Recommended Method of Analysis

CL 263,499 Herbicide: GC Method for the Determination of CL 263,499 Residues in Soybean Plant, Seed and Straw

A. Principle

CL 263,499 residues are extracted from soybean plant, seed and straw using acidic aqueous methanol. After filtration, the extract is pH-adjusted and partitioned with methylene chloride. The methylene chloride is partitioned with pH 9 buffer. After adjustment to acidic pH, the aqueous buffer is partitioned with methylene chloride. The methylene chloride is evaporated and the residue dissolved in methanol-water. The methanol-water solution is passed through a disposable micro ion exchange column and CL 263,499 eluted with dilute hydrochloric acid solution. The acidic aqueous solution is then partitioned with methylene chloride, the methylene chloride evaporated and the residue dissolved in a measured amount of methanol. The CL 263,499 residue is quantified using trimethylanilinium hydroxide, an on-column methylating agent, and gas chromatography. A 3% OV-17 column and a thermionic nitrogen-selective detector are used to measure residues versus an external standard. The validated sensitivity of the method is 0.1 ppm for each soybean commodity.

B. Reagents

1. Analytical Standard: CL 263,499 [2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-5-ethyl-3-pyridine carboxylic acid] obtained from American Cyanamid Company, Agricultural Research Division, P. O. Box 400, Princeton, New Jersey 08540.

2. Solvents, Specially Purified: "Distilled in Glass", Burdick and Jackson Laboratories, Incorporated, or equivalent.

   a. Methylene Chloride
   b. Methanol
   c. Acetone

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   a. Potassium Phosphate, monobasic, crystal
   
   b. Sodium Phosphate, dibasic, anhydrous powder
   
   c. Hydrochloric acid, concentrated
   
   d. Sodium Hydroxide, 50% w/w solution

4. **TMAH Methylation Reagent:** Trimethylanilinium hydroxide, 0.2M in methanol, Cat. No. 680400, Regis Chemical Company.

5. **Solutions:**
   
   a. **1M Hydrochloric Acid:** Add 84 ml of concentrated HCl to a 1-liter volumetric flask containing 500 ml of distilled water and dilute to the mark with distilled water.
   
   b. **Extraction Solvent, 1% 1M Hydrochloric Acid, 39% Water in Methanol:** Mix 40 ml of 1% 1M HCl with 1560 ml of distilled water and add to 2400 ml of methanol.
   
   c. **0.05M Hydrochloric Acid:** Add 4.2 ml of concentrated HCl to a 1-liter flask containing 500 ml of distilled water and dilute to the mark with distilled water.
   
   d. **25% w/w Sodium Hydroxide:** Mix 50 ml of a 50% w/w sodium hydroxide solution with 50 ml of distilled water.
   
   e. **pH 9.0 Buffer:** Dissolve 13.6 g of potassium phosphate, monobasic and 14.1 g of sodium phosphate, dibasic in 4 liters of distilled water. Using a pH meter and 25% sodium hydroxide solution, adjust the buffer solution to pH 9.0.

6. **GC Column Material:** 3% OV-17 on 100-120 mesh Supelcoport, Cat. No. 1-1754, Supelco, Incorporated.

C. **Apparatus**

1. **Balance, Analytical:** Mettler, weighing to nearest 10 mcg.

2. **Balance, Toploading Pan:** Sartorius, weighing to nearest 0.01 g.

3. **Assorted Glassware:** General laboratory.

4. **Sorvall Omni-Mixer and Mason Jars:** One-quart capacity, DuPont Instruments, Newton, Connecticut.

5. **Rotary Flash Evaporator:** Buchler Instruments, Model PF-10DN or equivalent equipped with a heated water bath maintained at 35°C in which the evaporation flask can be partially submerged.
6. **Filtering Flasks**: 500-ml capacity, Corning Glass Works.

7. **Filtering Funnels**: Buchner, porcelain, 11-cm diameter.

8. **Filter Paper**: Glass fiber, 11-cm diameter, Whatman, Incorporated.

9. **pH Meter**: Orion Research, Model 701A or equivalent.

10. **Separatory Funnels**: Squibb type with Teflon stopcocks, 1000-ml, 500-ml, 250-ml capacity, Kontes Glass Company.

11. **Plastic Syringe**: Becton-Dickinson, 30-cc, Disposable, Luer-loc tip.

12. **GC Syringe**: Hamilton, Model 701-N, 10-ml capacity.


14. **GC Column**: 182-cm x 2-mm I.D., glass, packed with 3% OV-17 on 100-120 mesh Supelcoport. The column was packed using a slight vacuum. Silylated glass-wool plugs were placed at each end. The column was conditioned overnight at 275°C with a carrier gas flow of 30 ml/minute.

15. **Solid Phase Extraction Column**: J. T. Baker Chemical Company, 3-ml quaternary amine ion-exchange, Cat. No. 7091-3.

**D. Preparation of Standard Solutions**

1. **Stock Solution**

   Weigh accurately 10 ± 0.01 mg of CL 263,499 analytical standard into a 100-ml volumetric flask. Dilute to the mark with acetone and mix well. This is Standard Solution A and contains 100 mcg/ml.

2. **Standard Fortification Solutions**

   a. Pipet a 20-ml aliquot of Standard Solution A into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution B and contains 20 mcg/ml.

   b. Pipet a 10-ml aliquot of Standard Solution A into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution C and contains 10 mcg/ml.
c. Pipet a 10-ml aliquot of Standard Solution C into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution D and contains 1 mcg/ml.

NOTE: All standard solutions for GC must contain 50 mcL of TMAH per milliliter.

E. Gas Chromatographic Conditions

1. Instrument: Tracor Model 550 gas chromatograph.

2. Detector: Model 702 thermionic nitrogen-phosphorus detector. Bead setting should be adjusted to give a peak height of approximately 30% full scale deflection (FSD) for a 5.0 ng injection of CL 263,499.

3. Column: 182 cm x 2 mm I.D., glass, packed with 3% OV-17 on 100-120 mesh Supelcoport.

4. Instrument Conditions:

   a. Column Temperature 220°C
   b. Injection Port Temperature 300°C
   c. Detector Temperature 270°C
   d. Outlet Temperature 250°C
   e. Hydrogen Flow Rate 2.5 ml/min
   f. Helium Carrier Flow Rate 35 ml/min
   g. Air Flow Rate 120 ml/min
   h. Retention Time approximately 4 min.
   i. Electrometer set to give a peak height of approx. 30% FSD for a 5.0 ng injection

5. Recording Integrator: Hewlett-Packard Model 3380A

   a. Chart Speed 0.5 cm/min
   b. Attenuation set to give approx. 30% FSD for a 5.0 ng injection of CL 263,499
   c. Area Reject 1000
   d. Slope Sensitivity 1 mV/min

F. Linearity Check

1. Pipet 5-, 10-, 15- and 20-ml aliquots of Standard Solution C into separate 100-ml volumetric flasks. Dilute to volume with acetone. These solutions contain 0.5, 1.0, 1.5 and 2.0 mcg/ml, respectively, of CL 263,499.
2. Pipet 2.0-ml aliquots of each concentration into separate vials, add 100 mc1 of TMAH and inject 5-mc1 aliquots of each solution into the GC.

3. Plot the height for each peak versus the nanograms injected to demonstrate the linearity of response. Significant departure from linearity over this range indicates instrumental difficulties which should be corrected before proceeding.

G. Sample Preparation

1. Pulverize sufficient dry ice in a Hobart food chopper to thoroughly chill the bowl and the blade.

2. Add the pre-frozen sample in portions of sufficiently small size to enable reduction to fine particle size. (It may be necessary to add small portions of dry ice during the chopping procedure to ensure that the samples remain in a frozen state.)

3. Allow the sample to stand in a freezer overnight for the dry ice to dissipate completely.

4. Keep all samples frozen until ready for analysis.

H. Recovery Test

The validity of the procedure should always be demonstrated by recovery tests before analysis of unknown samples is attempted. A fortified sample should also be processed with each set of samples analyzed.

1. Weigh a 20-g subsample of soybean control material into a quart-blender container.

2. Add by pipet a volume of standard fortification solution appropriate to the fortification level to be tested.

3. Add the solution dropwise and mix the sample well before adding the extraction solvent.

4. Continue with the extraction and cleanup steps as described in the following sections.

I. Extraction

1. Accurately weigh 20 g of soybean material into a 1-quart Mason jar.

2. Extract sample with 200 ml of 1% IM HCl, 39% water in methanol at medium speed for 15 minutes.
3. Vacuum filter the extract through a Buchner funnel fitted with a
11-cm glass fiber filter paper. Add 100 ml extractant to the
blendor jar, replace lid and shake to thoroughly rinse the jar.
Filter the rinsate.

4. Transfer the filtrate to a 500-ml beaker.

J. Methylene Chloride Partition

1. Adjust the filtrate to pH 2.0 (± 0.1) using a pH meter and 1M
   HCl.

2. Transfer the pH 2 solution to a 500-ml separatory funnel. Gently
   partition the extract with 100 ml of methylene chloride for
   30 seconds. Collect the lower methylene chloride layer in a 500-ml
   beaker*.

3. Repeat the extraction twice more using 100 ml of methylene chloride
   each time, combining the methylene chloride fractions in the beaker.
   Discard the upper aqueous methanol phase.

K. Buffer Partition Cleanup

1. Transfer the 300 ml of methylene chloride to a 1-L separatory funnel.
   Add 200 ml of pH 9 buffer to the separatory funnel and gently
   partition the methylene chloride, shaking for 1 minute. Allow a
   few minutes for the phases to separate*.

2. Discard the lower methylene chloride phase. Collect the upper
   aqueous buffer layer in a 500-ml beaker. Adjust the buffer to
   pH 2.0 (± 0.1) with 1M HCl using a pH meter.

3. Transfer the pH 2 solution to a 500-ml separatory funnel and partition
   with 100 ml of methylene chloride for 30 seconds. Collect the lower
   methylene chloride layer in a 1-L round bottom flask. Repeat the
   partition three more times using 100 ml of methylene chloride each
   time, combining the methylene chloride fractions in a 1-L round bottom
   flask.

* The lower methylene chloride layer may emulsify at this point. The emulsion
   can be broken by passing the methylene chloride phase through a powder
   funnel filled with glass wool. Repeat the process if necessary to
   thoroughly deemulsify the methylene chloride. Use the separatory funnel to
   remove water separated from the methylene chloride.
4. Evaporate the methylene chloride just to dryness on a rotary evaporator at 35°C. Dissolve the residue in 20 ml of methanol. Quantitatively transfer the methanol to a 100-ml round bottom flask. Rinse the 1-L flask twice more with 20 ml of methanol each time, using ultrasonication to dissolve and transfer the residue.

5. Evaporate the 60 ml of methanol on a rotary evaporator just to dryness.

L. Solid Phase Extraction Column Cleanup

1. Add 1 ml of methanol to the flask, carefully dissolving the residue. Add 24 ml of distilled water to the methanol and swirl.

2. Prepare a 3-ml Baker solid phase extraction column by passing 10 ml of methanol through the column followed by 10 ml of distilled water using a 30-cc disposable plastic syringe fitted to the top of the column. Do not allow the column to go dry.

3. Pass the 25-ml aqueous methanol sample solution from step L.1 through the extraction column using the 30-cc syringe. The eluant should not exceed a rate of about 2 drops per second. Discard the eluant. Add 15 ml of 0.05M HCl to the 30-cc syringe, passing the solution through the extraction column at a rate not to exceed about 2 drops per second. Collect the eluant in a 50-ml beaker.

M. Methylene Chloride Partition

1. Add 20 ml of distilled water to the eluant and ensure that the pH is 2.0 (+ 0.1) using a pH meter.

2. Transfer the pH 2 solution to a 250-ml separatory funnel. Partition the solution three times with 100 ml of methylene chloride, collecting the methylene chloride extracts in a 500-ml round bottom flask.

3. Evaporate the methylene chloride just to dryness on a rotary evaporator.

4. Dissolve the residue in 4.0 ml of methanol for analysis by GC.

N. Gas Chromatographic Analysis

1. Transfer a 2.0-ml aliquot of a 1.0 mcg/ml standard of Cl 263,499 to a small vial. Evaporate the acetone with the aid of a stream of nitrogen and pipet 2.0 ml of methanol into the vial. Add 100 mc1 of TMAH methylation reagent to the standard vial (all GC solutions should contain 50 mc1 TMAH/ml solution).
2. Transfer a 1.0-ml aliquot of the sample solution to a small vial.

3. After obtaining a stable GC response to the standard, add 50 mcl of TMAH to the sample solution vial and inject a 5.0-mcl aliquot of the sample into the GC. After addition of TMAH, the sample should be injected within 10 minutes.

4. Compare the peak height with that obtained from a 5.0-mcl injection of a GC solution containing 1.0 mcg/ml of CL 263,499 (external standard).

5. If the sample peak goes off scale, dilute to an appropriate volume with methanol and reinject. NOTE: All final (diluted) extracts prepared for GC should contain 50 mcl of TMAH per milliliter of solution.

6. Alternate injections of standard and sample and use the average peak height of the standards bracketing a sample for calculation.

O. Calculations

For each sample calculation, use the sample peak height and the average peak height value resulting from measurement of the external standard peak height obtained before and after the sample injection as follows:

\[
\text{ppm} = \frac{R(\text{SAMPLE}) \times (V1) \times (V3) \times C(\text{STD}) \times (V5) \times (DF)}{R(\text{STD}) \times (W) \times (V2) \times (V4)}
\]

Where:

R(SAMPLE) = Peak height of sample
R(STD) = Average peak height of working standard
W = Weight of sample taken for analysis in grams
V1 = Volume of extracting solvent added to sample in ml
V2 = Aliquot of extract taken for analysis in ml
V3 = Volume of methanol added to dissolve the residue for chromatographic analysis in ml
V4 = Volume of sample solution injected in mcl

V5 = Volume of working standard solution injected in mcl

C(STD) = Concentration of working standard solution in mcg per ml

D.F. = Dilution factor

Figures M-1586.A to M-1586.C show typical chromatograms for determining CL 263,499 residues in soybean plants, straw and seed, respectively.
Figure M-1586.A: Typical Chromatograms of Fortified and Control Soybean Plant
Figure M-1586.B: Typical Chromatograms of Fortified and Control Soybean Straw

- Standard CL 263,499 (5.0 mg Injected)
- Soybean Straw Fortified with CL 263,499 at 0.1 ppm (25 mg Injected), 81% Recovery
- Control Soybean Straw (25 mg Injected)
Figure M-1586.C: Typical Chromatograms of Fortified and Control Soybean Seed

- Standard CL 263,499 (5.0 mg Injected)
- Soybean Seed Fortified with CL 263,499 at 0.1 ppm (25 mg Injected), 85% Recovery
- Control Soybean Seed (25 mg Injected)
| Sample Type: Soybean Plant | Analyzed For: CL 263.499 | Method Used: M-1586 | Validated Sensitivity: 0.10 PPM | Response Measured As: Peak Height in Millimeters | V4 = V5 = 5.0 |

**TABLE I**

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<th>Standards</th>
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-N.M.: not measurable (less than 2 units). Corresponding for control samples, to the limits given above in the APPARENT-residue column. These limits are calculated by insertion of the number 2 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

Control samples are indicated by a minus sign before the R(SAMP) value.
TABLE II

TYPE OF EXPERIMENT: RECOVERY STUDY  APP. PPM FOUND = ________________________ R(STD) = N x V2 x V4  PPM FOUND @ 100
SAMPLE TYPE: SOYBEAN SEED  PPM FOUND @ 100
ANALYZED FOR: CL 263,499
METHOD USED: M = 1586
VALIDATED SENSITIVITY: 0.10 PPM
RESPONSE MEASURED AS: PEAK HEIGHT IN MILLIMETERS  % RECOVERY = ________________________  PPM ADDED
V4 x V5 = 1  __________

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Control samples are indicated by a minus sign before the R(SAMP) value.
<table>
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<th>TABLE III</th>
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<th>TYPE OF EXPERIMENT:</th>
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