I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of the herbicide active ingredient s-metolachlor (Novartis code CGA-77102) and its degradates CGA-51202, CGA-354743, and CGA-37735 in soil and thatch, and for CGA-77102 in grass. The analytes are separated by high performance liquid chromatography (HPLC) and detected by mass spectrometry (LC/MS/MS) as unique precursor/product ion fragments. A turboionspray atmospheric pressure ionization (API) interface is used to introduce the HPLC effluent into the mass spectrometer for analyses. The chemical structures, chemical names, and Chemical Abstracts Registry numbers of the analytes are presented in Figure 1.

The limit of detection by (smallest standard amount injected during the chromatographic run) is 0.1 ng for all analytes. The limit of quantification (LOQ) (defined as the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) is 10 ppb for all analytes in soil and thatch, and 1 ppm for CGA-77102 in grass.

B. Principle

Soil/Thatch: A 10-gram subsample of soil or thatch is extracted two times with 70% (v/v) methanol/water, 1% (v/v) in ammonium hydroxide at room temperature using mechanical agitation. The sample is centrifuged and filtered. The methanol content is removed by rotary evaporation. The sample is made acidic and passed through an ENV SPE column. The analytes are eluted from the ENV SPE using basic methanol. The samples are adjusted to the desired final volume and organic content. LC/MS/MS is used for analysis of the samples. A flow diagram for the soil/thatch method is presented in Figure 2.

Grass: A 5-gram subsample of grass is extracted two times with 80% (v/v) acetonitrile/water at room temperature using mechanical agitation. The sample is centrifuged and filtered. A 5-mL aliquot of the extract is diluted with 5 mL of 0.1% formic acid in water. The extract is loaded onto a conditioned C18 SPE column. The analyte, which is retained on the SPE column, is washed with 5 mL of 40% acetonitrile/water (0.1% in formic acid). The analyte is eluted from the SPE using 80% (v/v) acetonitrile/water. The C18 eluate is concentrated to dryness using rotary evaporation and is reconstituted in 20% (v/v) acetonitrile/water. LC/MS/MS is used for analysis of the samples. A flow diagram for the grass method is presented in Figure 3.

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, polyallomer, wide mouth, 250-mL capacity (Fisher cat. #05-562-19A) or equivalent with cap (Fisher cat. #05-563-1D) or equivalent, appropriate size for extraction/centrifugation of the soil samples.

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

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

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

8.0 Filter paper, 24-cm prepleated circles, Whatman Reeve Angel 802 (Fisher cat. #09-901D) or equivalent.

9.0 Flasks, round bottom, 100-mL (Fisher cat. #10-067C), 250-mL (Fisher cat. #10-067E), 500-mL (Fisher cat. #09-552C) or equivalent.

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

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

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

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

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

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

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

17.0 Solid phase extraction (SPE) columns: (1) Mega Bond Elut ENV, 1 gram/6 mL capacity/volume (Varian cat. #1225-5012), and (2) Mega Bond Elut C18 (Varian cat. #1225-6001).

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

19.0 Ultrasonic bath (Fisher cat. #15-335-110) or equivalent.

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

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

B. Reagents and Analytical Standards

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

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

2.0 Acetic acid, 0.1% in water: mix 1.0 mL of acetic acid with 1000 mL of purified water.

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

4.0 Acetonitrile, 0.1% in acetic acid: mix 1.0 mL of acetic acid with 1000 mL of acetonitrile.

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

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

7.0 Ammonium hydroxide, 0.25% in water. Mix 2.5 mL of ammonium hydroxide with 1000 mL of purified water.

8.0 Ethylene glycol, certified ACS grade (Fisher cat. #E178-1) or equivalent.

9.0 Formic acid (90%), purified (Fisher cat. #A119P-1) or equivalent.

10.0 Formic acid, 0.1% in water. Mix 1.0 mL of formic acid with 1000 mL of purified water.

11.0 *Grass extraction solvent: 80% acetonitrile in water.* Mix 800 mL of acetonitrile with 200 mL of purified water.

12.0 Grass C18 wash solvent: 40% acetonitrile/water (0.1% in formic acid). Mix 400 mL of acetonitrile with 600 mL of purified water and 1 mL of formic acid.

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

14.0 Methanol, 0.1% in ammonium hydroxide. Mix 1 mL of ammonium hydroxide with 1000 mL of methanol.

15.0 o-Phosphoric acid, HPLC grade (Fisher cat. #A260-500) or equivalent.

16.0 Phosphoric acid, 0.1% in water. Mix 1 mL of phosphoric acid with 1000 mL of purified water.

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

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

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

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

21.0 Soil extraction solvent: 70% methanol in water, 1% in ammonium hydroxide. Mix 290 mL of purified water, 700 mL of methanol, and 10 mL of ammonium hydroxide.

22.0 Sample diluent: 20% acetonitrile in water. Mix 200 mL of acetonitrile with 800 mL of purified water.

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

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

25.0 CGA-77102 (Chemical Purity = 98.5%), CGA-51202 (98.8%), CGA-354743 (96.8%), CGA-37735 (99.2%), Novartis Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

C. Safety and Health

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

D. Analytical Procedure

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

1.0 Soil/Thatch Moisture Determination

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

1.2 Add approximately 10-20 g of soil/thatch 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/thatch.

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/Thatch

(Note: Samples must be homogenized prior to analysis using suitable sample preparation techniques.) Soil characterization data for the soils used in this validation study are presented in Table 1.

2.1 Weigh and record 10 ± 0.1 g of soil/thatch sample into a plastic extraction bottle.

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

2.3 Add 75 mL of the extraction solvent 1 (70% methanol/water, 1% in ammonium hydroxide) to the sample.

2.4 Place the sample on an orbital shaker and agitate the sample at high speed for thirty minutes at room temperature.

2.5 Centrifuge the sample at approximately 9,000 RPM for 7 minutes, or at an alternate speed and time if the results are considered satisfactory.

2.6 Decant the sample extract through filter paper into a 500-mL round bottom flask.

2.7 Repeat the extraction adding 75 mL of the extraction solvent. Repeat Steps 2.4 through 2.6, adding the second extract to the first contained in the round bottom flask from Step 2.6. (Note: If the extract is

visibly cloudy, the sample may be transferred to a plastic centrifugable bottle and the solids removed by centrifugation, as detailed in Step 2.5.)

2.8 Add approximately 10 mL of purified water to the round bottom flask containing the sample to help ensure the aqueous content does not become too low during the rotary evaporation step (Step 2.9).

2.9 Remove methanol from the sample until only aqueous remains via rotary evaporation with a water bath temperature of approximately 35 °C. Add water, if necessary, to prevent the sample from going dry. A minimum aqueous volume of approximately 25 mL should be maintained.

2.10 If the sample is cloudy with suspended particulates, transfer the sample to a centrifugable plastic bottle, using several mL of water to rinse the round bottom flask, adding this rinsate to the bottle. Centrifuge the sample at approximately 9,000 RPM for 7 minutes, or at an alternate speed and time if the results are considered satisfactory.

2.11 Acidify the sample by adding 0.2 mL of phosphoric acid. Swirl to mix.

2.12 Pass the sample through a Varian ENV SPE column (1-g), attached to an appropriate vacuum manifold. The flow rate through the column should be no faster than one drop per two seconds. (The SPE columns are preconditioned by passing 10 mL of methanol and then 10 mL of 0.1% phosphoric acid through each column. Discard the eluate.

2.13 When all of the sample from Step 2.12 has passed through the ENV SPE column, add approximately 5 mL of 0.1% phosphoric acid to the round bottom flask, swirl to rinse any residues still on the glass, and pass through the ENV SPE column. When all of this rinsate has passed through the column, pass an additional 2-3 mL of 0.1% phosphoric acid through the ENV SPE column. Discard the eluate.

2.14 Rinse the ENV SPE column by passing approximately 6 mL of 0.25% ammonium hydroxide in water through the column. Discard the eluate.

2.15 Rinse the ENV SPE column by passing approximately 6 mL of purified water through the column. Discard the eluate.

2.16 Elute the analytes into a pre-calibrated 50-mL concentration tube using 10 mL of methanol, 0.1% in ammonium hydroxide. (The concentration tube is calibrated by pipetting the desired final volume of sample diluent into the tube and then marking the meniscus with a marking pen. In these studies, a final sample volume of 5 mL was used for control samples and those fortified at the method LOQ of 10 ppb.)

2.17 Remove the organic content from the sample via rotary evaporation with a water bath temperature of approximately 35°C.

2.18 Add 1.0 mL of acetonitrile to the sample. Adjust the final volume to the calibration mark using purified water. The sample may be further diluted with sample diluent, if necessary. Transfer an aliquot of the sample to an appropriate sized HPLC sample vial. The sample should be stored refrigerated (<5°C) until the time of analysis. Samples should be stored frozen for long term storage (> 2 weeks).

2.19 Analyze the sample using LC/MS/MS.

3.0 Grass

Grass clippings were obtained from a control turf plot from a turf study conducted in California, Novartis Study 280-98, sample inventory number INV18146.2, sample number 236625.

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

3.1 Weigh and record 5 ± 0.1 g of grass sample into a plastic extraction bottle.

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

3.3 Add 100 mL of the extraction solvent 1 (80% acetonitrile/water) to the sample.

3.4 Place the sample on an orbital shaker and agitate the sample at high speed for thirty minutes at room temperature.

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

3.6 Decant the sample extract through filter paper into a 250-mL round bottom flask.

3.7 Repeat the extraction adding 50 mL of the extraction solvent. Repeat Steps 3.4 through 3.6, adding the second extract to the first contained in the round bottom flask from Step 3.6. Swirl the extracts in the flask to mix the contents well.

3.8 Transfer a 5.0-mL aliquot of the extract to a small beaker or Erlenmeyer flask. Add 5 mL of 0.1% formic acid. Mix well. (This step dilutes the extract with aqueous to ensure retention on the C18 SPE column.)

3.9 Pass the sample through a Varian C18 SPE column (1-g), attached to an appropriate vacuum manifold. The flow rate through the column should be no faster than one drop per two seconds. (The SPE columns are preconditioned by passing 5 mL of acetonitrile and then 5 mL of 40% acetonitrile/water (0.1% in formic acid) through each column.) The analyte is retained on the C18 SPE column. Discard the eluate.

3.10 Add approximately 5 mL of 40% acetonitrile/water (0.1% in formic acid) to the sample flask from Step 3.8 and swirl to rinse any residues from the glass walls. Pass this rinsate through the C18 SPE column just as the sample extract from Step 3.9 finishes passing through the column.

3.11 After the rinsate has passed through the column, increase the vacuum and pass air through the cartridge for approximately 30 seconds to remove residual water.

3.12 Elute the analyte from the C18 SPE column with 10 mL of 80% acetonitrile/water, collecting the sample in a 100-mL round bottom flask. Gravity or a gentle vacuum may be used to help pass the eluting solvent through the SPE column.

3.13 Evaporate the sample to dryness using a rotary evaporator with a water bath temperature of approximately 35-40 °C. Acetonitrile may be added to help azeotrope the sample to dryness.

3.14 Add 10 mL of 20% acetonitrile/water to the flask. Swirl the contents in the flask and sonicate to aid dissolution of the residue. The sample may be further diluted with sample diluent, if necessary. Transfer an aliquot of the sample to an appropriate sized HPLC sample vial. The sample should be stored refrigerated (<5°C) until the time of analysis. Samples should be stored frozen for long term storage (> 2 weeks).

3.15 Analyze the sample using LC/MS/MS.

E. Instrumentation

1.0 Description and Operating Conditions: HPLC

See Table 2 for a description of the reversed phase HPLC system and chromatographic conditions used for the analysis.

2.0 Description and Operating Conditions: LC/MS/MS

CGA-51202, CGA-354743, and CGA-37735 are monitored as negative ions. CGA-77102 is monitored as a positive ion. Triple quadrupole analysis (MS/MS) of the unique precurser/product ion pair is suggested. See Table 3 for a description of the mass spectrometer instrumentation and operating conditions.

3.0 Description and Operating Conditions: LC/MS/MS Turboionspray Interface

The optimized values for the turboionspray interface may vary with time and may need to be periodically re-optimized. The turboionspray split flow is adjusted so that a small wet spot is visible on the orifice plate at the initial mobile phase gradient composition. Typical turboionspray operating conditions are described in Table 3.

4.0 Calibration and Standardization: LC/MS/MS

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

4.2 Determine the specific ion to monitor for each analyte by infusion of an analyte test solution (approx. 2.5 ng/µL in 50% acetonitrile/water, 0.1% acetic acid) while scanning the Q1 quadrupole mass analyzer to find the optimum ion. Determine the specific product ion fragment to monitor for each analyte in the MS/MS mode by passing the characteristic precurser ion through Q1, fragmenting the ion in Q2, and scanning the resulting ion fragments in Q3. The selected product ion chosen to monitor will depend on the intensity of the ion fragment along with the possibility that an interference also has the same fragment ion. Table 4 lists the precurser/product ion pairs that are monitored for each analyte. Typical ionspray mass fragmentation spectra are presented in Figure 4.

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

4.4 Calibrate the instrument by constructing a calibration curve from detector response (chromatographic peak area is preferred) 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.

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. Trace levels (<0.1 ppb) of CGA-77102 are commonly seen in method blank and control samples.

2.0 Metolachlor (CGA-24705) has the same retention time and mass fragmentation spectra as s-metolachlor (CGA-77102). Metolachlor consists of two rotational isomers ("r" and "s"), differing only in spatial orientation of the substituent groups.

G. *Confirmatory Techniques*

1.0 No confirmatory analysis procedure is included in this method. This method employs highly specific LC/MS/MS for the detection mode, coupled with the characteristic retention time observed for the analyte on the appropriate HPLC column.

H. Time Required

1.0 One chemist can complete the sample extraction and cleanup procedure for a set of twelve samples in an eight-hour working day.

2.0 Each HPLC analysis requires approximately 21 minutes.

I. Modifications and Potential Problems

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

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

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

4.0 No analyte stability or solubility problems have been observed when standard solutions have been prepared and stored as detailed in Section II.J. During method development, degradation of CGA-354743 and CGA-51202 (10-35%) was observed in dilute solutions in acetonitrile that were stored refrigerated. Degradation was not observed for solutions in acetonitrile stored in a freezer or for acetonitrile/water solutions stored refrigerated.

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

6.0 This method will work with the PE Sciex API-365, API-2000, API-3000, and API-III+ LC/MS/MS systems using the turboionspray interface. The ability of LC/MS/MS systems from other manufacturers to satisfactorily perform the analysis is unknown.

7.0 Single quadrupole LC/MS systems may be suitable for performing these analyses, provided that the analyst first confirms that there are no significant interferences originating from the sample matrix.

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

9.0 Several injections (3-4) of samples or standards should be made into the LC/MS system prior to initiation of the sample run sequence. This is necessary to stabilize analyte retention times and the MS response.

10.0 A 3-mm i.d. HPLC column with a flow rate of 0.6 mL/min was used in this method. A larger diameter column, with a corresponding higher mobile phase flow rate, may also be used provided that the sensitivity is adequate.

11.0 CGA-51202 exhibits a split peak in the chromatography. For quantitation, it is recommended that the entire area under the split peak be integrated. The preferred method for integrating CGA-51202 is presented in Figure 5.

J. Preparation of Standard Solutions

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

1.0 Prepare individual 200 ng/µL stock solutions for each analyte. Weigh approximately 10.0 mg of analyte. Determine the appropriate volume of acetonitrile to add using the equation presented below. The concentration of the analytical standard is corrected for its chemical purity.

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

Where V is the volume of acetonitrile needed; w is the weight, in mg, of the solid analytical standard; P is the purity, in decimal form, of the analytical standard; C is the desired concentration of the final solution, in ng/µL; and 10^3 is a conversion factor. **(Note: Due to higher fortification levels, a stock solution level of 1000 ng/µL is prepared in acetontrile for CGA-77102 in grass samples.)**

For example:

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

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

2.0 For soil and thatch, prepare a 20 ng/µL mixed solution by pipetting 10.0 mL of each analyte's 200 ng/µL stock solution into a 100-mL volumetric flask and then diluting to the calibration mark with purified water. **For grass samples, the 1000 ng/µL stock solution of CGA-77102 is diluted to 50 ng/µL and 5 ng/µL in acetonitrile for use as fortification solutions.**

3.0 Mixed fortification and analytical standards for soil/thatch analyses are prepared by subsequent dilution of the 20 ng/µL mixed solution with 20% acetonitrile/water. Fortification solutions should be prepared such that no more than 1.0 mL of solution is added during fortification. (Example: For a 10-g sample, the addition of 1.0 mL of a 0.1 ng/µL fortification solution will result in a fortification level of 10 ppb.) **For grass samples, the 5 ng/µL solution of CGA-77102 in acetonitrile is diluted with 20% acetonitrile/water for analytical standards.**

K. Methods of Calculation

1.0 Determination of Residues in Samples

1.1 Inject the sample solution from Step II.D.2.18 (soil/thatch) or Step II.D.3.14 (grass) into the analysis system. The sample solution may be diluted if the analyte response exceeds the range of the calibration curve. The amount of analyte injected (ng) is determined by entering the value of the chromatographic peak height, or area, in the calibration response curve (Step II.E.4.4) and calculating (by computer, calculator, or manual means) the corresponding value of nanograms injected.

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.

2.1 Add an appropriate volume of a fortification solution (from Section II.J. to the sample prior to any of the extraction/isolation steps. The total volume of the added fortification solution should not exceed 1.0 mL.

2.2 Proceed with the sample extraction/isolation procedure (Step II.D.2.3 for soil/thatch or Step II.D.3.3 for grass).

3.0 Calculations

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

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

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

where R is the recovery factor expressed in decimal form (i.e., 0.8 = 80%) and is calculated from equation (4), and the chemical purity of the analytical standard has been accounted for in the preparation of the standard solutions. **The use of the recovery correction factor "1/R" is left to the discretion of the study director.**

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

$$(2)\ \text{grams soil injected} = g \times \frac{V_i}{V_f}$$

where, g is the grams of sample (wet weight) used, V_i is the volume (mL) injected onto the HPLC column, and V_f is the final volume (mL) of the cleaned-up sample (from Step II.D.2.18).

The grams of sample injected for grass is calculated from equation (3).

$$(3)\ \text{grams grass injected} = g \times \frac{V_a V_i}{V_e V_f}$$

where, g is the grams of sample (wet weight) used, V_a is the aliquot volume (mL) of sample which is processed, V_e is the total volume of grass extract (mL), V_i is the volume (mL) injected onto the HPLC column, and V_f is the final volume (mL) of the cleaned-up sample (from Step II.D.3.14).

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

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

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

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

Residues of degradates found in test samples may also be expressed as parent equivalents by multiplying the amount found by the ratio of the molecular weight of CGA-77102 to that of the metabolite (equation (6)).

$$(6)\ \text{ppb CGA - 77102 equiv.} = \text{ppb metabolite} \times \frac{MW\,(p)}{MW\,(m)}$$

where MW(p) is the average molecular weight of CGA-77102 (283.8) and MW(m) is the average molecular weight of the metabolite, 279.3 for CGA-51202, 329.4 for CGA-354743, and 193.3 for CGA-37735.

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.

3.3 The confidence limits at 95% are determined for each fortification level in each substrate using equation (6)

$$(6)\ \text{C.L.}\ (95\%) = X \pm \frac{ts}{\sqrt{n}}$$

where "X" is the mean recovery, "s" is the standard deviation, "n" is the number of samples, and "t" is the "t-test" value for (n-1) degrees of freedom. In this report, values for "t" of 2.776 and 3.182 were used for 4 and 3 degrees of freedom (for 5 samples and 4 samples), respectively for the 95% confidence limit.

TABLE 2. HPLC SYSTEMS AND OPERATING CONDITIONS

Instrumentation:

Perkin-Elmer Series 200 Gradient Pump
Perkin-Elmer Series 200 Autosampler with Peltier Cooling Tray
Eppendorf Model CH-30 Column Heater

Operating Conditions:

Column Heater: 30°C
Injection Volume: 20 µL
Mobile Phase Flow Rate: 0.6 mL/min
Column: Zorbax SB-C8 (Hewlett-Packard, #863954-306, 15 cm x 3.0 mm, dp = 3.5 µm, equipped with an Upchurch (#A-318) pre-column filter (2.0 µm)
Mobile Phase A: 0.1% (v/v) acetic acid in acetonitrile
Mobile Phase B: 0.1% (v/v) acetic acid in purified water
Mobile Phase Gradient Program:

Time (min.)	% A	% B	Curve
0.0	20	80	-
10.0	100	0	1
3.0	100	0	-
1.0	20	80	1
7.0	20	80	-

Total Run Time: 21 min.

TABLE 3. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS

Instrumentation:

PE Sciex API-365 Triple Quadrupole Mass Spectrometer
TurbolonSpray Liquid Introduction Interface
Instrument Control and Data Collection: Apple MacIntosh Power PC Computer, Model 8500/180

Software:

Apple System 8.1

PE Sciex Software:

MassChrom v. 1.1.1
LC2Tune v. 1.4
Sample Control v. 1.4
MacDAD v. 1.4
MacQuan v. 1.6
Multiview v. 1.4
Bundler v. 1.4
File Translator v. 1.6.1
Downloader v. 1.2
Firmware (332) v. M3L1103
Firmware (340) v. M401100

Experiment Information:

Experiment Name: 77102 NEG
Scan Type: MRM
Scan Time: 0.615 sec/scan
Pause Time: 5.0 msec

Mass Range Information

	Q1 (amu)	Q3 (amu)	Dwell (msec)	Param	Start	Stop
Mass Range 1	192.3	134	200			
Mass Range 2	278.2	206	200	R02	23.0	23.0
Mass Range 3	328.1	121	200	R02	33.0	33.0

TABLE 3. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS (Continued)

Experiment Name: 77102 POS
Scan Type: MRM
Scan Time: 0.206 sec/scan
Pause Time: 6.0 msec

Mass Range Info

	Q1 (amu)	Q3 (amu)	Dwell (msec)
Mass Range 1	284.1	252.1	200

Method Information

Command	Description		Time (sec)	Duration (min)	Elapsed Time (min:sec)
Pause			240	4.0	4:00
Scan	Mode:	Profile	0.615	6.0	10:00
	Threshold:	1.0 x 10 E1 cps			
	Pause:	1.0 sec			
	Expt:	77102 NEG			
	State:	77102 NEG			
Scan	Mode:	Profile	0.206	2.5	12:30
	Threshold:	1.0 x 10 E1 cps			
	Pause:	1.4 sec			
	Expt:	77102 POS			
	State:	77102 POS			
Scan	Mode:	Profile	0.615	0.1	12:36
	Threshold:	1.0 x 10 E1 cps			
	Pause:	1.0 sec			
	Expt:	77102 NEG			
	State:	77102 NEG			

(Note: Switch back to "77102 NEG" state file after run completion to reset turboinspray temperature.)

TABLE 3. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS: LC/MS/MS (Continued)

Typical State File Values

77102 NEG		77102 POS	
Negative Ions		Positive Ions	
Gasses		**Gasses**	
NEB	12	NEB	12
CUR	10	CUR	10
CAD	1	CAD	2
Controls		**Controls**	
IS	-4900	IS	5200
TEM	420	TEM	350
OR	-13	OR	18
RNG	-95	RNG	85
Q0	10	Q0	-10
IQ1	12	IQ1	-12
ST	18	ST	-14
RO1	13	RO1	-11.5
IQ2	20	IQ2	-17
RO2	30	RO2	-30
IQ3	42	IQ3	-48
RO3	36.5	RO3	-35
DF	200	DF	-200
CEM	2900	CEM	2900
Q1 Resolution		**Q1 Resolution**	
Mass	**Offset**	**Mass**	**Offset**
30	0.030	30	0.036
100	0.045	100	0.042
1000	0.163	1000	0.160
2000	0.280	2000	0.275
Q3 Resolution		**Q3 Resolution**	
Mass	**Offset**	**Mass**	**Offset**
30	0.020	30	0.025
100	0.020	100	0.030
1000	0.075	1000	0.100
2000	0.070	2000	0.070

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

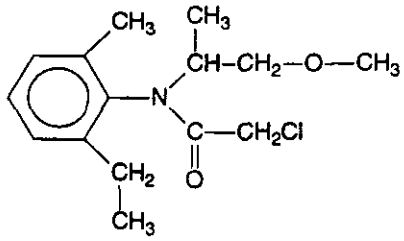
TABLE 4. TYPICAL ANALYTE MONITORING IONS

Analyte	Exact Molecular Weight	Q1 Molecular Ion	Q3 Product Ion
CGA-77102	283.1	284.1	252.1
CGA-51202	279.1	278.2*	206.0*
CGA-354743	329.1	328.3*	121.0*
CGA-37735	193.1	192.3*	134.0*

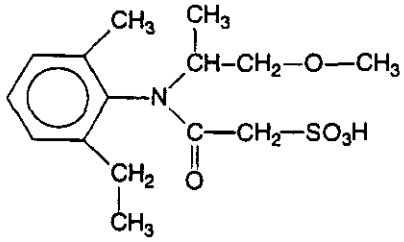
Masses used for Q1 and Q3 based on where optimum response was observed during infusion optimization. Optimum masses may vary slightly from calculated exact masses.

* Monitored as negative ion.

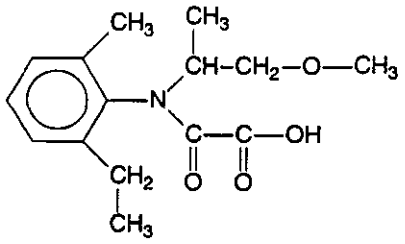
FIGURE 1. CHEMICAL NAMES AND STRUCTURES



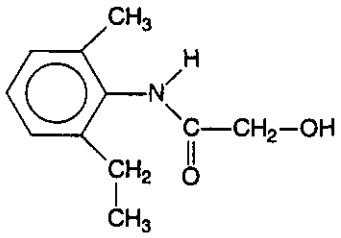
CGA-77102
CAS Number: 87392-12-9
CAS Name: Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-, (S)-
Purity: 98.5%



CGA-354743
CAS Number: Not Assigned
CAS Name: Ethanesulfonic acid, 2-[(2-ethyl-6-methylphenyl)(2-methoxy-1-methylethyl)amino]-2-oxo-, sodium salt
Purity: 96.8%



CGA-51202
CAS Number: 152019-73-3
CAS Name: Acetic acid, [(2-ethyl-6-methylphenyl)(2-methoxy-1-methylethyl)amino]oxo-
Purity: 98.8%



CGA-37735
CAS Number: 97055-05-5
CAS Name: Acetamide, N-(2-ethyl-6-methylphenyl)-2-hydroxy-
Purity: 99.2%

FIGURE 2. METHOD FLOW DIAGRAM FOR SOIL/THATCH

Measure 10-gram sample of soil or thatch
(Fortify, if necessary.)
Extract with 75 mL of 70% methanol/water, 1% in ammonium hydroxide for 30 minutes at room temperature using mechanical agitation.
Centrifuge/filter.
Repeat extraction with additional 75 mL of the extraction solvent.
Centrifuge/filter and combine extracts.

↓

Remove organic content via rotary evaporation.

↓

Acidify sample.
Pass through pre-conditioned Varian ENV SPE column.
(Analytes are retained.)
Rinse with basic and neutral purified water.

↓

Elute analytes from ENV SPE with basic methanol, collect in a concentration tube.
Remove organic via rotary evaporation until only aqueous remains.
Dilute to precalibrated mark.
Analyze by LC/MS/MS.

FIGURE 3. METHOD FLOW DIAGRAM FOR GRASS

Measure 5-gram sample of grass.
(Fortify, if necessary.)
Extract with 100 mL of 80% acetonitrile/water for 30 minutes at room temperature using mechanical agitation.
Centrifuge/filter.
Repeat extraction with additional 50 mL of extraction solvent
Centrifuge/filter and combine extracts.

↓

Remove 5-mL aliquot of sample extract.
Dilute with 5 mL of 0.1% formic acid.
Pass through pre-conditioned Varian C18 SPE column.
(Analytes are retained.)
Rinse with 40% acetonitrile/water (0.1% in formic acid).

↓

Elute analytes from SPE with 80% acetonitrile/water, collect in a r.b. flask.
Evaporate sample to dryness via rotary evaporation.
Dissolve residue in 20% acetonitrile/water.
Analyze by LC/MS/MS.