I. INTRODUCTION/SUMMARY

A. Scope

A method for the analytical determination of CGA-173506 and degradates (CGA-339833, CGA-192155, CGA-265378) in soil was developed by ABC Laboratories, Inc. (ABC), Columbia, MO. The limit of quantitation (LOQ) for all analytes is 0.010 µg/g (ppm) based on fortification experiments. The limit of detection (LOD) is 0.0025 µg/g (ppm) for CGA-339833 and CGA-192155 based on the lowest standard concentration injected with an 8 g/mL soil extract concentration. The LOD is 0.0050 µg/g (ppm) for CGA-265378 and CGA-173506 based on the lowest standard concentration injected with a 4 g/mL soil extract concentration. A two column switching HPLC system is used to quantitate samples with ultraviolet (UV) detection.

B. Principle

Extraction of analytes from the soil is accomplished by shaking for two, 10 minute periods with 90% acetonitrile (ACN):9% reagent grade water:1% glacial acetic acid (HOAc) containing 50 mg/L butylated hydroxy toluene (BHT). Solids are separated from the liquid extract by centrifugation. The extraction solvent is reduced to between 0.5 to 1 mL using vacuum rotary evaporation. The extract is then diluted to a 5 mL volume for injection onto the HPLC system for quantitation of CGA-265378 and CGA-173506. A 3 mL aliquot of the extract injected for CGA-265378 and CGA-173506 is then concentrated with a stream of nitrogen to a 1.5 mL volume prior to injection onto the HPLC system for quantitation of CGA-339833 and CGA-192155.

II. MATERIALS AND METHODS

A. Apparatus

Similar apparatus may be used for this method if deemed acceptable.

1.0 Balance, analytical (Sartorious 1702 MP8) or equivalent
2.0 Balance, top loading (Mettler PM 200)
3.0 Bottle, polyethylene, Silgan disposable 8 oz round, wide mouth, disposable
4.0 Centrifuge, IEC Centra GP8
5.0 Centrifuge tubes, 15-mL graduated
6.0 Culture tubes, 16 X 125 mm glass
7.0 Cylinder, graduated 50-mL
8.0 Cylinder, graduated 1000-mL

9.0 Cylinder, graduated 2000-mL
10.0 Flasks, flat bottom, 500-mL
11.0 Funnels, powder, 75 mm
12.0 Glass wool (Fisher cat. # 11-388)
13.0 Pasteur pipets, disposable
14.0 Pipettes, glass, class A certified, various volumes
15.0 Pipettes, repeater pipetters, various volumes
16.0 Rotary evaporator, Strong Innovations
17.0 Syringe, Hamilton, 500 μL
18.0 Vacuum pump (Welch 1402)
19.0 Ultrasonic bath (Branson 3200)
20.0 Vials, autosampler, clear, 12 X 32 mm, 2-mL
21.0 Vortex mixer (Thermolyne Maxi Mix II)

B. Reagents and Analytical Standards

Other brands of reagents may be substituted provided they are of equivalent purity, produce adequate recovery of all analytes, and a reagent blank produces no interferences. Use of other reagents has not been explored by ABC.

1.0 Acetic Acid, glacial, Fisher Scientific
2.0 Acetonitrile, HPLC grade, Burdick and Jackson
3.0 Ammonium Acetate, ACS reagent grade, Sigma
4.0 Butylated Hydroxy Toluene, Sigma
5.0 Dimethyl Sulfoxide, certified, Fisher Scientific
6.0 Reagent grade water (Type 1 water), Labconco Purification System
7.0 Phosphoric Acid, ACS reagent grade, J.T. Baker
8.0 CGA-339833, Standard reference material (Ciba-Geigy), Greensboro, NC
9.0 CGA-192155, Standard reference material (Ciba-Geigy), Greensboro, NC
10.0 CGA-265378, Standard reference material (Ciba-Geigy), Greensboro, NC
11.0 CGA-173506, Standard reference material (Ciba-Geigy), Greensboro, NC

C. Reagent Solutions and Mixtures

1.0 Extraction Solution. Combine 1800 mL of ACN with 100 ± 10 mg of BHT and mix to dissolve the BHT. After the BHT is dissolved, combine 180 mL reagent grade water and 20 mL of acetic acid (HOAc) with the ACN:BHT mixture and mix. Make fresh daily.

2.0 Dilution Solvent for Standards. Combine 100 mL of ACN, 395 mL of reagent grade water, and 5 mL of HOAc and mix.

3.0 Mobile Phase 1. Prepare 40% ACN:60% reagent grade water:5 mL/L HOAc (used for the isocratic chromatography of CGA-265378 and CGA-173506 on the first column, a Zorbax SB-CN HPLC column). For each liter of mobile phase, combine 400 mL of ACN with 600 mL of reagent grade water and add 5 mL/L HOAc. Mix the solution by stirring. Filter through a 0.45 µm filter membrane using a vacuum or sparge with helium for a minimum of 30 minutes before use.

4.0 Mobile Phase 2. Prepare 20% ACN:80% reagent grade water:5 mL/L HOAc:0.1 to 1.5 g/L NH_4OAc (used for the isocratic chromatography of CGA-339833 and CGA-192155 on the first column, a Zorbax SB-CN HPLC column). For each liter of mobile phase, combine 200 mL of ACN with 800 mL of reagent grade water, add 5 mL/L of HOAc and an amount of NH_4OAc that will give separation of CGA-339833 and CGA-192155 with a minimum of a 5 mL retention volume for the CGA-339833 peak. Mix the solution by stirring. Filter through a 0.45 µm filter membrane using a vacuum or sparge with helium for a minimum of 30 minutes before use.

5.0 Mobile Phase 3. Prepare 90% ACN:10% reagent grade water:5 mL/L HOAc (used to regenerate the first column, a Zorbax SB-CN HPLC column). For each liter of mobile phase, combine 900 mL of ACN with 100 mL of reagent grade water and add 5 mL/L of HOAc. Mix the solution by stirring. Filter through a 0.45 µm filter membrane using a vacuum or sparge with helium for a minimum of 30 minutes before use.

6.0 Mobile Phase 4. Prepare 40% ACN:60% reagent grade water:5 mL/L HOAc:0.20 g/L NH_4OAc (used isocratically on the second columns, which are Prodigy ODS2 for the determination of CGA-339833 and CGA-192155). For each liter of mobile phase, combine 400 mL of ACN with 600 mL of reagent grade water. Add 5 mL of HOAc and 0.20 g NH_4OAc for each liter of mobile phase. Mix the solution by stirring. Filter through

a 0.45 μm filter membrane using a vacuum or sparge with helium for a minimum of 30 minutes before use.

7.0 Mobile Phase 5. Prepare 50% ACN:50% reagent grade water:5 mL/L HOAc (used isocratically on the second columns, a Zorbax ODS and Spherisorb ODS2 HPLC columns for the determination of CGA-256378 and CGA-173506, respectively). For each liter of mobile phase, combine 500 mL of ACN with 500 mL of reagent grade water and add 5 mL/L of HOAc for each liter of mobile phase. Mix the solution by stirring. Filter through a 0.45 μm filter membrane using a vacuum or sparge with helium for a minimum of 30 minutes before use.

D. HPLC Columns and Supplies

Substitution of HPLC column brands may be acceptable if similar retention characteristics and freedom from matrix interferences can be demonstrated. If other columns are substituted, the secondary separation characteristics (ammonium ion concentration) for the substituted columns will have to be explored. The secondary interactions related to the SB-CN column are strong for all the analytes, while secondary interactions for CGA-339833 and CGA-192155 are also very strong on the ODS columns used. See the section, K.1.0, **Discussion of Special Chromatographic Separations Achievable**, for help regarding HPLC column selection.

1.0 4.6 X 150 mm Zorbax™ SB-CN (5μm) column (cat no. 883975.905)
2.0 4 X 12.5 mm Zorbax SB-CN (5μm) cartridge guard column (cat no. 820674.916)
3.0 Zorbax Reliance™ guard column hardware kit (cat no. 820529.901)
4.0 4.6 X 250 mm Zorbax ODS (5μm) column (cat no. 880952.702)
5.0 4.6 X 250 mm Phenomenex Prodigy™ 5 ODS2 (5μm) column (cat no. 006-3300-E0)
6.0 4.6 X 250 mm Spherisorb™ ODS2 (5μm) cartridge column (Phase Separations part no. 839540)
7.0 Column end fittings for Spherisorb cartridge columns (Phase Separations part no. 614100)

E. Safety and Health

As the reference substances used in this analysis have not been completely evaluated at this time, general laboratory safety is advised (e.g., safety glasses, gloves, etc. should be used).

F. Standard Solutions

After preparation, standard solutions are to be stored in brown glass bottles. Structures for each of the four analytes are presented in Figure 1.

1.0 Primary Stock Standard Solutions. Each primary stock standard solution is prepared individually by weighing the respective reference material into a weigh boat to achieve an approximate 10 mg weight. The weight of the individual reference material is recorded (to 0.1 mg), and the material is transferred to a 50 mL volumetric flask using 10 mL of ACN. The standard for CGA-173506 is diluted with ACN to volume.

The remaining standard solutions have 30 to 35 mL reagent grade water added to each; then 0.5 mL HOAc added to the volumetric, and the volumetric swirled to mix the contents. The solution is then made to volume with reagent grade water. The solutions are again mixed by inversion or an ultrasonic bath may be used to assure the reference materials are in solution. The concentration of each stock standard solution, corrected for percent purity, is calculated. Primary stock solutions are to be stored in a freezer (approximately -20 °C) when not in use.

2.0 Stock Mixed Standard and Spiking Solutions. Mixed standard and spiking solutions are made by diluting the primary stock standards using dilution solvent (20% ACN:79% reagent grade water:1% HOAc). The stock mixed standard is prepared by adding an aliquot of the individual stock standard solution containing 1000 μg of the reference standard to a 100-mL volumetric flask. The 100-mL flask is then diluted to volume with dilution solvent to give a 10 μg/mL mixed standard solution. The 10 μg/mL mixed standard solution is also diluted to make a 1 μg/mL mixed standard spiking solution. All mixed standard solutions should be stored refrigerated (approximately 4 °C) when not in use. Make fresh every three months.

3.0 Standard Curve Solutions. The standard curve, used to calibrate the instrument, is prepared by dilution of the 10 μg/mL mixed standard. Standards are prepared by dilution with standard diluent to be 1.0, 0.50,

0.20, 0.050, and 0.020 μg/mL. All mixed standard solutions should be stored refrigerated (approximately 4 °C) when not in use. Make fresh monthly.

The 0.020 μg/mL concentration should be used in the standard curve for the determination of CGA-265378 and CGA-173506. The 0.020 μg/mL standard level may be omitted from the standard curve for CGA-339833 and CGA-192155 determinations as the matrix concentration for these analytes is double that of the CGA-265378 and CGA-173506 screening levels. The different standard concentrations are injected onto the chromatograph to bracket and develop a standard response curve for a defined group of samples. The standard curve solutions are also used to spike fortified samples.

4.0 Individual Analyte Spiking and Standard Curve Solutions. Individual standard solutions are prepared by pipetting an aliquot of primary standard solution, equivalent to 400 μg, into a 100-mL volumetric flask and diluting with dilution solvent. This 4 μg/mL standard is then serially diluted to give an individual HPLC injection standard by diluting 10 mL of the 4 μg/mL standard solution to 50 mL with dilution solvent. This 0.80 μg/mL standard solution may be used to identify retention times for the individual analytes.

G. Sample Extraction

Step by step instructions follow for extraction of soil and preparation of the concentrated extract for injection onto an HPLC system. Figure 2 gives the steps for extract production in a flow chart format.

1.0 Weigh 20 ± 0.1 g of soil into a 250-mL plastic bottle.

2.0 Fortification solutions are added at this time to fortify samples at the appropriate level. Allow the fortification solutions to remain on the matrix for 5 to 10 minutes prior to addition of extraction solvent.

3.0 Add 100 mL of extraction solvent (90% ACN:9% reagent grade water:1% HOAc + 50 mg/L BHT) to each sub sample contained in a 250 mL bottle. Cap the bottles and place on a linear oscillating shaker for 10 minutes on high (280 oscillations/minute).

4.0 Remove the bottles from the shaker and place into groups of two. Balance each pair of bottles by adding extraction solvent to have the gross weight

agree to within 0.1 g. Place each group across from each other on the centrifuge.

5.0 Centrifuge at 3000 rpm for 2 minutes or a longer period as required to produce a clear supernatant.

6.0 Remove the bottles from the centrifuge. Decant the extraction liquid through a powder funnel plugged with glass wool (to remove the floating particulate matter) fitted on top of a 500-mL flat bottom flask.

7.0 Add 50 mL of extraction solvent and resuspend the solids by capping the extraction bottle and shaking by hand vigorously. Then place the bottles on the shaker on high for a second, 10 minute shaking period.

8.0 Again, balance the pairs of extraction bottles with extraction solvent as indicated in step 4 above and centrifuge as in step 5 above.

9.0 The liquid is decanted through the same glass wool plug as the initial extraction solvent into the same 500-mL flat bottom.

10.0 The glass wool plug is rinsed with 10 mL of extraction solvent and the glass wool plug is discarded.

11.0 The volume of the extract is reduced to 0.5 to 1 mL by vacuum rotary evaporation using a 20 to 40 °C water bath. By starting out the evaporation in a colder water bath, foaming and bumping may be avoided. After most of the ACN has been evaporated, drain the evaporation traps, and warm the water bath to speed the evaporation of the aqueous portion of the extract.

Note that in order to achieve acceptable recoveries, steps 12 to 15 must be followed!

12.0 Transfer the remaining extract volume to a 15-mL graduated centrifuge tube. Pipet 2.0 mL of ACN into the 500-mL flat bottom flask.

Grasp the neck of the flask and hold the flask so that the neck is in a horizontal position. While in this position, turn and swirl the flask in an ultrasonic bath so that the solids on the sides of the flask come into contact with the ACN while in the ultrasonic bath. The flask should be rotated a minimum of three revolutions over a 30 second period while holding the flask in a horizontal position, in order to dislodge the solids deposited in a

ring during rotary evaporation. The flask is then turned so that the flat bottom portion of the flask rests on the bottom of the ultrasonic bath, and the ACN and solids contained in the flask are sonicated for another 15 seconds.

The ACN rinse from the flat bottom flask is then transferred to the 15-mL centrifuge tube containing the original extract. Two milliliters of reagent grade water is then added to the flat bottom flask and the sonication process in the paragraph above is repeated.

The aqueous rinse from the flat bottom flask is added to the 15-mL centrifuge tube. If the volume of the tube goes above 5.0 mL, reduce the volume with a stream of nitrogen and a 30 to 40 °C water bath to achieve a 5.0 mL volume. If the volume of the tube is below 5.0 mL, add reagent grade water to achieve a 5.0 mL volume. Vortex the 15-mL centrifuge tube briefly and centrifuge for 10 minutes on a table top centrifuge.

13.0 Transfer 3.0 mL of the extract from step 12 above to a fresh 15-mL centrifuge tube using care not to disturb the solids. Use a stream of nitrogen and a 30 to 40 °C water bath to reduce the volume of the extract to 1 to 1.5 mL. Bring the volume of the extract to 1.5 mL with reagent grade water, vortex, and centrifuge for 10 minutes on a table top centrifuge. Place the extract into an injection vial and analyze for CGA-339833 and CGA-192155.

14.0 Transfer an aliquot of the solution from step 12 above to an injection vial. Use care to avoid transfer of solids and analyze for CGA-265378 and CGA-173506.

15.0 If sample extract dilutions are required for analyte response to be within the analytical curve, then dilute with dilution solvent used for the standards (20 ACN:79 reagent grade water:1 HOAc).

H. HPLC Chromatography (General Discussion)

A Shimadzu HPLC system was used to chromatograph the extracts and standards for this method. Similar instruments are acceptable substitutes. The system used employs three analytical HPLC columns with column to column switching and UV detection. A schematic of the hydraulic configuration for the analytical system is shown in Figure 3. General instrument parameters are given below.

Adjustment of mobile phase conditions may be required to provide resolution from matrix peaks and produce desirable retention times. In general, mobile phases must produce retention times providing 4 to 10 column void volumes. The retention volume for the first analyte peak on the first column should be 3 to 5 column volumes. The last analyte peak should apex at no more than 12 column volumes for the first column. All columns used as the second column should provide 4 to 8 column volumes. If the columns specified do not provide these retention volumes, then the mobile phases should be adjusted. See the section, K.1.0, Discussion of Special Chromatographic Separations Achievable in this report to afford easier mobile phase component adjustments.

Individual instrument parameters are included with each analytical run. Conditions may vary from the general description given below to account for variations in mobile phase preparation and the retention time variation of individual columns.

1.0 Injection Volume. Inject 200 µL for CGA-265378 and CGA-173506 or 300 µL for CGA-339833 and CGA-192155. If adequate signal to noise is achieved for the lowest standard, lower injection volumes may be used.

2.0 Autosampler Rinse Solution. 40% ACN:60% reagent grade water.

3.0 UV Detection.

Compound	Wavelength
CGA-339833	275 nm
CGA-192155	285 nm
CGA-265378	268 nm
CGA-173506	268 nm

I. HPLC Analysis of CGA-265378 and CGA-173506 (non-polar analytes)

1.0 First Column Specifications

A Zorbax SB-CN 4.6 mm internal diameter by 15 cm length with 5μ CN packing material with a Zorbax SB-CN 4 X 12.5 mm cartridge guard column was used as the first column. A similar column may be used if analyte and matrix peaks are demonstrated to be resolved adequately. Since this is the initial column used, recovery of analytes and separation of matrix coextractive peaks through both columns must be demonstrated.

2.0 First Pump Flow Rate

A flow rate of 0.7 to 1.5-mL/min may be used for this analysis. The normal flow rate is 1.0 mL/min, but may be lowered or raised depending on the back pressure of the analytical columns during the column switching time when the two columns are connected in series.

3.0 First Column Mobile Phase for CGA-265378 and CGA-173506

A 40% ACN:60% reagent grade water with 5 mL/L HOAc (Mobile Phase 1) is used to elute the analytes isocratically from the first column. After the analytes have eluted from the first column, the mobile phase is immediately taken to 90% ACN:10% reagent grade water with 5 mL/L HOAc at 2 mL/min (Mobile Phase 3) for 5 minutes to remove the strongly retained soil coextracted compounds. After 5 minutes of pumping mobile phase 3, mobile phase 1 is again pumped for 10 minutes at 2 mL/min to equilibrate the column prior to the next injection. The 2 mL/min flow rate is lowered to 1 mL/min for at least 1 minute prior to the next injection to allow for flow rate consistency during the next injection.

4.0 Normal Retention Times for Column 1 at 1 mL/min flow

Compound	Time (min)
CGA-265378	7 - 8
CGA-173506	9 - 11

An example chromatogram for the two analytes with the mobile phase and conditions specified above is shown as Figure 4. The peaks in this chromatogram for CGA-192155 and CGA-339833 are shown to demonstrate their elution order relative to the non-polar compounds. Variations in columns or mobile phase can cause these two compounds to

move such that they may co-elute. They will be separated from the non-polar compounds on the ODS2 columns.

5.0 Establishment of Switching Valve Times

The SB-CN column is eluted with 1.0 mL/min of mobile phase 1. The eluant from the column is directed to a UV detector set at 268 nm wavelength. Chromatograms are acquired for three replicate injections of the 1.0 μg/mL standard. Retention times for each of the analytes must be within 0.1 minutes for each of the three injections. If retention times are not within $\pm$ 0.05 minutes, the system should be evaluated and/or repaired before analysis is started. The column should be timed for the volume of injection to be used for analysis. Different injection volumes will influence the retention times. To correctly set the peak window, the same injection volume must be used for timing as used for the analysis.

The peak tailing factor (calculated at 5% of peak height)[1] should be less than 1.8 for all of the analytes eluting from the SB-CN column. If excessive tailing is observed, replacement of the guard column is indicated. If peak tailing is still unsatisfactory, replacement of the analytical column is indicated.

Establishment of switching valve times for the SB-CN column should be performed routinely after any of the following have occurred; 1) The column has not been used for a day, 2) A new mobile phase is made, 3) Any suggestion of retention time shifting such as drifting peak height response after the second column, or 4) Injection volume is changed.

Switching valve times for transfer of the eluant from the first column to the second column are set so that the transfer times will encompass all three injections made during the column timing procedure. This is accomplished by overlaying chromatograms acquired during column timing. The switching valve initiation time is set to be just as the analytical peak rises from the base line. The switching valve return time is set to be just as the peak tail comes back to base line.

The switching valves are placed in series (see Figure 3) so that the flow from the SB-CN column can enter the first valve (Valve A) and can be directed to a second column. When Valve A is in the initial position, the flow continues through Valve A and into Valve B. As either valve position is changed, the flow from the SB-CN column is directed onto the second column.

6.0 Second Columns

Two, 4.6 mm internal diameter x 25 cm length ODS (5μm), columns are used as the second column for the analysis of both CGA-265378 and CGA-173506. The two columns are used by connecting each one of the columns to a switching valve (see Figure 3). A Spherisorb ODS2 cartridge column (CGA-173506 cut) or a Zorbax ODS column (CGA-265378 cut) have both been used successfully as second columns to chromatograph these extracts for the respective compounds.

7.0 Pump Flow Rates for Second Columns

A flow rate of 1 to 2-mL/min may be used for these columns. The normal flow rate is 1.0 mL/min, but may be lowered or raised depending on the back pressure of the analytical column.

8.0 Second Column Mobile Phase

The mobile phase used for both columns is 50% ACN:50% reagent grade water with 5 mL/L HOAc (Mobile phase 5).

J. HPLC Analysis of CGA-339833 and CGA-192155 (polar analytes)

1.0 First Column

Specifications of the first column are the same as that column listed under section I.1.0. A similar column may be used if analyte and matrix peaks are demonstrated to be resolved adequately. Since this is the initial column used, recovery of analytes and freedom from matrix coextractive peaks through both columns must also be demonstrated.

2.0 First Pump Flow Rate

A flow rate of 0.7 to 1.5-mL/min may be used for this analysis. The normal flow rate is 1.0 mL/min, but may be lowered or raised depending on the back pressure of the analytical columns during the column switching time when two columns are connected in series.

3.0 First Column Mobile Phase for CGA-339833 and CGA-192155

A 20% ACN:80% reagent grade water with 5 mL/L HOAc and 0.1 to 1.5 g/L NH_4OAc (Mobile Phase 2) is used to elute the analytes isocratically from the first column. After the analytes have eluted from the first

column, the mobile phase is immediately taken to 90% ACN:10% reagent grade water with 5 mL/L HOAc at 2 mL/min (Mobile Phase 3) for 5 minutes to remove the strongly retained soil coextracted compounds. After 5 minutes of pumping mobile phase 3, mobile phase 2 is again pumped for 10 minutes at 2 mL/min to equilibrate the column prior to the next injection. The 2 mL/min flow rate is lowered to 1 mL/min for at least 1 minute prior to the next injection to allow for flow rate consistency during the next injection.

4.0 Normal Retention Times for Column 1 at 1 mL/min flow

Compound	Time (min)
CGA-339833	7 - 8
CGA-192155	9 - 11

An example chromatogram for the two analytes with the mobile phase and conditions specified above is shown as Figure 5.

5.0 Establishment of Switching Valve Times

The SB-CN column is eluted with 1.0 mL/min of mobile phase 2 and the eluant from the column directed to the UV detector set at 268 nm wavelength. Chromatograms are acquired for three replicate injections of the 1.0 µg/mL standard. Retention times for each of the analytes must be within 0.1 minutes for each of the three injections. If retention times are not within ± 0.05 minutes, the system should be evaluated and/or repaired before analysis is started. The column should be timed for the volume of injection to be used for analysis. Different injection volumes will influence the retention times, and the peak window may be set incorrectly.

The peak tailing factor (calculated at 5% of peak height)[1] should be less than 1.8 for all of the of analytes eluting from the SB-CN column. If excessive tailing is observed, replacement of the guard column is indicated. If peak tailing is still unsatisfactory, replacement of the analytical column is indicated.

Establishment of switching valve times for the SB-CN column should be performed routinely after any of the following have occurred; 1) The column has not been used for a day, 2) A new mobile phase is made, 3) Any suggestion of retention time shifting such as drifting peak height response after the second column, or 4) Injection volume is changed.

Switching valve times for transfer of the eluant from the first column to the second columns are set so that the transfer times will encompass all three injections made during the column timing procedure. This is accomplished by overlaying chromatograms acquired during column timing. The switching valve initiation time is set to be just as the analytical peak rises from the base line. The switching valve return time is set to be just as the peak tail comes back to base line.

The switching valves are placed in series (see Figure 3) so that the flow from the SB-CN column can enter the first valve (Valve A) and can be directed to a second column. When Valve A is in the initial position, the flow continues through Valve A and into Valve B. As either valve position is changed, the flow from the SB-CN column is directed onto the second column.

6.0 Second Columns

Two, 4.6 mm internal diameter x 25 cm length (5μm) Prodigy ODS2, columns are used as the second column for the analysis of both CGA-339833 and CGA-192155. The two identical columns are used by connecting each one of the columns to a switching valve (see Figure 3). Best results have been obtained by using the specified ODS2 columns.

7.0 Pump Flow Rates

A flow rate of 1 to 2 mL/min may be used for these columns. The normal flow rate is 1.0 mL/min, but may be lowered or raised depending on the back pressure of the analytical column.

8.0 Second Column Mobile Phase

The mobile phase used for both columns is 40% ACN:60% reagent grade water with 5 mL/L HOAc and 0.2 g/L NH_4OAc (Mobile phase 4).

K. Modifications and Potential Problems

1.0 Discussion of Special Chromatographic Separations Achievable

All of these special conditions are discussed to enable the chromatographer to prepare mobile phases that may be used to resolve analytes from matrix peaks if needed. Additionally, when starting with new columns, the chromatographer may not be able to use the same mobile phases indicated in this method for the SB-CN or the Prodigy ODS2 columns. Hopefully, this discussion will be a valuable aid in establishing acceptable chromatography conditions with new columns, or provide an analyst unfamiliar with this method some insight into the special separation characteristics related to the SB-CN and the Prodigy ODS2 columns.

Chromatographic separation and retention times are dependent on ACN concentration for all analytes on all columns and all compounds do respond in a reverse phase manner. Additionally, separation and retention time for CGA-339833 and CGA-192155 rely heavily on ammonium ion concentration (0 to 2 g/L NH_4OAc) on both the Zorbax SB-CN column and the Prodigy ODS2 under acidic conditions (5 mL/L HOAc). CGA-339833 and CGA-192155 retention times on both columns decrease as the ammonium ion concentration increases with the acidity and ACN levels held constant. The retention time effect of ammonium ion concentration is greater for CGA-339833 on both the Prodigy ODS2 and the SB-CN column than for CGA-192155. The difference in ammonium ion effect for CGA-339833 and CGA-192155 on both these columns (SB-CN and the Prodigy ODS2) is so great that the elution order of CGA-339833 and CGA-192155 can be inverted. Additionally, CGA-339833 may not elute from these columns in the absence of ammonium ion. It has been found that all of these conditions identified for individual columns are able to provide stable retention times for analysis.

2.0 Separations on the SB-CN Column

To find the proper concentration of ammonium ion, it is easier to prepare a 20% ACN:80% reagent grade water mobile phase with 5 mL/L HOAc, remove a portion of that mobile phase and add enough NH_4OAc to attain a 2 g/L concentration. By using the gradient pumps to mix these mobile phases, one can quickly identify the proper concentration of NH_4OAc to achieve acceptable retention and resolution for CGA-339833 and CGA-192155 on the SB-CN column.

By using different concentrations of NH_4OAc, the retention times for CGA-339833 will move greatly, while CGA-192155 will move to a lesser extent. Separation of the CGA-339833 and CGA-192155 from each other, or from matrix interferences may be achieved by adjusting the NH_4OAc and ACN concentrations in the mobile phase.

Retention times for CGA-265378 and CGA-173506 are not affected by NH_4OAc. When using the 20% ACN mobile phase discussed in this section, the retention times for both the CGA-265378 and CGA-173506 analytes is greater than 20 column volumes, however, if the mixed standards are used to time the columns, these compounds will eventually elute.

3.0 Separations on ODS Columns

Evaluation of ammonium ion concentration on ODS columns gave a more pronounced effect for CGA-339833 than for CGA-192155. An ammonium ion effect was not noted for CGA-265378 and CGA-173506. It has been found that the use of the Prodigy ODS2 column does not provide the resolution for matrix peaks for both CGA-265378 and CGA-173506 that the Zorbax ODS and Spherisorb ODS2 columns provide, respectively.

All of these ammonium ion interactions, in combination with ACN concentration on both the SB-CN and the ODS columns, may be used to enhance resolution from matrix peaks (interferences) if the need arises.

4.0 Regeneration of SB-CN HPLC Columns

After extended use of the SB-CN column, it has been found that the peak shape deteriorates. This is evidenced by tailing or split peaks found during timing of the SB-CN column. These columns have been successfully restored to obtain the original peak shapes for all analytes by pumping 40 to 60 mL of 100% dimethyl sulfoxide through the column with the flow reversed through the column.

5.0 Loss of Analytes

Recovery of analytes can be influenced by the technique used when reconstituting the rotary evaporated soil extract.. It is imperative that the reconstitution method be followed as indicated in this method and the soil extracts be used and generated as indicated in this method.

CGA-173506 and CGA-265378 partition into the soil coextracted material and must be removed by the addition of ACN first, then sonication used to remove the analytes from the precipitated soil coextracts. This ACN rinse is then transferred from the flask, and finally the flask rinsed with reagent grade water.

The concentrated extracts injected for the polar metabolites (CGA-339833 and CGA-192155), should not be used for the analysis of the non-polar metabolites. The non-polar metabolites partition into the precipitated soil coextractives generated by removing the ACN during concentration.

The non-polar metabolite extracts should not be used for analysis of the polar metabolites as the level of ACN will cause the polar metabolites to move from the retention window established for column switching on the first column.

During development of this method, it was noted that CGA-265378 recoveries were severely reduced in the presence of acetone or ethyl ether. It was hypothesized that there was an oxidative conversion to some other unidentified analyte. Low recoveries were also noticed with increased soil organic matter such as found in the thatch-sod (0 to 7.5 cm) from a turf plot study. In order to potentially buffer this loss, the initial method contained 50 ppm BHT in the extraction solution.

6.0 Confirmatory Techniques

No confirmatory analysis procedure has been developed at this time.

7.0 Time Requirement

A set of 12 samples can be extracted during a six hour period by one person.

Chromatography is accomplished using automated equipment. A set of twelve samples will require 20 injections of extracts and standards at a run time of about 30 minutes each. An entire set of extracts can be injected in about 10 hours. Data reduction requires an additional 2 hours.

Injection of the set must be repeated in order to acquire data for the second group of analytes. The same amount of time will be required for the

second injection set. The total instrument time required for a set of 12 samples is approximately 20 hours.

L. Data Acquisition and Calculations

MULTICHROM™ is a computer program in use at ABC which allows for data acquisition, data analysis, results reporting, and information management. Peak response for standards and sample extracts are measured using the MULTICHROM system. The MULTICHROM system uses the concentration (µg/mL) versus peak response to form a regression curve and from that interpolate the concentration of analytes in the extracts. This value is then converted to parts per million (ppm) in the sample by entering the final sample volume, any dilution factors, and the initial sample weight into the MULTICHROM program. MULTICHROM calculates sample concentrations according to the following equation:

$$ppm\ found = \frac{(\mu g/mL\ found)\ x\ (final\ volume\ mL)\ x\ (dilution\ factor)}{sample\ weight\ (g)}$$

The dilution factor if required, is calculated as follows. If no dilution is needed, then a 1 is entered into the calculation.

$$dilution\ factor = \frac{final\ volume\ (mL)}{aliquot\ volume\ (mL)}$$

Data from the MULTICHROM program are then entered into Excel® to calculate the recovery of fortified samples. Recovery of the fortified samples is calculated by subtracting the peak response for the control (average if more than one control is in a sample set) from the peak response of the fortified samples and then interpolating the concentration in the fortified extract. Recovery is then calculated according to the following formula:

$$percent\ recovery = \frac{(ppm\ found\ x\ 100)}{ppm\ fortified}$$

Moisture determinations for samples are performed according to the ABC soil moisture determination SOP FC 1.7.3. This SOP requires that samples be dried in an oven at 105 °C overnight, removed and weighed. Samples are returned to the oven for a second drying period and reweighed to prove that the second dry weight is within 1% of the first dry weight. If the first dry weight and the second dry weight do not agree, then the SOP provides for a third drying period with the

expected 1% agreement still in effect. The actual calculation for soil moisture is defined by the formula with all weights recorded in grams:

$$\text{soil moisture \%} = \frac{[(\text{Wet sample} + \text{container wt.}) - (\text{Dry sample} + \text{container wt.})] \times 100}{[(\text{Wet sample} + \text{container wt.}) - (\text{weight of container})]}$$

Sample moisture is then used to correct sample residue levels for moisture content on a dry weight basis. The formula used to correct for moisture content is given by:

$$\text{ppm corrected for moisture} = \frac{\text{ppm found} \times 100}{100 - \text{soil moisture \%}}$$

Figure 1. Chemical Structures

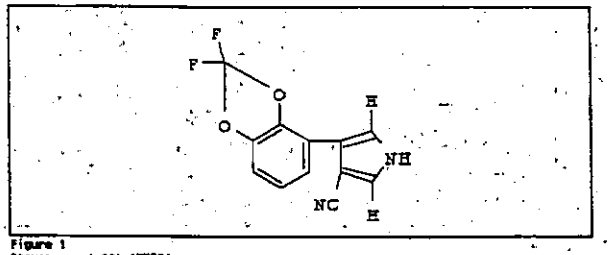

Figure 1
Structure of CGA-173506

Ciba Code: CGA-173506
Molecular Formula: $C_{12}H_4F_2N_2O_2$
Molecular Weight: 248.191
CAS Number: 131341-86-1
CAS Name: 1H-Pyrrole-3-carbonitrile, 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-

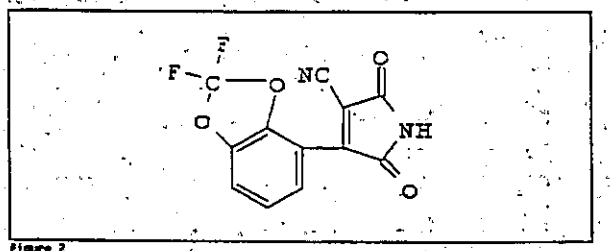

Figure 2
Structure of CGA-265378

Ciba Code: CGA-265378
Molecular Formula: $C_{12}H_4F_2N_2O_4$
Molecular Weight: 278.173
CAS Number: not assigned
IUPAC Name: 4-(2,2-Difluoro-benzo[1,3]dioxol-4yl)-2,5-dioxo-2,5-dihydro-1-*H*-pyrrole-3-carbonitrile

Figure 1.(con't) Chemical Structures

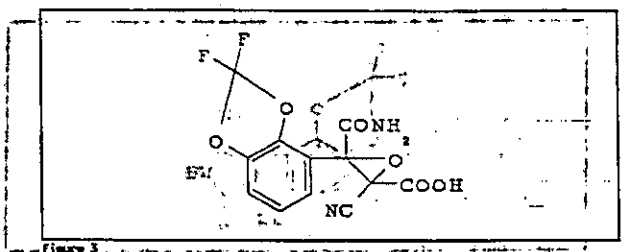

Figure 3
Structure of CGA-339833

Ciba Code:	CGA-339833
Molecular Formula:	$C_{12}H_6F_2N_2O_6$
Molecular Weight:	312.188
CAS Number:	not assigned
CAS Name:	Oxiranecarboxylic acid, 3-(aminocarbonyl)-2-cyano-3-(2,2-difluoro-1,3-benzodioxol-4-yl)-, cis

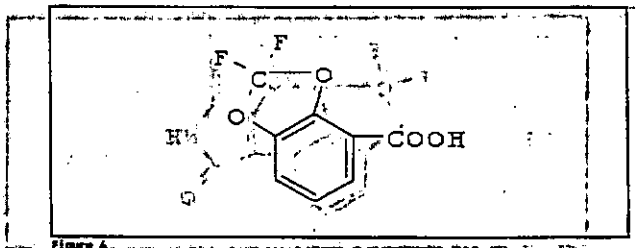

Figure 4.
Structure of CGA-192155

Ciba Code:	CGA-192155
Molecular Formula:	$C_8H_4F_2O_4$
Molecular Weight:	202.115
CAS Number:	126120-85-2
CAS Name:	1,3-Benzodioxole-4-carboxcylic acid, 2,2-difluoro-

Figure 2. Extraction and Analysis Scheme

Weigh 20 g matrix into a 250 mL polyethylene bottle
(Fortify if required)

↓

Add 100 mL of 90 ACN:9 H_2O:1 HOAc with 50 mg/L BHT

↓

Shake at 280 oscillations/min (high) for 10 minutes

↓

Balance opposing bottles with extraction solvent

↓

Centrifuge 2 minutes @ 3000 rpm to remove suspended soil

↓

Decant liquid through a glass wool plugged powder funnel into a 500 mL flat bottom

↓

Extract a second 10 minute period with 50 mL of extraction solvent

↓

Balance opposing bottles with extraction solvent

↓

Centrifuge 2 minutes @ 3000 rpm to remove suspended soil

↓

Decant liquid through the same glass wool plugged powder funnel into the same 500 mL flat bottom

↓

Rotary evaporate the extraction liquids in the 500 mL flat bottom to 0.5 to 1 mL using a 20 to 40 °C water bath

↓

Transfer the remaining liquid to a 15 mL graduated centrifuge tube

↓

Pipet 2 mL of ACN to the 500 mL flat bottom and sonicate while rotating the flask to bring all previously wetted surfaces into contact with the ACN and transfer this completely to the 15 mL centrifuge tube

↓

Use 2 mL of reagent grade water to wash and complete the transfer, then bring the volume to 5 mL for the extract

↓

Vortex the 15 mL tube and then centrifuge to clarify

↓

Sample extract is ready to inject for CGA-265378 and CGA-173506 onto a column switching HPLC with UV detection

↓

Transfer 3 mL of the extract to a 15 mL graduated tube and take the volume to 1.0 to 1.5 mL with a stream of nitrogen

↓

Add reagent grade water to make a 1.5 mL volume

↓

Sample extract is ready to inject for CGA-339833 and CGA-192155 onto a column switching HPLC with UV detection