I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of GS-23199 and CGA-294849, metabolites of CGA-215944, 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 are presented in Figure 1.

The limit of detection (smallest standard amount injected during the chromatographic run) is 2 ng for both analytes for the C18 analytical analyses and 1 ng for both analytes for the CN confirmation analyses. The limit of determination (the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) is 10 ppb in soil.

This is the second method issued for the analysis of the metabolites GS-23199 and CGA-294849 in soil (the first method issued was AG-653). After issuance of method AG-653, several problematic soils were encountered which contained significant interferences for the analytes. In this method, additional cleanup steps have been added that remove more of the potential interferences, in addition to a new reversed phase HPLC analysis system which permits the resolution of those interferences which still are present after the additional cleanup steps. While method AG-653 performed well with the soils used for method validation, this new method will do a much better job of removing and/or resolving interferences that may occur when different soils are encountered.

B. Principle

Soil samples (20 g) are reflux extracted with 20% (v/v) water/methanol. The samples are centrifuged and filtered. Methanol is removed via rotary evaporation until approximately 20 mL of extract remains. The extract is made basic with ammonium

hydroxide and then passed through a SAX solid phase extraction (SPE) column (the analytes are not retained). The non-retained fraction is placed on a rotary evaporator and methanol is removed until only aqueous remains. The aqueous is acidified and then passed through a C18 SPE attached piggy back style to a SCX SPE (the analytes are not retained.) The non-retained eluate is placed on a rotary evaporator and solvent is removed until only 2-4 mL of water remains. Methanol is added and the residue is transferred to a concentration tube where the methanol is removed until only aqueous remains. Water is added to adjust the final sample volume to a precalibrated mark. The sample is injected onto a reversed phase HPLC system with the analytes detected by UV absorbance. 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.

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 extractions. Must be centrifugable.

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) 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, Reeve Angel 802 (Fisher cat. #09-832D) or equivalent.

9.0 Filter, sample, for filtering final sample prior to analysis, Whatman Anotop 25 Inorganic Membrane Filter, 0.2 µm pore, 25 mm diameter (Whatman cat. #6809-2022).

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

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

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

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

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

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

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

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

18.0 Vials, 1.5-ml (Sun Brokers, Inc. cat. #200-002) or equivalent, with Teflon-lined, crimp-top seals (Sun Brokers, Inc. cat. #200-152) or equivalent.

B. Reagents and Analytical Standards

All reagents 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 Ammonium hydroxide, certified ACS plus grade (Fisher cat. #A669S-500) or equivalent.

3.0 Extraction solvent: 20% (v/v) water in methanol. Add 800 mL of methanol to 200 mL of purified water. 400 100

4.0 C18 SPE extraction column, 1 gram size (Varian cat. #1225-6001) or equivalent.

5.0 SAX SPE extraction column, 1 gram size (Varian cat. #1225-6013) or equivalent.

6.0 SCX SPE extraction column, 1 gram size (Varian cat. #1225-6011) or equivalent.

7.0 Formic acid, 90%, laboratory grade (Fisher cat. #A119P-500) or equivalent.

8.0 Formic acid, 0.1%: mix 1.0 mL of formic acid with 999 mL of purified water.

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

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

11.0 Mobile phase (A) for C18 column: 3% methanol/water. Mix 30 mL of methanol with 970 mL of purified water.

12.0 Mobile phase (B) for C18 column: 30% water/acetonitrile. Mix 700 mL of acetonitrile with 300 mL of water.

13.0 Mobile phase (A) for CN column: 5/10/85% methanol/1-propanol/hexane. Mix 50 mL of methanol with 100 mL of 1-propanol. Add 850 mL of hexane to this mixture and mix the contents.

14.0 Mobile phase (B) for CN column: 20/20/60% methanol/1-propanol/hexane. Mix 200 mL of methanol with 200 mL of 1-propanol. Add 600 mL of hexane to this mixture and mix the contents.

15.0 1-Propanol, certified grade (Fisher cat. #A414-4), or equivalent.

16.0 Rinse solution for C18 SPE: 0.1/5/94.9% formic acid/methanol/water. Mix 1.0 mL of formic acid with 50 mL of methanol and 950 mL of water.

17.0 Rinse solution for SAX SPE: 0.5/20/79.5% ammonium hydroxide/water/methanol. Mix 5.0 mL of ammonium hydroxide with 995 mL of the soil extraction solvent (20/80% water/methanol).

18.0 Sample diluent for C18 analytical analysis: 5% methanol/water.

19.0 Sample diluent for CN confirmation analysis: 5/10/85% methanol/1-propanol/hexane. Combine 10 mL of 1-propanol with 5 mL of methanol. Add 85 mL of hexane. Mix the contents.

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

21.0 GS-23199 and CGA-294849, Ciba-Geigy Corp., 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). Formic acid and ammonium hydroxide are irritants and should be used in a well-ventilated area (i.e., a fume hood).

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 the beaker or pan. Record the weight of the container plus wet soil.

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

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

1.5 Record the weight of the container plus dry soil.

1.6 Calculate the moisture content using the equation:

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

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

2.0 Soil 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 a 250-mL round bottom flask.

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

2.3 Add 100 mL of the soil extraction solvent. Swirl the contents briefly. Attach a reflux condenser to the flask and heat under reflux for one hour. Permit the extract to cool prior to centrifugation and filtering.

2.4 Transfer the sample to an appropriate size polypropylene centrifugeable bottle. Centrifuge the sample at approximately 9,000 RPM for 10 minutes, or at an alternate speed and time if the results are considered satisfactory.

2.5 Decant the sample extract through filter paper into a 100-mL graduated cylinder. Record the volume of extract.

2.6 Transfer the sample to a 250-mL round bottom flask. Rinse the graduated cylinder with approximately 5 mL of methanol and add to the sample. Add approximately 5 mL of water to each

sample to help prevent it from going dry during the rotary evaporation step.

2.7 Place the sample on a rotary evaporator with a water bath temperature of approximately 40 to 45°C. Use a solvent trap to minimize losses due to bumping. (Note: Periodic venting of the sample is required to prevent losses due to bumping.) Remove the methanol until approximately 20 mL of extract remains.

2.8 Remove the sample from the rotary evaporator. Add 100 µL of ammonium hydroxide.

2.9 Pass the sample through a preconditioned SAX SPE column, collecting the non-retained eluate in a 100-mL round bottom flask. A vacuum SPE reservoir may be used to improve flow through the SPE column. The sample loading speed should not exceed a fast drip rate. (Preconditioning of the SAX SPE is done by passing one column volume of methanol and then one column volume of the basic SAX rinse solution through the column. Do not permit the column to go dry before passing the sample through the SPE column.) Add approximately 5 mL of the basic SAX SPE rinse solution to the round bottom flask previously containing the sample. Swirl to rinse and dissolve any residues remaining on the glass. Transfer the rinsate via disposable pipette to the SAX SPE column and pass through, collecting in the 100-mL round bottom flask with the rest of the sample.

2.10 Place the sample on a rotary evaporator, water bath temperature of approximately 40 to 45°C, and remove methanol until only aqueous remains. Do not permit the extract to dry. A final volume of approximately 10 mL is desired.

(Purified water may be added to ensure the extract does not dry during this step.)

2.11 Remove the sample from the rotary evaporator. Add 50 µL of formic acid to each sample. Sonicate and vortex samples to ensure all residue is in solution.

2.12 Load the sample onto a preconditioned C18 SPE extraction column attached piggy back style to a SCX SPE column. Collect the non-retained eluate, which contains both analytes, in a 100-mL round bottom flask. (Note: The SPE columns are preconditioned by passing one column volume each of methanol and 0.1% formic acid through the columns. Discard the rinse solutions. Add approximately 2 mL of 0.1% formic acid to the lower SCX column to ensure it will not dry while the sample is loaded and eluted.) The sample loading speed should not exceed a fast drip rate.

(C18 rinse)

2.13 Add approximately 5 mL of 0.1/5/94.9% formic acid/methanol/water to the 100-mL round bottom flask in which the rotary evaporation step was done. Vortex the solvent along the sides of the flask to dissolve any residues. Load this rinse onto the SPE columns and collect the eluate in the flask containing the sample from Step 2.12. Disconnect the C18 SPE column from the SCX column and then rinse the SCX column with one column volume of methanol, collecting the eluate with the sample in the 100-mL round bottom flask.

2.14 Place the sample on a rotary evaporator with a water bath temperature of approximately 40 to 45°C and remove solvent until approximately 3-5 mL of solvent remains. Do not permit the

sample to go dry. Use methanol to azeotrope the water, if needed.

2.15 Add 5 mL of methanol to the sample. Sonicate and vortex the flask well to ensure all of the residue is dissolved. Transfer the sample to a precalibrated 50-mL concentration tube using a disposable Pasteur pipet. Repeat with a second 5-mL rinse of the flask with methanol and add the rinsate to the sample. (Precalibration of the concentration tube is done by marking the meniscus line on each concentration tube after adding 4.0 mL of the sample diluent.)

2.16 Remove methanol from the sample until 2-3 mL of water remains. Water may be added as needed to prevent the sample from drying. (Note: It is important that all of the methanol be removed from the sample to ensure good chromatographic peak shape on the C18 analysis column.)

2.17 Add 0.2 mL of methanol to the sample. Dilute the sample with water to the 4.0-mL calibration mark. Additional dilution of the sample may be done using 5% methanol/water, if necessary.

2.18 Analyze using the reversed phase C18 HPLC system with UV detection. Refrigerate the sample if it will not be analyzed the same day the sample was processed.

2.19 For confirmation analyses using the CN normal phase HPLC system, first measure an aliquot of sample (from Step 2.17) and place in a 100-mL round bottom flask (a minimum volume of 3 mL is recommended). Add 20 mL of acetonitrile to the sample. (This is done to azeotrope the water from the sample.)

Remove the solvent from the sample via rotary evaporation with a water bath temperature of approximately 35°C until approximately 2-3 mL of sample remain. **(Warning: Do not let the sample go dry or significant losses of CGA-294849 will occur.)** Add approximately 20 mL of acetonitrile and evaporate solvent until approximately 2-3 mL remains. Repeat a third time with another 20 mL of acetonitrile, removing solvent until 2-3 mL remains. Add 5 mL of methanol to the sample, swirl the contents, and transfer to a 50-mL concentration tube via disposable pipette that has been precalibrated for the volume of sample being processed. Rinse the round bottom with 5 mL of methanol and transfer to the sample tube. Add approximately 1 mL of 1-propanol to the sample. Place the sample on a rotary evaporator with a water bath temperature of approximately 35°C and remove solvent until a volume of 1-propanol remains that is approximately 15% of the total sample volume that was processed. (Example: For a 3.0 mL sample, evaporate solvent until approximately 0.45 mL remains.) Add several drops (3-5) of methanol to the sample. Dilute to the calibration volume mark using hexane. Sonicate and vortex mix the sample thoroughly. Additional dilutions may be done using the CN sample diluent, if desired. Filter the sample with an Anotop sample filter, if necessary. (Note: A white suspended residue is frequently observed in soil samples prior to filtration. This residue will precipitate to the bottom of the vial in a short period of time and may not require filtration.) Analyze the sample by normal phase HPLC with the CN column and UV detection. Refrigerate the sample if it will not be analyzed the same day the sample was processed.

E. Instrumentation

1.0 Description and Operating Conditions: HPLC

See Tables II and III for descriptions of the HPLC systems for the C18 analytical and CN confirmation systems.

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. 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 will be presented in Tables IV - IX for the analytical and confirmation systems. Typical standard chromatograms will be presented in Figure 3 for the C18 column and in Figure 7 for the CN column.

F. Interferences

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

G. Confirmatory Techniques

1.0 Confirmation of residues can be obtained by analysis of the samples on a second HPLC

system which utilizes a cyano (CN) column. See Table III for a description.

H. Time Required

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

2.0 Each HPLC analysis requires approximately 20 minutes for the C18 analysis system and 19 minutes for the CN confirmation system.

I. Modifications and Potential Problems

1.0 Analytical Method AG-666 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. A 500-mL round bottom flask may be necessary for the initial evaporation of solvent from the raw extract (Step 2.7) if severe bumping or foaming occurs in a 250-mL round bottom flask.

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

4.0 No analytes have been observed binding to the Whatman Anotop 25 sample filters during the final sample filtration step. It is unknown whether the analytes will bind to other brands/types of sample filters.

5.0 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 this method if columns of different manufacture are used.

6.0 During method development trials it was noted that interferences were encountered with certain soils which did not permit quantitation with the CN confirmation analysis system. The interferences were resolved from the analytes, however, with the reversed phase C18 analysis system.

7.0 Both analytes are very polar and have minimal retention on reversed phase columns. The YMC ODS-AQ column offers superior retention and peak shape over all other brand columns that have been tried.

8.0 Significant losses of CGA-294849 may occur if the samples are permitted to go to complete dryness during rotary evaporation steps.

J. Preparation of Standard Solutions

Stock solutions are stored in amber bottles in a freezer (<-10°C) when not in use. Fortification and HPLC standards are stored refrigerated in amber bottles when not in use. No analyte stability or solubility problems have been observed in the standard solutions used in this study.

1.0 Prepare individual 100 ng/µL stock solutions for each analyte. Weigh approximately 10.0 mg of analyte. Determine the appropriate volume of methanol 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 methanol 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 methanol required to dilute 9.9 mg of an analyte, of 98.0% purity, to a final concentration of 100 ng/uL is:

$$V\,(mL) = \frac{9.9\ mg \times 0.98}{100\ ng/uL} \times 10^3 = 97.02\ mL$$

2.0 Prepare a 20 ng/µL mixed standard solution in methanol by pipetting 10.0 mL of each analyte (from its 100 ng/µL stock solution in Step 1.0) into a 50-mL volumetric flask and diluting to the mark with methanol. Store the solution in an appropriate size amber bottle. This solution is used to prepare all subsequent dilutions.

3.0 Fortification standards are prepared by dilution of the 20 ng/ul mixed standard with methanol. The concentrations 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.)

4.0 <u>C18 analytical standard.</u>
A 1.0 ng/µL analytical standard for the C18 HPLC system is prepared by pipetting 1.0 mL of each 100 ng/µL stock solution along with 3.0 mL of methanol into a 100-mL volumetric flask and then diluting to the mark with purified water. Subsequent dilutions are prepare by dilution of this solution with 5% methanol/water.

5.0 CN confirmation standard.
A 1.0 ng/µL analytical standard for HPLC calibration use is prepared by pipetting 0.5 mL from each 100 ng/µL stock solution into a 50-mL volumetric flask, adding 1.5 mL of methanol, 5.0 mL of 1-propanol, and diluting to the 50-mL mark with hexane. Subsequent serial dilutions are made with 5/10/85% methanol/1-propanol/hexane to prepare additional calibration standards.

K. Methods of Calculation

1.0 Determination of Residues in Samples

1.1 Inject the sample solution from Step II.D.2.17 or II.D.2.19 into the analysis system. The sample solution may be diluted if the analyte response exceeds the range of the calibration curve. The amount of analyte injected (ng) is determined by entering the value of the chromatographic peak height, or area, in the calibration response curve (Step II.E.4.2) and calculating (by computer, calculator, or manual means) the corresponding value of nanograms injected. Typical chromatograms for control and fortified soil samples will be presented in Figures 4-6 and 8-10 for the C18 and CN columns, 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 in soil.

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. The use of the "1/R" recovery correction factor is optional and left to the discretion of the study director.

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

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

where, g is the grams of soil (wet weight) used, V_a is the aliquot volume of extracted sample used for analysis, V_e is the volume of extract solvent used, V_{std} is the volume (mL) of fortification standard added (if any), V_i is the volume (mL) injected onto the HPLC column, m is the percent moisture in the sample, expressed in decimal form

(ex. 0.1 = 10%), and V_f is the final volume (mL) of the cleaned-up sample (from Step II.D.2.17 or II.D.2.19). (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.

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-215944 to that of the metabolite (equation (5)).

$$(5)\ \text{ppb CGA-215944 equiv.} = \text{ppb metabolite} \times \frac{MW(p)}{MW(m)}$$

where MW(p) is the average molecular weight of CGA-215944 (217.2) and MW(m) is the average molecular weight of the metabolite, 127.1 for GS-23199, and 142.1 for CGA-294849.

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: C18 ANALYTICAL COLUMN

Instrumentation:

Perkin-Elmer Model Series 410 Gradient Pump
Perkin-Elmer Model ISS 200 Autosampler
Eppendorf Model CH-30 Column Heater
Perkin Elmer Model LC-95 UV Absorbance Detector

Operating Conditions:

Column Heater: 30°C
Detection Wavelength: 265 nm
Injection Volume: 100 µl
Mobile Phase Flow Rate: 1.5 ml/min
Column: YMC ODS-AQ (YMC, Inc.), 25 cm x 4.6 mm, dp = 5 µm, equipped with an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent, and a YMC ODS-AQ guard column.
Mobile Phase A: 3% methanol/water
Mobile Phase B: 70% acetonitrile/water

Gradient Program:

Time	%A	%B
0	100	0
8	100	0
8.5	0	100
11.5	0	100
12	100	0
21.5	100	0

Total Run Time: 21.5 min.
Analyte Retention Times:

CGA-294849	6.3 min
GS-23199	9.9 min

TABLE III. HPLC SYSTEM AND OPERATING CONDITIONS: CN CONFIRMATION COLUMN

Instrumentation:

Perkin-Elmer Model Series 4 Gradient Pump
Perkin-Elmer Model ISS 200 Autosampler
Eppendorf Model CH-30 Column Heater
Perkin Elmer Model LC-95 UV Absorbance Detector

Operating Conditions:

Column Heater: 30°C
Detection Wavelength: 265 nm
Injection Volume: 50 µl
Mobile Phase Flow Rate: 1.5 ml/min
Column: Spherisorb CN (Phase Separations, Inc.), 25 cm x 4.6 mm, dp = 5 µm, equipped with an Upchurch (#A-318) pre-column filter (0.5 µm), or equivalent
Mobile Phase A: 5/10/85% Methanol/1-Propanol/Hexane
Mobile Phase B: 20/20/60% Methanol/1-Propanol/Hexane

Gradient Program:

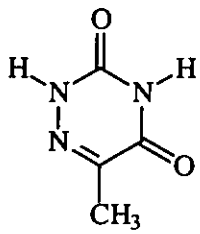
Time	%A	%B
0	100	0
6.5	100	0
7	0	100
11	0	100
11.5	100	0
19	100	0

Total Run Time: 19 min.

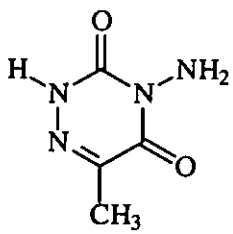
Analyte Retention Times:

GS-23199	4.5 min
CGA-294849	7.5 min

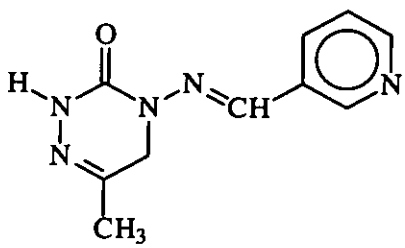
FIGURE 1. CHEMICAL NAMES AND STRUCTURES



GS-23199
CAS Name: 6-Methyl-1,2,4-triazine-3,5(2H,4H)-dione
CAS No.: 932-53-6



CGA-294849
CAS Name: 4-Amino-6-methyl-1,2,4-triazine-3,5(2H,4H)-dione
CAS No.: 16077-52-4



CGA-215944
CAS Name: (E)-4,5-Dihydro-6-methyl-4-[(3-pyridinyl methylene)amino]-1,2,4-triazin-3(2H)-one
CAS No.: 123312-89-0

FIGURE 2. AG-666 FLOW DIAGRAM FOR SOIL

Weigh 20 gram soil sample. Fortify, if necessary.
Add 100 mL of 20% water/methanol.
Reflux extract for one hour.
Centrifuge and filter sample.
Measure and record volume of filtered extract.

Remove methanol from sample via rotary evaporation until approximately 20 mL remains.
Basify sample with ammonium hydroxide.
Pass through SAX SPE.
Collect non-retained fraction in round bottom flask.

Remove methanol from sample via rotary evaporation until only water remains. Do not permit to go dry. Ensure 5-10 mL of water is present in sample.
Acidify sample with formic acid.
Pass through C18 SPE piggybacked to a SCX SPE.
Collect non-retained fraction in round bottom flask.

Remove all solvent via rotary evaporation until only 3-5 mL of water remains. Do not permit to go dry.
Add approximately 5 mL of methanol, swirl and transfer to a pre-calibrated concentration tube.
Remove solvent via rotary evaporation until approximately 2-3 mL of water remains.
Dilute sample with water to 4-mL mark, or more for samples with high level residues.

Analyze by HPLC with UV detection.