.0 N-EVAP evaporator model 111 (Organomation cat #11155-P) or equivalent.

13.0 Oxford 400 series Dispensers (1 to 10 mL capacity) (Fisher cat. #13-687-75) or equivalent.

14.0 Pasteur pipette, disposable (Fisher cat. #13-678-7C) or equivalent.

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

16.0 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.)

17.0 Repipet Jr. Dispensers (Fisher cat. #13-687-59A) or equivalent.

18.0 Rotary evaporator, Buchi (Fisher cat. #09-548-105F) or equivalent, with rotary evaporator trap (Fisher cat. #K570210-0124) or equivalent.

19.0 Shaker, Eberbach 6010 two-speed (Baxter cat. #S1105) with utility carrier (Baxter cat. #S110) or equivalent

20.0 Ultrasonic bath, (Fisher cat. #15-336-6) or equivalent.

21.0 Vials, 1.8-mL (Perkin-Elmer cat. #N930-1385) or equivalent, with polyethylene caps (Perkin-Elmer cat. #0494-8532) or equivalent.

22.0 Vacuum manifold (Alltech cat. #210351) or equivalent.

B. Reagents and Analytical Standards

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

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

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

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

4.0 Extraction solvent: 10% (v/v) water/methanol. Add 900 mL of methanol to 100 mL of purified water.

5.0 Phosphoric acid, 85%, HPLC grade (Fisher cat. #A260-500) or equivalent.

6.0 0.1 M Acetic acid, Pipette 5.7 mL of Acetic acid, ACS grade (Fisher cat. #A38S-500) into 1-L volumetric flask and dilute to volume with purified water.

7.0 0.1% Ammonium hydroxide, Pipette 1 mL of Ammonium hydroxide, ACS grade (Fisher cat. #A669S-212) into 1-L volumetric flask and dilute to volume with purified water.

8.0 Ethyl acetate, HPLC grade (Fisher cat. #E195-4) or equivalent.

9.0 Methyl-tert butyl ether, HPLC grade (Fisher cat. #E127-4) or equivalent.

10.0 Hexane, HPLC grade (Fisher cat. #H302-4) or equivalent.

11.0 Mobile phase A: 50% (v/v) water/ acetonitrile + 0.1% phosphoric acid. Mix 500 mL of purified water with 500 mL of acetonitrile. Add 1 mL of phosphoric acid to this mixture and mix the contents.

12.0 Mobile phase B: 50% (v/v) methanol/ acetonitrile + 0.1% phosphoric acid Mix 500 mL of methanol with 500 mL of acetonitrile. Add 1 mL of phosphoric acid to this mixture and mix the contents.

13.0 Mobile phase C: water + 0.1% phosphoric acid Add 1 mL of phosphoric acid into 1000 mL of purified water and mix the contents.

14.0 Mobile phase D: acetonitrile + 0.1% phosphoric acid
Add 1 mL of phosphoric acid into 1000 mL of acetonitrile and mix well.

15.0 Mobile phase E: 45% (v/v) acetonitrile/ water + 0.1 % phosphoric acid
Mix 450 mL of acetonitrile with 550 mL of purified water. Add 1 mL of phosphoric acid to this mixture and mix the contents.

16.0 Mobile phase F: 30% (v/v) water/ acetonitrile + 0.1 % phosphoric acid
Mix 700 mL of acetonitrile with 300 mL of purified water. Add 1 mL of phosphoric acid to this mixture and mix the contents.

17.0 10-mL Spec Plus MP1 extraction disc cartridge (Ansys cat. #502-11-70) or equivalent.

18.0 10-mL Aminopropyl (NH_2) extraction disc cartridge (Ansys cat. #501-07-35) or equivalent.

19.0 0.5-g Silica gel cartridge (Varian cat. #1210-2037) or equivalent.

20.0 Sodium chloride, ACS grade (Fisher cat. #S271-1) or equivalent.

21.0 CGA-279202, CGA-357261, CGA-357262, CGA-331409, CGA-321113 and CGA-373466, 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 evaporation steps of all solvents should be done in a fume hood. Acetic acid and ammonium hydroxide are irritants and should be used in a well ventilated area (i.e., a fume hood) during reagent preparation.

D. Analytical Procedure

1.0 Soil Moisture Determination

Soil characterization data for the soils used in this validation study are presented in Table I.

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 a 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:

$$m = \frac{W_{1.2} - W_{1.3}}{W_{1.2} - W_{1.1}}$$

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 $W_{1.3}$ is the weight of the dry soil plus container (from Step 1.5).

2.0 Soil Extraction/Cleanup

Soil samples must be homogenized prior to analysis using suitable sample preparation techniques.

2.1 Weigh and record 20 ± 0.1 g of soil sample and place in an appropriate-sized, centrifugable polypropylene bottle.

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 the extraction solvent. Swirl the contents briefly. Place the bottle in a mechanical shaker and agitate the sample at room temperature at high speed for approximately 30 minutes.

2.4 Centrifuge the sample at approximately 9,000 RPM for approximately 10 minutes, or at an alternate speed and time if the results are considered satisfactory.

2.5 Decant the sample extract through a 2V filter paper into a 500-mL round bottom flask. Use a disposable pipette to transfer the extract left at the bottom of the bottle if necessary.

2.6 Pour the second 100 mL of the extraction solvent into the plastic bottle containing the sample, extract, centrifuge, and filter the sample as detailed in Steps 2.3-2.5. Combine this extract with the extract from Step 2.5 in the same round bottom flask.

2.7 Place the flask on a rotary evaporator with a water bath temperature of approximately 40°C to 50°C. Use a solvent trap to minimize losses due to bumping. (Note: To prevent losses due to bumping, evaporate the solvent at room temperature for the first 3-5 minutes and periodically vent the sample, as required.) Remove the solvent until approximately 0.5-1.0 mL of water remains.

2.8 Remove the flask from the rotary evaporator. Add 2 mL of methanol into the flask and use a disposable pipette to rinse the inside wall thoroughly. Swirl the flask briefly to dissolve the residue into the solution. Sonicate the flask briefly, if required.

2.9 Add 5 mL of 0.1 M acetic acid and swirl the flask briefly to mix the contents. Add 10 mL of 0.1 M acetic

acid into the flask. Mix the contents briefly.

2.10 Condition a 10-mL Spec Plus MP1 extraction disc cartridge by connecting the cartridge to a vacuum manifold with the vacuum off. Open the valve under the cartridge. Pipette approximately 2 mL of 65% (v/v) ethyl acetate/hexane to rinse the cartridge. Use a pipette bulb to push the solvent from the top if necessary. Add approximately 2 mL of methanol into the cartridge, pass it through the disc until the level of methanol is approximately 1 mm from the disc surface and then close the valve. **Do not allow the disc to dry.** Add approximately 0.25 mL of methanol into the cartridge and allow it to soak the disc for approximately 1 minute. Add approximately 1 mL of 0.1 M acetic acid and allow it to soak the disc for approximately 1 min.

2.11 Use a disposable pipette to transfer the sample solution (from the 500-mL round bottom flask) into the cartridge, open the valve and turn on the vacuum (~ 5-10 " Hg) to assist the elution of the solution through the disc at a dropwise rate. Turn off the valve underneath the cartridge to stop the flow when the liquid level is approximately 0.2-0.5 mL on top of the disc so the disc is still wet.

2.12 Rinse the inside wall of the round bottom flask thoroughly with approximately 2 mL of methanol, swirl the flask briefly, then add approximately 15 mL of 0.1 M acetic acid. Swirl the flask well.

2.13 Transfer the solution into the cartridge. Turn on the valve underneath the cartridge to continue the elution. Save the disposable pipette for Step 2.20.

2.14 Dry the disc with vacuum ~20" Hg for approximately 1 minutes.

2.15 Move the cartridge from the vacuum manifold into a 50-mL concentration tube (A). Pipette approximately 6 mL of 65% (v/v) ethyl acetate/hexane and collect the eluate. Use a pipette bulb to push the solvent through the disc and assist the flow (dropwise). A small amount of water left in the disc will be pushed along with the solvent.

2.16 Evaporate the solvent in the tube using an N-EVAP evaporator at 40-50°C with a gentle flow of nitrogen until only a small drop of water is left in the tube. This step takes only 2-3 minutes. (**Do not allow it to dry**). The N-EVAP is used to evaporate several tubes at the same time unattendedly without bumping problem like the rotary evaporator does. However, a rotary evaporator may also be used with care if it is available.

2.17 Pipette 1 mL of methanol into the tube (A) and mix briefly on a vortex mixer then put it in the sonicator for approximately 1 minute. Add 2 mL of 0.1% ammonium hydroxide and mix well on the vortex mixer.

2.18 Condition a 10-mL Aminopropyl extraction disc cartridge by connecting the cartridge to the vacuum manifold with vacuum off. Open the valve under the cartridge. Add approximately 3 mL of methanol into the cartridge and use a pipette bulb to push the solvent from the top

until the level of methanol is approximately 1 mm from the disc surface. Close the valve. **Do not allow the disc to dry.** Add approximately 0.25 mL of methanol and allow it to soak for approximately 1 minute. Add approximately 0.5 mL of water and allow it to soak for approximately 1 minute.

2.19 Move the cartridge from the vacuum manifold into a 50-mL concentration tube (B).

2.20 Use the disposable pipette (from Step 2.13) to transfer the solution from the concentration tube (Step 2.17) into the Aminopropyl extraction disc cartridge. Let the sample drip dropwise from the cartridge into the tube by gravity. The analytes are unretained. Note: Do not permit the sample to pass through the cartridge at a fast flow rate.

2.21 Repeat Steps 2.17 and 2.20 one more time.

2.22 After all of the solution has passed through the disc, pipette approximately 1 mL of 50% (v/v) methanol/water into the cartridge and allow it to drip into the tube. Use a pipette bulb to push the residue liquid from the disc into the tube.

2.23 Add approximately 1 mL of water saturated with sodium chloride into the tube (B).

2.24 Add approximately 5 mL of hexane into the tube (B), close the tube opening with a Teflon® stopper and mix the contents vigorously using the vortex mixer for approximately 1 minute.

2.25 Allow the phases to separate. Use a new disposable pipette to transfer the hexane portion (top layer) into a 50-mL concentration tube (C).

2.26 Repeat Steps 2.24 and 2.25 one more time. Save the aqueous portion for Step 2.37.

2.27 Evaporate hexane in the tube (C) to dryness using the N-EVAP at approximately 40-50°C with a gentle flow of nitrogen. The N-EVAP is highly recommended over the rotary evaporator at this step because of bumping problems that may occur with rotary evaporator.

2.28 Add approximately 0.5-1.0 mL of methanol into the tube (C) and evaporate methanol to dryness (to get rid of water residue left in the tube). This step may be omitted if there is no water residue left in the tube after Step 2.27 (to save time).

2.29 Pipette approximately 1 mL of hexane into the tube (C), sonicate for approximately 30 seconds and mix well on the vortex mixer for approximately 30 seconds.

2.30 Prepare a 3-mL silica cartridge by connecting the cartridge to the vacuum manifold with vacuum off. Open the valve under the cartridge. Add approximately 2 mL of 40% (v/v) methyl-butyl ether/hexane into the cartridge and use a pipette bulb to push the solvent through the disc. Add approximately 2 mL of hexane and allow it to pass through the cartridge until the level of liquid is approximately 1-2 mm above the silica surface. Close the valve. The cartridge is ready for the next step.

2.31 Use a disposable pipette to transfer the hexane from Step 2.29 to the silica gel cartridge. Open the valve and let the solvent drip by gravity into a collecting vessel. The analytes are retained on the cartridge. Discard the hexane.

2.32 Repeat Steps 2.29 and 2.31 three more times.

2.33 Move the silica cartridge from the vacuum manifold into a 50-mL concentration tube (D).

2.34 Pipette approximately 3-4 mL of 40% (v/v) methyl-tert butyl ether/hexane into the cartridge and let the solvent drip by gravity. Collect the eluate containing the analytes in the tube (D).

2.35 Evaporate the solvent in the tube (D) to dryness using the N-EVAP at 40-50°C with a gentle flow of nitrogen. The rotary evaporator may be used if it is available; however, the N-EVAP is more convenient to use with several tubes simultaneously.

2.36 Pipette 1 mL of acetonitrile using a volumetric pipette into the tube (D) and sonicate for approximately 10 minutes with an occasional mixing on the vortex mixer. Pipette 1 mL of distilled water into tube (D) and sonicate for approximately 5 minutes with an occasional mixing on the vortex mixer. The sample may be diluted further with 50% water/ acetonitrile if the analyte response exceeds the range of the calibration curve. Transfer the sample into an autosampler vial and analyze by HPLC with UV detection for the presence of CGA-279202, CGA-357261, CGA-357262, and CGA-331409 using the appropriate HPLC method (Tables II and IV).

2.37 Add 1 drop of 85% phosphoric acid into the tube (B) containing the aqueous portion after the hexane partitioning in Step 2.26.

2.38 Repeat Steps 2.24 and 2.25 three times, collecting the hexane portions in a 50-mL concentration tube (E).

2.39 Evaporate hexane to dryness using the N-EVAP at 40-50°C with a gentle flow of nitrogen. The rotary evaporator may be used if it is available; however, the N-EVAP is more convenient to use with several tubes simultaneously.

2.40 Pipette 1 mL of acetonitrile using a volumetric pipette into the tube (E) and sonicate for approximately 10 minutes with occasional mixing on the vortex mixer. Pipette 1 mL of distilled water into tube (E) and sonicate for approximately 5 minutes with occasional mixing on the vortex mixer. The sample may be diluted further with 50% water/acetonitrile if the analyte response exceeds the range of the calibration curve. Transfer the sample into an autosampler vial and analyze by HPLC with UV detection for the presence of CGA-373466 and CGA-321113 using the HPLC method in Table III.

2.41 Store the sample in a refrigerator (approximately 2-7°C) if it will not be analyzed the same day the sample is processed (freezing is not recommended). Before the analysis, samples must be permitted to reach to room temperature and mixed well.

E. Instrumentation

1.0 Description and Operating Conditions: HPLC

See Tables II and V for descriptions of the HPLC systems for the analysis of CGA-279202 and its stereoisomers using Inertsil ODS-2 and Resolve C-18 columns. See Table III and VI for descriptions of the HPLC systems for the analysis of CGA-373466 and CGA-321113 using Inertsil ODS-2 and Resolve C-18 columns. See Table IV for a description of the HPLC system for the analysis of CGA-279202 and CGA-357262, using a Spherisorb CN/Resolve C-18 column switching system.

2.0 Calibration and Standardization

2.1 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.

2.2 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 50 ng (50 µL injections). 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 standard calibrations are presented in Tables VII-XV for the analytical and confirmation systems. Typical standard chromatograms are presented in Figures 5 and 17 for the Inertsil ODS-2 column, Figures 9 and 21 for the Resolve C-18 column and Figure 13 for the Spherisorb CN/Resolve C-18 column switching systems.

2.3 Column switching method is used to confirm the presence of CGA-279202 and CGA-357262 in soil samples, particularly at the level near the limit of determination. First, establish the retention time of CGA-279202 and CGA-357262 on column 1 (Sperisorb CN) using mobile phase E at a flow rate of 0.8 mL/min. Connect the outlet of the CN column to the detector and inject 50 uL of CGA-293343+CGA-357262(1 ng/uL) to determine their retention time. The retention times for the analytes may not vary any more than 0.1 minutes from the mean value obtained for three consecutive injections. (**Note**: CGA-279202 coelutes with CGA-357262 under this condition). Establishing the switching valve time should be performed daily just before the analysis of any set of samples. The switching valve time is set so that the entire peak for the analytes are encompassed. The switching valve duration is set to begin just as the analytical peak rises from the baseline and end as the peak tail comes back to the baseline (see Figure 4). Once the switching time and switch duration between load and inject position are set, connect the outlet of the Spherisorb CN column (column one) to the inlet of the Resolve C-18 (column two) via the switching valve and connect the outlet of the Resolve C-18 column to the detector (see Figure 3). An old HPLC column should be connected at the waste outlet of the switching valve to create a back pressure between 500 to 1000 psi. This will prevent the pump (2) from shutting down prematurely due to the low pressure limit of the pump (approximately 50 psi) at the inject position. A flow rate of 0.8 mL/min

is used for both columns but different flow rate may be used if the results are considered satisfactory. Inject the sample or standard solution into the CN column while the switching valve is in the load position. Switch the valve to the inject position (by relay controller of the autosampler) at the time previously determined for a predetermined switching duration (approximately 0.8 minutes). CGA-279202 and CGA-357262 are transferred from column one to column two for further separation. The valve is then switched back to the load position (at the end of switching duration of 0.8 min) and the analysis continues to the end (approximately 30-35 minutes).

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. It is recommended to rinse all glassware and plastic bottles with methanol and dry before use.

G. Confirmatory Techniques

1.0 Either the Inertsil ODS-2 or Resolve C-18 column may be used as the analytical column depending upon the quality of the chromatograms of the soil samples. The column which is not selected as the analytical column is then used as the confirmation column.

2.0 Neither column was able to separate interfering peaks existing in the soil control from CGA-279202 and CGA-357262 with the soil samples used in this study. The column switching method using a Spherisorb

CN and a Resolve C-18 column (see details in Table IV and Figures 3-4) was used to solve this problem.

H. Time Required

1.0 The sample extraction and cleanup procedures can be completed for a set of approximately seven samples in an eight-hour working day.

2.0 HPLC analysis for CGA-279202 and its three stereoisomers requires approximately 60 minutes (method in Table II). HPLC analysis for CGA-373466 and CGA-321113 requires approximately 35 minutes (method in Table III). HPLC analysis for CGA-279202 and CGA-357262 using the column switching method (Table IV) requires approximately 35 minutes.

I. Modifications and Potential Problems

1.0 Analytical Method AG-654 was validated only for the soil types listed in the final method. Other soil types, or soil samples from different locations, may exhibit binding or interference problems which were not observed with these samples.

2.0 "Bumping" is 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. The alternative procedure is to evaporate the first 100 mL of extract after Step II.D.2.5 to approximately 2-3 mL then continue to Step II.D.2.6.

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

4.0 The compositions of the mobile phases were optimized for the columns used for the analytical and confirmation analysis systems. The compositions may need to be

altered from the conditions used in these methods if columns of different manufacturer or HPLC systems are used.

5.0 Before starting the HPLC analysis, a minimum of 2 blank runs should be performed to equilibrate the HPLC column and eliminate any organic contaminants from the previous analysis. This will also reduce the variation of retention times of the analytes.

6.0 If there are significant interferences in the sample, the gradient profile or solvent composition may be modified to obviate the coelution problem.

7.0 It is not recommended to stop at Steps 2.35 and 2.39 (i.e., put the tubes in a freezer for the next day analysis).

8.0 Sample and standard solutions that are stored in the refrigerator must be permitted to reach to room temperature and mixed well before analysis.

J. Preparation of Standard Solutions

All individual standards that are prepared in acetonitrile are stored in amber bottles in a freezer (<-10°C) when not in use. No analyte stability or solubility problems have been observed in the standard solutions that are prepared in acetonitrile used in this study.

1.0 Prepare individual 200 ng/µL stock solutions for each analyte. 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.

$$V\,(mL) = \frac{W(mg) \times P}{C\,(ng/uL)} \times 10^3$$

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/uL; and 10^3 is a conversion factor.

For example:

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

$$V\,(mL) = \frac{9.9\ mg \times 0.98}{200\ ng/uL} \times 10^3 = 48.51\ mL$$

2.0 Prepare a 20 ng/µL mixed standard solution in acetonitrile by pipetting 5.0 mL of each analyte (from its 200 ng/µL stock solution in Step 1.0) into a 50-mL volumetric flask and diluting to the mark with acetonitrile. Store the solution in an appropriate size amber bottle. This solution is used to prepare all subsequent dilutions. Store this mixed standard solution in a refrigerator (approximately 2-7°C) when not in use.

3.0 Fortification standards are prepared by diluting the 20 ng/uL mixed standard with acetonitrile. The concentration of the solutions to be prepared 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 20 g soil sample, the addition of 1.0 mL of a 0.2 ng/µL fortification solution will result in a fortification level of 10 ppb). Store this mixed standard solution in a refrigerator (approximately 2-7°C) when not in use.

4.0 A 1.0 ng/µL analytical standard for HPLC calibration use is prepared by pipetting 0.5 mL from the 20 ng/µL mixed standard

(Step II.J.2.0) into a 10-mL volumetric flask and diluting to the 10-mL mark with 50% (v/v) water/acetonitrile. Subsequent serial dilutions are made with 50% (v/v) water/acetonitrile to prepared additional calibration standards. It is recommended to prepare the HPLC analytical standards each day prior to use. If it is necessary, store this mixed standard solution in a refrigerator (approximately 2-7°C) when not in use.

K. Methods of Calculation

1.0 Determination of Residues in Samples

1.1 Inject the sample solution from Steps II.D.2.36 and 2.40 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.2.2) and calculating (by computer, calculator, or manual means) the corresponding value of nanograms injected. Typical chromatograms for control and fortified soil samples are presented in Figures 6-8, 10-12, 14-16, 18-20, and 22-24 for the Inertsil ODS-2 column, Resolve C-18 column and Spherisorb CN/Resolve C-18 column switching system, respectively.

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 10 ppb or more of each analyte.

3

2.1 Add an appropriate volume of a fortification solution (from Step II.J.3.0) 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).

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. (**Note**: Correcting the residue found in field samples for recovery of fortified samples is left to the discretion of the Study Director. If samples are not corrected for recovery, the "1/R" term is omitted from equation (1).

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

$$(2)\ \text{g sample injected} = \frac{g}{V_e + (m \times g)} \times \frac{V_a V_i}{V_f}$$

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 (mL) of extracting solvent used, 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.2.36 and Step II.D.2.40) (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. When the entire extract volume is used for the cleanup process, the term "V_e + (m x g)" will be equal the aliquot volume, V_a.).

The recovery factor, expressed as a percentage (R%), is calculated from fortification experiments and is presented in equation (3).

$$(3)\ R\% = \frac{\text{ppb analyte found - ppb analyte (control)}}{\text{ppb analyte added}} \times 100\%$$

The amount (ppb) of analyte found is calculated from equation (4).

$$(4)\ \text{ppb analyte found} = \frac{\text{ng analyte found}}{\text{g sample injected}}$$

Residues of metabolites found in test samples may also be expressed as parent equivalents by multiplying the amount found by the ratio of the molecular weight of CGA-279202 to that of the metabolite (equation (5)).

$$(5)\ \text{ppb CGA-279202 equiv.} = \text{ppb metabolite} \times \frac{MW(p)}{MW(m)}$$

Where MW(p) is the average molecular weight of CGA-279202 (408.38) and MW(m) is the average molecular weight

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of the metabolite, 408.38 for CGA-357262, CGA-357262, CGA-331409, and 394.35 for CGA-321113 and CGA-373466.

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: INERTSIL ODS-2 COLUMN (for CGA-279202, CGA-357261, CGA-357262, and CGA-331409)

Instrumentation:
Perkin-Elmer Model Series 4 Gradient Pump
Perkin-Elmer Model ISS 100 Autosampler
Eppendorf Model CH-30 Column Heater
Operating Conditions:
Column Heater: 30°C
Injection Volume: 50 µL
Column: Inertsil ODS-2 (Metachem cat. #296-250X030), 25 cm x 3.0 mm, dp = 5 µm, equipped with a guard column (cat. #296-CS3) and an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent.
Detector: Kratos Spectroflow UV detector at 254 nm
Data Acquisition: Microvax II(Q) operating system, VMS Version 5.3-1 Application Software VG Multichrom Version 2.0 Worksheet Version: Ws.pas 1.3.1
Mobile Phase A: 50% (v/v) water/acetonitrile + 0.1% phosphoric acid
Mobile Phase B: 50% (v/v) methanol/acetonitrile + 0.1% phosphoric acid
Mobile Phase Gradient:

Step	Time (min.)	Flow (mL/min.)	%A	%B	Curve
0	0	0.6	80	20	
1	0.5	0.6	80	20	
2	30	0.6	40	60	1
3	5	0.6	40	60	
4	0.1	1.0	0	100	1
5	5.0	1.0	0	100	
6	0.1	0.8	80	20	1
7	20	0.8	80	20	

Total Run Time - approximately 60 min.
Analyte Retention Times

CGA-357261	18.4 min.
CGA-279202*	23.9 min.
CGA-357262*	27.7 min.
CGA-331409	34.6 min.

* **Note:** Some soil samples may contain interfering peaks that coelute with CGA-279202 and CGA-357262 and make it difficult to quantify. If significant interferences are observed, the column switching method detailed in Table IV, should be used.

TABLE III. HPLC SYSTEM AND OPERATING CONDITIONS: INERTSIL ODS-2 COLUMN (for CGA-373466 and CGA-321113)

Instrumentation:

Perkin-Elmer Model Series 4 Gradient Pump
Perkin-Elmer Model ISS 100 Autosampler
Eppendorf Model CH-30 Column Heater

Operating Conditions:

Column Heater: 30°C
Injection Volume: 50 µL
Column: Inertsil ODS-2 (Metachem cat. #296-250X030), 25 cm x 3.0 mm, dp = 5 µm, equipped with a guard column (cat. #296-CS3) and an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent.
Detector: Kratos Spectroflow UV detector at 254 nm
Data Acquisition: Microvax II(Q) operating system, VMS Version 5.3-1 Application Software VG Multichrom Version 2.0 Worksheet Version: Ws.pas 1.3.1

Mobile Phase A: 50% (v/v) water/acetonitrile + 0.1% phosphoric acid
Mobile Phase B: 50% (v/v) methanol/acetonitrile + 0.1% phosphoric acid
Mobile Phase Gradient:

Step	Time (min.)	Flow (mL/min.)	%A	%B	Curve
0	0	0.6	80	20	
1	0.5	0.6	80	20	
2	10	0.6	50	50	1
3	3	0.6	50	50	
4	0.1	1.0	0	100	1
5	5.0	1.0	0	100	
6	0.1	0.8	80	20	1
7	16	0.8	80	20	

Total Run Time approximately 35 min.
Analyte Retention Times:

CGA-373466	9.9 min.
CGA-321113	13.0 min.

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TABLE IV. HPLC SYSTEM AND OPERATING CONDITIONS: COLUMN SWITCHING SYSTEM WITH SPHERISORB CN AND RESOLVE C-18 (for CGA-279202 and CGA-357262)

Instrumentation: (see details of the method in Section II.G.3)
Waters 6000A Pump (pump 1)
Perkin-Elmer Model Series 4 Gradient Pump (pump 2)
Perkin-Elmer Model ISS 200 Autosampler
Eppendorf Model CH-30 Column Heater
Valco 6-port valve with electronic actuator or equivalent
Perkin-Elmer LC-95 UV detector

Operating Conditions:

Column Heater:	30°C
Injection Volume:	50 µL
Column 1:	Spherisorb CN (Metachem cat. 189-150X046),15 cm x 4.6 mm, dp = 5 µm, equipped with a guard column (cat. #189-CS) and Upchurch (cat. #A-318) pre-column filter (0.5 µm), or equivalent.
Column 2:	Resolve C18 (Waters Assoc. cat. #11740), 30 cm x 3.9 mm, dp = 5 µm, equipped with a guard column (cat. #46915) and an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent.
Mobile phase E:	55% water/acetonitrile + 0.1% phosphoric acid (for column 1)
Mobile phase F:	30% water/acetonitrile + 0.1% phosphoric acid(for column 2)
Flow rate:	0.8 mL/min (for both pumps)
Detector:	Perkin-Elmer LC-95 at 254 nm
Data Acquisition:	Microvax II(Q) operating system, VMS Version 5.3-1 Application Software VG Multichrom Version 2.0 Worksheet Version: Ws.pas 1.3.1
Cut time	approximately 4.5 min after the injection
Cut duration	approximately 0.8 min
Total Run Time	approximately 35 min.

Analyte Retention Times:

CGA-279202	17.3 min.
CGA-357262	19.6 min.

Note: See Figure 3 for the diagram of how to connect the columns to the switching valve.
See Figure 4 for chromatogram of analytes on column 1 and the establishment of the column switching time interval.

TABLE V. HPLC SYSTEM AND OPERATING CONDITIONS: RESOLVE C-18 CONFIRMATION COLUMN (for CGA-279202 CGA-357261, CGA-357262, and CGA-331409)

Instrumentation:
Perkin-Elmer Model Series 4 Gradient Pump
Perkin-Elmer Model ISS 200 Autosampler
Eppendorf Model CH-30 Column Heater
Operating Conditions:
Column Heater: 30°C
Injection Volume: 50 µL
Column: Resolve C18 (Waters Assoc. cat. #11740), 30 cm x 3.9 mm, dp = 5 µm, equipped with a guard column (cat. #46915) and an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent.
Detector: Perkin-Elmer LC-95 at 254 nm
Data Acquisition: Microvax II(Q) operating system, VMS Version 5.3-1 Application Software VG Multichrom Version 2.0 Worksheet Version: Ws.pas 1.3.1
Mobile Phase C: water + 0.1% phosphoric acid
Mobile Phase D: acetonitrile + 0.1% phosphoric acid
Mobile Phase Gradient:

Step	Time (min.)	Flow (mL/min.)	%C	%D	Curve
0	0	0.8	50	50	
1	0.5	0.8	50	50	
2	35	0.8	28	72	1
3	3	0.8	28	72	
4	0.1	1.5	0	100	1
5	5	1.5	0	100	
6	0.1	1.2	50	50	1
7	15	1.2	50	50	

Total Run Time approximately 60 min.
Analyte Retention Times:

CGA-357261	19.8 min.
CGA-279202*	23.7 min.
CGA-357262*	26.4 min.
CGA-331409	31.8 min.

* **Note** Some soil samples may contain interfering peaks that coelute with CGA-279202 and CGA-357262 and make it difficult to quantify. If significant interferences are observed, the column switching method detailed in Table IV, should be used.

TABLE VI. HPLC SYSTEM AND OPERATING CONDITIONS: RESOLVE C-18 CONFIRMATION COLUMN
(for CGA-373466 and CGA-321113)

Instrumentation:

Perkin-Elmer Model Series 4 Gradient Pump
Perkin-Elmer Model ISS 200 Autosampler
Eppendorf Model CH-30 Column Heater

Operating Conditions:

Column Heater: 30°C
Injection Volume: 50 µL
Column: Resolve C18 (Waters Assoc. cat. #11740), 30 cm x 3.9 mm, dp = 5 µm, equipped with a guard column (cat. #46915) and an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent.
Detector: Perkin-Elmer LC-95 at 254 nm
Mobile Phase C: water + 0.1% phosphoric acid
Mobile Phase D: acetonitrile + 0.1% phosphoric acid
Mobile Phase Gradient:

Step	Time (min.)	Flow (mL/min.)	%C	%D	Curve
0	0	0.8	50	50	
1	0.5	0.8	50	50	
2	10	0.8	30	70	1
3	3	0.8	30	70	
4	0.1	1.5	0	100	1
5	5	1.5	0	100	
6	0.1	1.2	50	50	1
7	15	1.2	50	50	

Total Run Time approximately 35 min.
Analyte Retention Times:

CGA-373466	11.1 min.
CGA-321113	12.6 min.