I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of CGA-173506 and degradation products, CGA-339833 and CGA-192155, (see Figure 1. for structures) in turf grass clippings and thatch-sod matrices defined in Ciba Protocol Nos. 32-95/33-95 (ABC Study Nos. 42162 and 42163). The limit of quantification (LOQ) for all analytes in grass and thatch-sod matrices is 0.050 ppm and 0.010 ppm, respectively, based on fortification experiments. The limit of detection (LOD) for all analytes in grass and thatch-sod matrices is 0.020 ppm and 0.0050 ppm, respectively. A two column switching HPLC system is used to quantitate samples with ultraviolet (UV) detection for all analytes. Alternatively, a single ODS column may be used for the quantitation of CGA-173506.

B. Principle

Extraction of analytes from the matrix is accomplished by shaking for two, 10-minute periods with 90% acetonitrile (ACN):9% 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 5 to 10 mL using vacuum rotary evaporation. The extract is then transferred to a 250-mL separatory funnel with 100 mL each of water and methylene chloride (DCM). The water and DCM are partitioned with both the aqueous and the DCM fractions retained.

The aqueous fraction is saturated with NaCl and CGA-339833 is partitioned into ethyl acetate (EtOAc). The EtOAc is removed and the extract taken to dryness using rotary evaporation. The dried extract is then brought to a 5-mL volume for injection onto a two column switching HPLC.

The DCM fraction is partitioned with pH 8.5 K_2HPO_4 aqueous buffer to remove the CGA-192155 from the DCM and leave the CGA-173506 in the DCM fraction. The aqueous portion is acidified with HOAc and the CGA-192155 is partitioned into DCM. The DCM is removed by rotary evaporation and the dried extract brought to a 5-mL volume for injection onto a two column switching HPLC.

The DCM fraction containing the CGA-173506 is taken to dryness by rotary evaporation and the extract is reconstituted with toluene for silica gel column cleanup. The extract is transferred to a dry silica gel column with toluene, matrix interferences removed by elution with toluene, and CGA-173506 eluted from the column with DCM. The DCM is removed using rotary vacuum evaporation and the dried extract reconstituted to 5 mL for injection onto an HPLC.

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 Chromatography columns, 10 mm I.D. bore glass with reservoir and teflon stopcock
8.0 Cylinder, graduated, 50-mL, 100-mL, 1000-mL, and 2000-mL
9.0 Flasks, Erlenmeyer, 250-mL
10.0 Flasks, flat bottom, 500-mL
11.0 Funnels, powder, 75-mm
12.0 Glass Wool (Fisher cat. # 11-388)
13.0 Magnetic stir plates
14.0 Magnetic stir bars, teflon coated
15.0 Pasteur pipets, disposable
16.0 Pipets, glass, class A certified, various volumes
17.0 Pipets, repeater pipettes, various volumes
18.0 Rotary evaporator, Strong Innovations
19.0 Separatory funnels, 250-mL and 500-mL
20.0 Syringe, Hamilton, 500-µL
21.0 Vacuum pump (Welch 1402)
22.0 Ultrasonic bath (Branson 3200)
23.0 Vials, autosampler, clear, 12 X 32-mm, 2-mL
24.0 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, ACS plus, Fisher Scientific
2.0 Acetonitrile, HPLC grade Burdick and Jackson
3.0 Ammonium Acetate, ACS reagent grade, Sigma
4.0 L-Ascorbic Acid, Fisher Scientific

5.0 Butylated Hydroxy Toluene (BHT), Sigma
6.0 Dimethyl Sulfoxide, Certified, Fisher Scientific
7.0 Ethyl Acetate, Pesticide grade, Burdick and Jackson
8.0 Methylene Chloride, Pesticide grade, Burdick and Jackson
9.0 Reagent grade water (Type 1 water), Labconco Purification System
10.0 Potassium Phosphate (dibasic), ACS Reagent grade, J.T. Baker
11.0 Potassium Hydroxide, ACS Reagent grade, J.T. Baker
12.0 Phosphoric Acid, ACS reagent grade, J.T. Baker
13.0 Sodium Chloride, ACS Reagent grade, J.T. Baker
14.0 Sodium Sulfate, anhydrous, ACS Reagent grade, J.T. Baker
15.0 Silica Gel 60, EM Science catalog no. 7734-5
16.0 Toluene, Pesticide grade, Burdick and Jackson
17.0 CGA-339833, Standard reference material (Ciba-Geigy), Greensboro, NC
18.0 CGA-192155, Standard reference material (Ciba-Geigy), Greensboro, NC
19.0 CGA-173506, Standard reference material (Ciba-Geigy), Greensboro, NC

C. Reagent Solutions and Mixtures

1.0 Extraction Solution. Combine 1800-mL 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 HOAc with the ACN/BHT mixture and mix. Make fresh daily.

2.0 Saturated NaCl solution. Prepare by adding 1000 g of NaCl slowly to 2500-mL reagent grade water while stirring the water. Stir for 30 minutes to attain a saturated solution. Solid NaCl should be observed after stirring.

3.0 0.1 M KH_2PO_4 (pH=8.5) buffer. Add 17.4 g/L of potassium phosphate dibasic to 1-L reagent grade water. Stir to dissolve and adjust the pH to 8.5 ±0.1 with phosphoric acid and/or potassium hydroxide.

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

5.0 Reconstitution Solvent for Extracts. Combine 2-mL glacial acetic acid with 98-mL reagent grade water.

6.0 Mobile Phase 1. Prepare 40% ACN:60% reagent grade water with 5-mL/L HOAc (used for the isocratic chromatography of CGA-173506 on the first column, a Zorbax SB-CN HPLC column, and isocractic chromatography of GCA-192155 on the second column, a Prodigy ODS-2 HPLC column). For each liter of mobile phase, combine 400-mL 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 as mobile phase 1.

7.0 Mobile Phase 2. Prepare 20% ACN:80% reagent grade water with 5-mL/L HOAc and 0.75-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 ACN with 800 mL of reagent grade water and add 5-mL/L of HOAc plus 0.75-g/L NH_4OAc. 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 as mobile phase 2.

8.0 Mobile Phase 3. Prepare 90% ACN:10% reagent grade water with 5-mL/L HOAc (used to regenerate the first column, a Zorbax SB-CN HPLC column or the ODS column when used as a single column system). For each liter of mobile phase, combine 900-mL 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 as mobile phase 3.

9.0 Mobile Phase 4. Prepare 40% ACN:60% reagent grade water:5-mL/L HOAc:0.10-g/L NH_4OAc (used isocratically on the second column, a Prodigy ODS-2 for the determination of CGA-339833). For each liter of mobile phase, combine 400-mL ACN with 600 mL of reagent grade water. Add 5 mL of HOAc and 0.10-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 as mobile phase 4.

10.0 Mobile Phase 5. Prepare 50% ACN:50% reagent grade water with 5 mL/L of HOAc (used isocratically on the Spherisorb ODS2 HPLC column for the determination CGA-173506). For each liter of mobile phase, combine 500-mL 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 as mobile phase 5.

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 section P., "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 Phenomenex Prodigy™ 5 ODS-2 (5μm) column (cat no. 006-3300-E0)
5.0 4.6 X 250 mm Spherisorb™ ODS2 (5μm) cartridge column (Phase Separations part no. 839540)
6.0 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

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 must be diluted with ACN to volume in order to prevent precipitation of solid CGA-173506. Water is not added to this stock solution of CGA-173506.

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 and 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 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 to be at 1.0, 0.50, 0.20, 0.050, and 0.020 µg/mL. Each standard curve point is also made to contain 50-µg/mL L-ascorbic acid by addition of 4 mL of a 1.25 mg/mL aqueous solution to each 100 mL of standard preparation. All standard curve solutions should be stored refrigerated (approximately 4 °C) when not in use. Make fresh every month.

The different standard concentrations are injected onto the chromatograph to bracket and develop a standard response curve for a defined group of 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.

After preparation, all standard solutions are to be stored in brown glass bottles.

G. Extraction from Matrix (Figure 2.)

1.0 Weigh 5 ± 0.1 g of grass (20 ± 0.1 g of thatch-sod) into a 250-mL polyethylene plastic bottle.

2.0 Fortification solutions are added at this time to provide spiked 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% water:1% HOAc + 50 mg/L of BHT) to each sub-sample contained in a 250-mL bottle. Cap the bottles and place on the 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 4 and 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 5 to 10 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.

H. Partitioning into Fractions A (CGA-339833), B (CGA-192155), and C (CGA-173506) (Figure 2. con't)

1.0 Add 100 mL of water to the concentrated extraction liquid in the 500-mL flat bottom and swirl to rinse the flask. Transfer the aqueous extraction liquid to a 250-mL separatory funnel. Rinse the concentration flask with 100 mL of DCM by swirling, and place in the 250-mL separatory funnel also. Add 5-mL saturated NaCl solution to the separatory funnel.

2.0 Shake the separatory funnel for 1 minute and allow the phases to separate. Drain the lower phase into a second 500-mL separatory funnel.

3.0 Add 50 mL of DCM to the aqueous portion and shake for 1 minute.

4.0 After the phases have separated, drain the lower organic phase into the 500-mL separatory funnel containing the first organic portion. Reserve the aqueous portion as fraction A containing CGA-339833. The separatory funnel containing the DCM phase contains fractions B and C (CGA-192155 and CGA-173506, respectively).

I. Partitioning for Fraction A (Figure 2. con't)

1.0 Drain fraction A into a 250-mL Erlenmeyer flask containing 40-cm^3 solid granular NaCl and stir slowly for a minimum of 10 minutes to saturate the aqueous solution.

2.0 Decant the liquid, leaving excess solid NaCl in the flat bottom flask, into the original 250-mL separatory funnel and add 100 mL of EtOAc. Partition by shaking for 1 minute and allow the phases to separate. Drain the lower phase into the 250-mL Erlenmeyer flask used to saturate the aqueous and place the organic layer into a 500-mL flat bottom flask.

3.0 Return the aqueous portion to the 250-mL separatory funnel by decanting and add 100 mL of EtOAc. Shake for 1 minute to extract and allow the phases to separate. Drain the lower phase into the 250-mL Erlenmeyer flask used to saturate the aqueous and place the organic layer into a 1000-mL flat bottom flask.

4.0 Repeat the partition with ethyl acetate once more, for a total of three partitions.

5.0 Discard the aqueous portion and add the organic portion to a 1000-mL flat bottom.

6.0 Rotary evaporate to dryness using a 20 to 40 °C water bath and a vacuum. **Note that a large amount of NaCl will be present; this does not cause a problem.**

7.0 Reconstitute by pipetting 1-mL ACN into the flask. Turn the flask on its side to wet the inside with the ACN while sonicating. Then pipet 4 mL of 2% HOAc aqueous and sonicate the flask while wetting the sides. This is done by holding the flask on its side and turning in the ultrasonic bath for 10 to 20 seconds for each solvent addition. The extract is ready for HPLC injection to determine CGA-339833.

If extracts are deemed to have excessive particulate matter, they may be centrifuged and/or filtered to clarify.

J. Partitioning of Fraction B and C (Figure 2. con't)

1.0 The separatory funnel containing the DCM phase contains fractions B and C. The DCM is partitioned against 100 mL of 0.10 M K_2HPO_4 aqueous adjusted to pH 8.5 plus 5-mL saturated NaCl solution. After shaking for 1 minute, the phases are allowed to separate and the lower organic phase is drained into a 500-mL flat bottom. The aqueous layer is drained into a 250-mL Erlenmeyer flask and retained for CGA-192155 analysis.

2.0 The DCM from 1.0 above is returned to the 250-mL separatory funnel originally containing that fraction. Add 50 mL of 0.10 M K_2HPO_4 aqueous adjusted to pH 8.5 to the DCM. Shake for one minute and allow the phases to separate. Drain off the lower DCM layer into the 500-mL flat bottom that had contained it previously and retain for fraction C (containing CGA-173506).

3.0 Add the 100 mL of aqueous pH 8.5 buffer contained in the 250-mL Erlenmeyer flask and retained for CGA-192155 back into the 250-mL separatory funnel containing the 50 mL of pH 8.5 buffer for that sample. Retain the pH 8.5 buffer for the partitioning of fraction B.

K. Partitioning for Fraction B

1.0 Acidify fraction B (aqueous pH 8.5 buffer) with 5-mL glacial HOAc. Partition fraction B with 100-mL DCM for 1 minute and allow the phases to separate. Drain the lower organic phase into a 500-mL flat bottom flask.

2.0 Extract the aqueous with a second 100 mL of DCM by shaking for 30 seconds and allowing the phases to separate. Drain the lower organic phase into the original 500-mL flat bottom and discard the aqueous.

3.0 Rotary evaporate to dryness using a 20 to 40 °C water bath and a vacuum.

4.0 Reconstitute by pipetting 1-mL ACN into the flask. Turn the flask on its side to wet the inside with the ACN while sonicating. Then pipet 4 mL of 2% HOAc aqueous and sonicate the flask while wetting the sides. This is done by holding the flask on its side and turning in the ultrasonic bath for 10 to 20 seconds after each solvent addition. The extract is ready for HPLC injection to determine CGA-192155.

If extracts are deemed to have excessive particulate matter, they may be centrifuged and/or filtered to clarify.

L. Column Cleanup of Fraction C (Figure 2. con't)

1.0 Evaporate the DCM to dryness using a rotary vacuum evaporator and a 20 to 40 °C water bath. This includes the HOAc partitioned into the DCM remaining in the flask after the DCM has evaporated.

2.0 Prepare a 10 mm I.D. silica gel column by plugging the outlet end with glass wool. Add 10 mL of dry silica gel to the column and tap to pack the

silica gel. Add enough anhydrous Na_2SO_4 to provide a layer 10 to 25 mm deep on the top of the column.

3.0 Transfer the contents of the flask to the dry silica gel column by swirling with 2 X 5 mL portions of toluene.

4.0 Rinse the upper portion of the column into the column bed with a third 5-mL portion of toluene.

5.0 Elute the column with 25 mL more of toluene to remove potential interferences and discard all toluene fractions.

6.0 Elute CGA-173506 from the column with 50 mL of DCM into a 125-mL flat bottom flask.

Note that a column profile should be performed when using a new lot of silica. Removal of the non polar fractions from the column with the toluene rinses and elution is required and the volume of toluene may be increased to the point where CGA-173506 starts to elute from the column. The volume of DCM may be increased to not more than 100 mL if recovery of the CGA-173506 is not greater than 90% as indicated by a column profile. Alternatively, the elution solvent can be modified to be more polar with acetone.

7.0 Evaporate the elution solvents in the 125-mL flat bottom flask to dryness using vacuum rotary evaporation with a 20 to 40 °C water bath.

8.0 Reconstitute by pipetting 2-mL ACN into the flask. Turn the flask on its side to wet the inside with the ACN while sonicating. Then pipet 3 mL of 2% HOAc aqueous into the flask and sonicate while wetting the sides. This is done by holding the flask on its side and turning it in the ultrasonic bath for 10 to 20 seconds for each solvent addition. Transfer the contents of the flask to a 16 X 125 mm culture tube and cap. Refrigerate the reconstituted contents overnight to allow precipitation of the matrix coextractive materials.

9.0 Centrifuge or filter the extracts to produce clear extracts for injection onto the HPLC. Vial the extracts for injection onto the HPLC.

M. HPLC Chromatography(General Discussion)

A Shimadzu HPLC system is used to chromatograph the extracts and standards for this method. Similar instruments are acceptable substitutes. The primary system used employs two analytical HPLC columns with column to column switching and ultraviolet (UV) detection for CGA-339833 and CGA-192155. A schematic of the hydraulic configuration for the analytical system can be found as Figure 3. A single column HPLC system with UV detection may be used for analysis of CGA-173506 extracts if matrix interferences are minimal. Alternatively, a two column switching HPLC system may be used for the analysis of CGA-173506. General instrument parameters are given below.

Adjustment of mobile phase conditions may be required to provide resolution from matrix peaks and desirable retention times. First column mobile phases must produce retention times providing 4 to 10 column void volumes. 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 P., "Discussion of Special Chromatographic Separations Achievable," in this method 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. 200 µL for 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-173506	268 nm

N. Analysis of CGA-173506 (non-polar analyte)

1.0 First Column. A 4.6-mm internal diameter by 15-cm length with 5µ CN packing material has been used successfully. The specific column used is a 4.6 X 150 mm Zorbax™ SB-CN column with a 4 X 12.5 mm cartridge guard column containing the same packing material. 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 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 the two columns are connected in series.

3.0 First Column Mobile Phase for CGA-173506. A 40% ACN:60% 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% water with 5-mL/L HOAc at 2 mL/min (mobile phase 3) for 5 minutes and the flow rate of 2 mL/min is maintained. After 5 minutes of pumping mobile phase 3, mobile phase 1 is again pumped for 10 minutes at 2 mL/min. Prior to the next injection the 2-mL/min flow rate is lowered to 1 mL/min for at least 1 minute.

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

Compound	Time (min)
CGA-173506	10.0

An example chromatogram for the three analytes with the mobile phase and conditions specified above may be found in the appendix as Figure 4.

5.0 Establishment of Switching Valve Times. The SB-CN column is eluted with 1.0 mL/min of mobile phase 1 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 0.1 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. Retention values for 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 column switching.

The peak tailing factor (0.05)[3] 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 or regeneration 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) 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 aquired 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 be just as the peak tail comes back to base line.

6.0 Second Column. A 4.6-mm internal diameter x 25-cm length ODS (5μm) is used as the second column for the analysis of CGA-173506. A Spherisorb ODS2 cartridge column has been used successfully for the analysis of CGA-173506 as the second column to chromatograph these extracts. This column has also been used successfully as a single column system to analyze extracts.

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 is 50% ACN:50% water with 5-mL/L HOAc (mobile phase 5) for both the single column system and the column switching system.

The second column and the second column mobile phase may be connected directly to the autoinjector to chromatograph standards and extracts using mobile phase 3 in an isocratic mode to elute CGA-173506, then using mobile phase 2 to remove strongly retained compounds in a manner similar to that of the first column.

Minimal interferences in control extracts and recovery of analyte from fortified control extracts must be demonstrated to use this single column HPLC system.

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

1.0 First Column. A 4.6-mm internal diameter by 15-cm length with 5-μm CN packing material has been used successfully as the first column. The specific column used is a 4.6 X 150 mm Zorbax™ SB-CN column with a 4 X 12.5 mm cartridge guard column containing the same packing material. 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 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 the two columns are connected in series.

3.0 First Column Mobile Phase for CGA-339833 and CGA-192155. A 20% ACN:80% water with 5-mL/L HOAc and 0.75-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% water with 5-mL/L HOAc at 2 mL/min (mobile phase 3) for 5 minutes and maintained at a flow rate of 2 mL/min to remove strongly retained analytes. After 5 minutes of pumping mobile phase 3, mobile phase 2 is again pumped for 10 minutes prior to the next injection. Note that the 2-mL/min flow rate is lowered to 1 mL/min at least 1 minute prior to the end of the chromatographic sequence to allow flow rate equilibration.

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

Compound	Time (min)
CGA-339833	7.5
CGA-192155	9.8

An example chromatogram for the two analytes with the mobile phase and conditions specified above may be found in the appendix 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 0.1 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. Retention values for different injection volumes will influence the retention times, and the peak window may be set incorrectly.

The peak tailing factor (0.05)[3] 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 or regeneration 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 aquired 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 be just as the peak tail comes back to base line.

6.0 Second Columns. A 4.6-mm internal diameter x 25-cm length (5μm) Prodigy 5 ODS-2 column is used as the second column for the analysis of both CGA-339833 and CGA-192155.

7.0 Pump Flow Rates. A flow rate of 1 to 2 mL/min may be used for this column. 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 Phases.

8.1 Mobile phase used for CGA-339833 analysis is 40% ACN:60% water with 5-mL/L HOAc and 0.1-g/L NH_4OAc (mobile phase 4).

8.2 Mobile phase used for CGA-192155 analysis is 40% ACN:60% water with 5-mL/L HOAc (mobile phase 1).

P. 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 interference 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 5 ODS2 columns. The following discussion will be a valuable aid in establishing acceptable chromatography conditions for 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 5 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 of NH_4OAc) on both the Zorbax™ SB-CN column and the Prodigy 5 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 when the acidity and ACN levels remain constant. The retention time effect of ammonium ion concentration is greater for CGA-339833 on both the Prodigy 5 ODS2 and the SB-CN column than CGA-192155. The difference in ammonium ion effect for CGA-338933 and CGA-192155 on both these columns (SB-CN and the Prodigy 5 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 are able to provide stable retention times for analysis when identified for individual columns.

1.0 Separations on the SB-CN Column. To find the proper concentration of ammonium ion, it is easier to prepare a 20% ACN:80% 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 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-173506 are affected minimally by NH_4OAc. When using the 20% ACN mobile phase discussed in this section, the retention time for CGA-173506 is greater than 20 column volumes, however, if mixed standards are used to time the columns, CGA-173506 will eventually elute and may interfere with subsequent injections used for timing purposes.

2.0 Separations on ODS Columns. Evaluation of ammonium ion concentration on ODS columns gives a more pronounced effect for CGA-339833 than CGA-192155. Ammonium ion effect was not noted for CGA-173506.

3.0 Regeneration of SB-CN HPLC Columns

After extended use of the SB-CN column, it has been found 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 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.

4.0 Loss of Analytes

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

Analytes partition into the matrix coextracted material and must be removed from the matrix by adding ACN first, then sonicating to remove the analytes from precipitated grass coextracts. This ACN rinse is transferred from the flask and the flask then rinsed with aqueous acetic acid.

5.0 Confirmatory Techniques

No confirmatory analysis procedure has been developed at this time.

6.0 Time Requirement

A set of 12 samples can be processed during an eight hour day by three people.

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. This must be repeated for each analyte, thus total chromatography time will be approximately 30 hours.

Data reduction requires an additional 2 hours per analyte, or 6 hours for one group of data.

Q. 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 responses for standards and sample extracts are measured using the MULTICHROM system. The MULTICHROM system uses the µg/mL versus peak response to form a regression curve and from that to interpolate the concentration of analytes in the extracts. This value is then converted to parts per million 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:

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

The dilution factor, if required, is calculated as follows. If a dilution is not 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) and interpolating the concentration in the extract for the fortified sample. Recovery is then calculated according to the following formula:

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