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Study Title

CL 263,499 :Validation of Capillary Electrophoresis (CE) Determinative and LC/MS Confirmatory Method for CL 263,499, CL 288,511, and CL 182,704 Residues in Alfalfa and Clover Commodities (Alfalfa Green Forage, Hay, Seed and Process Meal and Clover Hay, Forage, Seed and Seed Screenings)

Data Requirement

Guideline No. 171-4(c)

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Study Identification Number

RES 93-140

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C).

Company: American Cyanamid Company

Company Agent: Desiree L. Little Date: 11/11/93

Product Registrations Manager
Title Desiree Little
Signature

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

This study was conducted according to the published Good Laboratory Practices (GLP) for tests of substances regulated under the Federal Insecticide, Fungicide, and Rodenticide Act (40 CFR, Part 160). The study director's statement(s) of compliance with GLP (40 CFR 160) are located on page(s) 57.

Sponsor: American Cyanamid Company

Submitter: American Cyanamid Company

Company Agent: Desiree L. Little

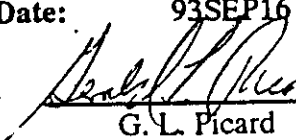
Date: 11/11/93

Product Registrations Manager
Title

Desiree L. Little
Signature



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CL Numbers:	CL 263,499; CL 288,511; CL 182,704; MAE-CL 182,704	Approved by:	 G. L. Picard
Reported By:	M. Safarpour		
Work Done By:	M. Safarpour; S. Chiu; S. Fletcher		

TITLE:

CL 263,499: Validation of Capillary Electrophoresis (CE) Determinative and LC/MS Confirmatory Method for CL 263,499, CL 288,511, and CL 182,704 Residues in Alfalfa and Clover Commodities (Alfalfa Green Forage, Hay, Seed and Process Meal and Clover Hay, Forage, Seed and Seed Screenings).

PURPOSE:

To validate Method M 2261 for the determination of CL 263,499, CL 288,511, CL 182,704 and MAE-CL 182,704 (malonic acid ester of CL 182,704) residues in alfalfa and clover commodities (alfalfa green forage, hay, seed and process meal and clover hay, forage, seed and seed screenings).

SUMMARY:

Method M 2261 (see Appendix A) was found to be satisfactory for the determination of CL 263,499, CL 288,511, CL 182,704 and MAE-CL 182,704 (malonic acid ester of CL 182,704) residues in alfalfa and clover commodities (alfalfa green forage, hay, seed and process meal and CL over hay, forage, seed and seed screenings). Residues of the MAE-CL 182,704 in field samples are converted in Method M 2261 to CL 182,704 using the conditions specified in PD-M Volume 28-1/23, 29-30, 47, 1991 American Cyanamid Report. Raw data from this metabolism study indicates a 90% efficiency for the conversion of the MAE-CL 182,704 to CL 182,704 under the base-hydrolysis conditions specified. The validated sensitivity of the method is 0.50 ppm for each compound in each commodity.

Recoveries were run by fortifying eight different untreated alfalfa and clover commodities with solutions of analytical grade CL 263,499, CL 288,511 and CL 182,704 (see protocol PU93PT01 and amendment to protocol, Appendix B). The detailed analytical data for CL 263,499, CL 288,511 and CL 182,704 recoveries are presented in Tables I to IX. The overall average recoveries for CL 263,499, CL 288,511 and CL 182,704 were $86 \pm 12\%$ S.D., $96 \pm 11\%$ S.D., $85 \pm 8\%$ S.D., respectively. Fortification levels for alfalfa and clover commodities ranged from 0.50 ppm to 10.0 ppm. Apparent CL 263,499, CL 288,511 and CL 182,704 residues in control samples averaged less than 0.035, 0.034 and 0.031 ppm, respectively.

STUDY PERSONNEL:

A list of the American Cyanamid personnel associated with this study is attached as Appendix C.

STUDY INTEGRITY:

There were no known circumstances that may have affected the quality or integrity of the data.

GLP COMPLIANCE:

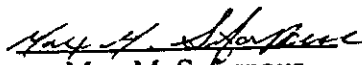
A statement of GLP compliance, signed by the Study Director, is attached as Appendix D.

QUALITY ASSURANCE:

A statement prepared and signed by the American Cyanamid Quality Assurance Unit with regard to this study is attached in Appendix E.

ARCHIVES:

All raw data, protocol along with any amendments or deviations, and appropriate samples, documentation, records and the final report and any subsequent amendments are stored in the Archives of the American Cyanamid Company, Agricultural Research Division, Princeton, New Jersey.


Max M. Safarpour
Study Director

9/16/93
Date

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APPENDIX A

American Cyanamid Method M 2261



AMERICAN CYANAMID COMPANY
AGRICULTURAL RESEARCH DIVISION
P. O. Box 400
Princeton, NJ 08543-0400 USA

Recommended Method of Analysis - M 2261

Imazethapyr (CL 263,499): Capillary Electrophoresis (CE) Determinative and LC/MS Confirmatory Method for CL 263,499, CL 288,511, and CL 182,704 Residues in Alfalfa and Clover Commodities (Alfalfa Green Forage, Hay, Seed and Process Meal and Clover Hay, Forage, Seed and Seed Screenings).

A. Principle

Residues of CL 263,499, CL 288,511, CL 182,704 and MAE-CL 182,704 (malonic acid ester of CL 182,704) are extracted from the sample with acidic water-methanol. The MAE-CL 182,704 is converted to CL 182,704 via base hydrolysis. The CL 263,499, CL 288,511 and CL 182,704 are subjected to suitable cleanup involving precipitation, centrifugation and solid phase extraction techniques. Measurement of CL 263,499, CL 288,511 and CL 182,704 residues is accomplished by capillary electrophoresis (CE) equipped with a UV detector, at 240 nm. Results are calculated as CL 263,499, CL 288,511 and CL 182,704 by the direct comparison of the peak heights to those of external standards. The validated sensitivity of the method is 0.50 ppm for each compound in each commodity.

Confirmation for total residues at the 3 ppm level is provided by thermospray LC/MS of the final extract.

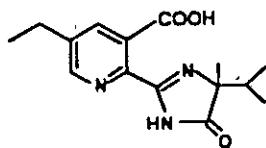
B. Reagents

(Items from manufacturers other than those listed may be used provided they are functionally equivalent.)

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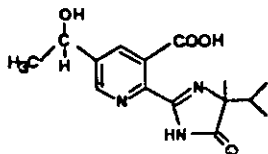
1. **Analytical Standards:** Analytical grade, known purity, American Cyanamid Company, Agricultural Research Division, P.O. Box 400, Princeton, New Jersey 08543-0400.

- a. CL 263,499: (+)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid



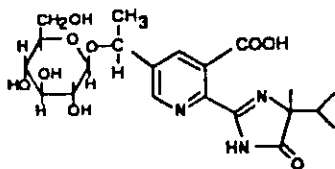
M.W. = 289

- b. CL 288,511: 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(1-hydroxyethyl)-3-pyridinecarboxylic acid



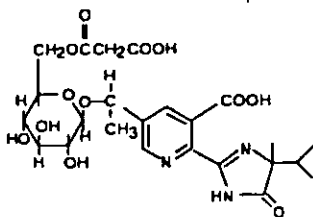
M.W. = 305

- c. CL 182,704: 5-[1-(beta-D-glucopyranosyloxy) ethyl]-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid



M.W. = 467

- d. MAE-CL 182,704: 5-[1-(beta-D-glucopyranosyloxy) ethyl]-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid



M.W. = 553

2. **Solvents:** B & J Brand High Purity Solvents (UV grade), Baxter Burdick and Jackson.
 - a. Acetonitrile
 - b. Methanol
 - c. Isopropyl Alcohol
 - d. Hexane
 - e. Methylene Chloride
 - f. Acetone
3. **Water, Deionized:** Water passed through Millipore's Milli-Q Plus Ultra Pure Water System. Use this water for all steps requiring deionized water.
4. **Chemicals:**
 - a. Aldrich Electrophoresis Grade
 - 1.) Sodium Tetraborate Decahydrate 96% Purity (Cat. No. 25,136-4)
 - 2.) Tetrabutylammonium Dihydrogen Phosphate, 97% Purity (Cat. No. 26,809-7)
 - b. "Baker Analyzed" Reagents, J. T. Baker Company.
 - 1.) Hydrochloric Acid, Concentrated (Cat. No. 9535-1)
 - 2.) Potassium Chloride (Cat. No. 3040-01)
 - 3.) Sodium Hydroxide, Pellets, Purity 98.8% (Cat. No. 3722-01)
 - 4.) Sodium Hydroxide Solution, 50% (Cat. No. 3727-01)
 - c. Supelco Separation Technologies
Reagent Grade, Celite 545 AW (Cat. No. 2-0199)
 - d. VWR Scientific
Lead (II) Acetate 3-Hydrate, 99.5% Purity (Cat. No. EM 7374-1)
 - e. Sigma Chemical Company
 - 1.) Sodium Phosphate, Monobasic, 99% Purity (Cat. No. S-3522)
 - 2.) Sodium Phosphate, Dibasic, 99% Purity (Cat. No. S-3397)
 - 3.) 1-Hexanesulfonic Acid, 98% Purity (Cat. No. H-5269)
 - 4.) Cholic Acid, 98% Purity (Cat. No. C-1254)
 - 5.) Borax (Sodium Tetraborate Decahydrate), 99% Purity (Cat. No. B-9876)

- 6.) D-(+) Glucose, 96% α -anomer and 4% β - anomer Purity
(Cat. No. G-7021)
- 7.) Tricine (N-tris[Hydroxymethyl]-methylglycine), 99% Purity
(Cat. No. T-0377)
- 8.) DTAB (Dodecyltrimethylammonium bromide), 99% Purity
(Cat. No. D-8638)

f. Calbiochem Corporation

TRIS [tris-(hydroxymethyl)-aminomethane], 99% Purity
(Cat. No. 648309), LaJolla, California

g. Bethesda Research Laboratories (BRL)

Sodium Dodecyl Sulfate (SDS), Electrophoresis Grade, Purity > 99% (Cat. No. 5525UB)

5. Solutions:

- a. 6 N Hydrochloric Acid: Add 250 mL of concentrated hydrochloric acid to 250 mL of Milli-Q water.
- b. 1 N Hydrochloric Acid: Add 83 mL of concentrated hydrochloric acid to 500 mL of Milli-Q water in a 1-liter volumetric flask and dilute to 1 liter with Milli-Q water.
- c. 0.05 N Hydrochloric Acid: Dilute 50 mL of reagent B.5.b to 1 liter with Milli-Q water.
- d. 0.005 N Hydrochloric Acid: Dilute 100 mL of reagent B.5.c to 1 liter with Milli-Q water.
- e. Extraction Solvent-Hydrochloric acid: Water: Methanol : Mix 40 mL of 1 N hydrochloric acid with 1560 mL of Milli-Q water and 2400 mL of methanol.
- f. Saturated Potassium Chloride-Methanol: Add 50 g of potassium chloride to 1 liter of methanol, stir for 5 minutes, allow excess potassium chloride to settle.
- g. 10% Sodium Hydroxide: Dilute 100 mL of 50% sodium hydroxide solution to 500 mL with Milli-Q water.
- h. 6 N Sodium Hydroxide: Dissolve 240 g of sodium hydroxide pellets (98.8%) in approximately 600 mL of Milli-Q water and dilute to 1 liter.
- k. 1 N Sodium Hydroxide: Dissolve 40 g of sodium hydroxide pellets (98.8%) in approximately 600 mL of Milli-Q water and dilute to 1 liter.

- l. 10% Lead Acetate: Dissolve 10 g of lead acetate in 100 mL of Milli-Q water and filter through 0.22 μ m Corning Nylon Filter System. Store at room temperature.
- m. 100 mM Sodium Hydroxide: Dissolve 2 g of sodium hydroxide pellets (98.8%) in approximately 100 mL of Milli-Q water and dilute to 500 mL.
- n. 1% 1 N Hydrochloric Acid in Methanol: Dilute 10 mL of 1 N hydrochloric acid to 1 liter with methanol.
- o. 1 M D-(+)-Glucose: Dissolve 1.8 g of D-(+)-glucose in 10 mL Milli-Q water.
- p. Separation Buffer (pH=9.2), Current approximately 85 μ A: 180 mM sodium dodecyl sulfate (SDS)/20 mM sodium tetraborate decahydrate: Add 10.38 g of sodium dodecyl sulfate (SDS) and 1.53 g of sodium tetraborate decahydrate to 200 mL of Milli-Q water, stir for approximately 5 minutes, filter through 0.22 μ m Corning Nylon Filter System. Store at room temperature (See Note 1, Note 2 and Note 3).

C. Apparatus

(Items from other manufacturers may be used provided they are functionally equivalent).

1. Capillary Electrophoresis: Applied Biosystems Model 270A-HT or Model 270A equipped with a high sensitivity optical flow cell and UV detector.
2. High Sensitivity Optical Cell: Applied Biosystems, Cat. No. 401536, Foster City, California.
3. Integrator: Chromjet Recording Integrator, Spectra-Physics, Fremont, CA.
4. Centrifuge: Sorval Model RC 5C Centrifuge, Rotor Model SS34 and Rotor Model GSA, Dupont, Wilmington, DE.
5. Oak Ridge Centrifuge Tube: 50-mL tube, Nalgene, Cat. No. 3119-0050.
6. Dryspin Polypropylene Bottle: 250-mL, Sorval Instruments, Cat. No. 03069.
7. Balance: Pan, Sartorius, Model L 610, precision \pm 5 mg.
8. Balance: Analytical, Sartorius Model R200D, precision \pm 0.05 mg, Scientific Products, Edison, NJ.
9. Assorted Glassware: General laboratory. NOTE: All glassware, and centrifuge tubes should be rinsed with Milli-Q water and then methanol and dry before use.
10. Flasks: 24/40 T 100-, 150-, 200-, 300- and 500-mL pear shaped flasks, Kontes, Scientific Glassware/Instruments, Vineland, NJ.
11. Filtering Flasks: 500-mL capacity, Corning Glass Works.
12. Filtering Funnels: Buchner, porcelain, 9-cm diameter.

13. Filter Paper: 9-cm diameter, glass-fiber filter, 934-AH, Whatman, Incorporated.
14. Rotary Evaporator: Buchler Instruments Model RE-121C equipped with a cold finger dry ice trap and a heated water bath set to approximately 35-40° C, Westbury, NY.
15. Constant Temperature Water Bath and Circulator: NESLAB Instruments, Model GP-200, NESLAB Instruments Inc. Newington, New Hampshire.
16. Ultrasonic Cleaner: Branson Model 3200, Branson Ultrasonics Corporation, Danbury, CT.
17. VacMaster-20 Vacuum Manifold Sample Processing Station: IST, Cat. No. 121-2016, Jones Chromatography, Lakewood, CO.
18. PTFE stopcock/needle unit: IST Cat. No. 121-0001, Jones Chromatography, Lakewood, CO.
19. PTFE ISOLUTE Column Adaptors: IST, Cat. No. 120-1100, Jones Chromatography, Lakewood, CO.
20. Empty Reservoirs: IST, 70-mL nominal capacity, Cat. No. 120-1008-F and 25-mL capacity Cat. No. 120-1007-E, Jones Chromatography, Lakewood, CO.
21. Luer Stopcocks: Cat. No. 1213-1005, Varian, Harbor City, CA.
22. Omni Mixer Homogenizer: Model 17105, OCI Instruments, Omni International Gainsville, VA.
23. pH Meter: Orion Model 701A, Orion Research, Boston, MA.
24. MILLEX -GS Filter Unit: Sterile, 0.22 um filter unit, Cat. No. SLGS0250S, Millipore Product Division, Bedford, MA.
25. Vacuum Filtration Device: A 500-mL suction flask fitted with 9 -cm diameter Buchner porcelain funnel by means of a rubber adapter.
26. Vortex Mixer: S/P Vortex Mixer, Cat. No. S8223-1, American Scientific Products, Edison, NJ.
27. Corning Filter System: 150-mL Filter System, 0.22 um Nylon, Cat. No. 25934-200, Corning Incorporated, Corning, NY.
28. Buchi Vacuum Pump and Controller: Vacobox, Model B-171, Cat. No. 1550705-5, Brinkman, Instruments, Inc., Westbury, NY.
29. Filter Support Collars: Cat. No. K389730, J. & H. Berge, Inc., South Plainfield, NJ.

30. Solid Phase Extraction Cartridges:

- a. **€18:** ISOLUTE, 500 mg/3-mL tube, unencapped, International Sorbent Technology (IST), Cat. No. 220-0050-B, distributed by Jones Chromatography, Lakewood, CO.
- b. Aromatic Sulfonic Acid (SCX): ISOLUTE, 1000 mg/6-mL tube, International Sorbent Technology (IST), Cat. No. 530-0100-C, distributed by Jones Chromatography, Lakewood, CO.

D. Preparation of Standard Solutions

1. Stock Solutions (Prepare bimonthly, store in amber bottles with polyseal caps in refrigerator).

- a. CL 263,499: Weigh accurately a known amount (approximately 15 mg) of CL 263,499 into an aluminum boat and place into a 50-mL volumetric flask. Dilute to the mark with acetone and mix well. Calculate and record the exact concentration of CL 263,499, correcting for the standard purity.
- b. CL 288,511: Weigh accurately a known amount (approximately 15 mg) of CL 288,511 into an aluminum boat and place into a 50-mL volumetric flask. Dilute to the mark with acetone and mix well. Calculate and record the exact concentration of CL 288,511, correcting for the standard purity.
- c. CL 182,704: Weigh accurately a known amount (approximately 15 mg) of CL 182,704 into an aluminum boat and place into a 50-mL volumetric flask. Add 25 mL methanol. Dilute to the mark with Milli-Q water and mix well. Calculate and record the exact concentration of CL 182,704, correcting for the standard purity.

The concentration of CL 182,704 should then be expressed as CL 288,511 equivalents by converting it using the ratio of molecular weights (0.65) described by the following equation:

$$\text{mcg/mL CL 182,704} \times \frac{\text{M.W. CL 288,511 (305)}}{\text{M.W. CL 182,704 (467)}} = \text{mcg/mL CL 288,511 equivalents}$$

2. Mixed Standard Fortification Solutions

- a. Pipet into a single 100-mL volumetric flask, an appropriate amount of each stock solution D.1.a., D.1.b. and D.1.c. to deliver 5,000 mcg of each compound, CL 263,499, CL 288,511 and CL 182,704, expressed as CL 288,511 equivalents. Dilute to the mark with acetone and mix well. This Mixed Standard Fortification Solution contains 50 mcg/mL each of CL 263,499, CL 288,511 and CL 182,704, expressed as CL 288,511 equivalents.
- b. Pipet into a single 100-mL volumetric flask, a 20-mL aliquot of the Mixed Standard Fortification Solution prepared in D.2.a. Dilute to the mark with acetone and mix well. This Mixed Standard Fortification Solution contains 10 mcg/mL each of CL 263,499, CL 288,511 and CL 182,704, expressed as CL 288,511 equivalents.

3. Mixed Calibration Standard Solutions
(Prepare monthly, store in amber bottles with polyseal caps in refrigerator).

a. 5.0 mcg/mL

Pipet into a 100-mL pear-shaped flask, a 10-mL aliquot of the Mixed Standard Fortification Solution prepared in D.2.a. Evaporate the organic solvents from the mixture on a rotary evaporator (some water will remain in the flask). Rinse the pear-shaped flask with 3 x 25 mL Milli-Q water and transfer to a 100-mL volumetric flask. Bring the volume up to the mark with Milli-Q water, stopper the flask and mix well. This Mixed Calibration Standard solution contains 5 mcg/mL of each compound.

b. 1.0 mcg/mL

Pipet 20 mL of Mixed Standard solution D.3.a. into a 100-mL volumetric flask. Dilute to the mark with Milli-Q water, stopper and mix well. This Mixed Calibration Standard solution contains 1.0 mcg/mL of each compound.

c. 0.50 mcg/mL

Pipet 10 mL of Mixed Calibration Standard solution D.3.a. into a 100-mL volumetric flask. Dilute to the mark with Milli-Q water, stopper and mix well. This Mixed Calibration Standard solution contains 0.50 mcg/mL of each compound. This standard is the working standard.

d. 0.25 mcg/mL

Pipet 5 mL of Mixed Calibration Standard solution D.3.a. into a 100-mL volumetric flask. Dilute to the mark with Milli-Q water, stopper and mix well. This Mixed Calibration Standard solution contains 0.25 mcg/mL of each compound.

e. 0.125 mcg/mL

Pipet 2.5 mL of Mixed Calibration Standard solution D.3.a. into a 100-mL volumetric flask. Dilute to the mark with Milli-Q water, stopper and mix well. This Mixed Calibration Standard solution contains 0.125 mcg/mL of each compound.

Use the 1.0, 0.50, 0.25, and 0.125 mcg/mL Mixed Calibration Standard solutions for the linearity check (See Section F).

E. Capillary Electrophoresis (CE) Conditions

1. Wavelength: 240 nanometers.
2. Polarity: (+)
3. Voltage: 15 KV
4. Injection: Vacuum, 4 seconds (approximately 73.8 nL)
5. Temperature: 29 °C
6. Capillary: Bare Fused Silica, approximately 72 cm length, approximately 75 μ m i.d., 280 μ m o.d., (approximately 50 cm from inlet to detector); (approximately 22 cm from detector to outlet).
7. Attenuation: 16
8. Chart Speed: 0.5 cm/min
9. Range: 0.010
10. Rise Time: 0.50
11. Conditioning a New Capillary:
 - a). Wash with 1 N NaOH, for 20 minutes.
 - b). Wash with 0.1 N NaOH, for 5 minutes.
 - c). Rinse with Milli-Q water, for 5 minutes.
 - d). Rinse with separation buffer, for 5 minutes.

Discard solutions in capillary vials after daily use.

Buffer, Milli-Q water and 0.10 N NaOH need to be filtered through 0.22 μ m filter prior to use.

12. Equilibration: 2 minutes, 100 mM sodium hydroxide wash followed by 5 minutes with the run buffer.
13. Separation Buffer: 20 mM sodium tetraborate decahydrate, 180 mM sodium dodecyl sulfate, pH 9.2.

NOTE: Capillary needs to be rinsed with Separation Buffer for 5 minutes prior to using the CE for any sample analysis.

14. Retention Time: Approximately 8 min. for CL 182,704, 9.5 min for CL 288,511 and 9.8 min for CL 263,499.
15. Sensitivity: Instrument sensitivity should be set so that a 4-second load of the 0.5 mcg/mL Mixed Calibration Standard gives an electrophoretic peak height of approximately 61 mm for CL 182,704, 61 mm for CL 288,511 and 71 mm for CL 263,499 (approximately 30%-40% full scale deflection).

F. Linearity Check

The Capillary Electrophoresis should be checked for linearity of response whenever new High Sensitivity Optical Cell, capillary or instrument is used for analysis.

1. Adjust the CE conditions for 1.0, 0.50, 0.25, and 0.125 mcg/mL Mixed Calibration Standard solutions injection. The 0.50 mcg/mL working standard should give approximately 30%-40% full scale deflection.
2. Plot the height for each peak versus the picogram injected for each compound to show linearity of response. Significant departure from linearity over this range indicates "system" difficulties which should be corrected before proceeding.

G. Sample Preparation

1. Pulverize sufficient dry ice in a food chopper (Hobart Model 84185-D) to chill the bowl and blade thoroughly.
2. Add the prefrozen alfalfa and clover commodities in small portions 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. For analysis of alfalfa seed and clover seed samples, blend the whole sample with dry ice for several minutes in a Waring Blendor to break the grain and pulverize the sample.
4. Allow the samples to stand in a freezer overnight for the dry ice to dissipate completely.
5. Keep all samples frozen until ready for analysis.

H. Recovery Test Samples

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 daily set of samples analyzed.

1. Weigh a 20-g subsample of control into a 1-quart Mason jar.
2. Add by pipet a volume of standard fortification solution appropriate to the fortification level to be tested.
3. Add the fortification solution dropwise and mix the sample well before adding the extraction solvent.
4. Continue with the extraction and cleanup steps as described in the method.

I. Extraction, Precipitation, Centrifugation Preliminary Cleanup (Important: See Note 4 for Stopping Points within the method)

1. Weigh 20 g of sample into a 1-quart Mason jar.
2. Add 300 mL of extraction solvent (Hydrochloric Acid:Water:Methanol) (B.5.e) and blend at medium speed for 5 minutes using an Omni mixer.
3. Add approximately 5 g of Celite 545 AW to the mixture in the Mason jar, swirl to mix, and filter the extract by vacuum through a double layer of filter paper prepared on a 9-cm Buchner funnel into a 500-mL filtration flask.
4. Pipet a 15-mL aliquot of the extract into a 150-mL pear-shaped flask. Discard the remaining extract.
5. Evaporate the aliquot to dryness using a rotary evaporator and Vacobox.
6. Dissolve the residue in 5 mL 1% 1 N hydrochloric acid in methanol (B.5.n). Stopper, swirl and sonicate. Add 50 mL acetonitrile, stopper, shake and transfer to a 250-mL centrifuge bottle. Rinse the flask with 50 mL acetonitrile and transfer to the centrifuge bottle. Rinse the flask with an additional 50 mL acetonitrile, stopper, shake and transfer to the centrifuge bottle.
7. Adjust the centrifuge bottles to approximately equal weight with acetonitrile using a pan balance.
8. Using centrifuge rotor model GSA, centrifuge at 10,000 rpm (16,266 x g) for 20 minutes at approximately 4 °C.
9. Transfer the supernatant to a 500-mL pear-shaped flask. Evaporate the acetonitrile to dryness using a rotary evaporator and Vacobox. Discard the precipitate. (See Note 4).
10. Dissolve the residue in 10 mL of 1N sodium hydroxide solution (B.5.k). Stopper, swirl and transfer to 100-mL pear-shaped flask. Rinse the 500-mL pear-shaped flask with an additional 10 mL of 1N sodium hydroxide solution. Stopper, swirl and transfer to the 100-mL pear-shaped flask. Rinse the 500-mL pear-shaped flask with an additional 3 mL of 1N sodium hydroxide solution. Stopper, swirl and transfer to the same 100-mL pear-shaped flask.
11. Stopper and place the 100-mL pear-shaped flask into a constant temperature water bath at approximately 45 °C for 40 minutes. Remove the 100-mL pear-shaped flask from the water bath and allow to cool for approximately 3 minutes in an ice bath. (See Note 5).
12. Transfer the solution into a 50-mL beaker and add 500 mcL of 1 M D-(+)-glucose (B.5.o) into the mixture. Rinse the 100-mL pear-shaped flask with 3 mL of 10% lead acetate solution (B.5.l). Stopper, swirl and pour into the same beaker. Rinse the same 100-mL pear-shaped flask with 3 mL of Milli-Q water. Stopper, swirl and pour into the beaker.
13. Add 2.5 mL of 6 N hydrochloric acid to the mixture. Using a pH meter, a magnetic stirrer and stirring bar, adjust the pH to approximately pH 11.5 with

- drops of 6 N and 1 N hydrochloric acid. Transfer the pH 11.5 mixture into a 50-mL centrifuge tube. Rinse the beaker with 2 mL of Milli-Q water and pour into the same centrifuge tube. Retain the 50-mL used beaker for use in step I.15.
14. Adjust the centrifuge tubes to approximately equal weight with Milli-Q water using a pan balance. Using centrifuge rotor model SS-34, centrifuge at 20,000 RPM (47,813 x g) for 10 minutes at approximately 4 °C. Transfer the supernatant into a 100-mL beaker.
 15. Dissolve the precipitate, in the 50-mL centrifuge tube, in 10 mL of 1N sodium hydroxide solution . Add 2 mL of 50% sodium hydroxide solution to the mixture. Stopper, swirl, and vortex to re-dissolve the precipitate and transfer into the 50-mL beaker retained in step I. 13. Rinse the centrifuge tube with 2 mL of Milli-Q water and pour into the same 50-mL beaker. Retain the centrifuge tube. Add 7.5 mL of 6 N hydrochloric acid. Using a pH meter, a magnetic stirrer and a stirring bar, adjust the pH to approximately pH 11.5 with drops of 6 N and 1 N hydrochloric acid.
 16. Pour the solution into the same 50-mL centrifuge tube retained in step I.15. Rinse the 50-mL beaker with 2 mL of Milli-Q water and pour into the same centrifuge tube.
 17. Adjust the centrifuge tubes to approximately equal weight with Milli-Q water using a pan balance. Centrifuge at 20,000 RPM (47,813 x g) for 10 minutes at approximately 4 °C.
 18. Transfer the supernatant into the 100-mL beaker of step I.14 to combine the solutions (See Note 6).
 19. Add 0.5 mL of 6 N hydrochloric acid. Using a pH meter, a magnetic stirrer and a stirring bar adjust the pH to approximately pH 1.75-1.85 using 6 N and 1 N hydrochloric acid.

J. Solid Phase Extraction Cleanup

1. Prepare an International Sorbent Technology (IST) ISOLUTE C-18 cartridge (500 mg/3-ml tube) using an IST, VacMaster Processing Station by washing the cartridge with two full column lengths (approximately 3 mL each time) of methanol followed by two full column lengths of 0.05 N hydrochloric acid. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.
2. Add 1/2 column length (approximately 2 mL) of 0.05 N hydrochloric acid to the C-18 cartridge then attach a 70-mL, non-fritted reservoir onto the top of the prepared IST C-18 cartridge using an adapter.
3. Pass the sample from step I.19 through the C-18 cartridge using the VacMaster Processing Station at the rate of approximately 1 drop per second and discard the eluate.
4. Rinse the 100-mL beaker, reservoir and C-18 cartridge with 5-mL of 0.05 N hydrochloric acid and pass the rinse through the cartridge at the rate of approximately 1 drop per second and discard the rinse. Do not allow the liquid level to drain below the top of the sorbent bed during rinse.

5. Remove the reservoir and adapter and wash the C-18 cartridge with two full column lengths of 0.005 N hydrochloric acid followed by one full column length of Milli-Q water at the rate of approximately 1 drop per second and discard the washes. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.
6. Assemble a clean 25-mL, non-fritted reservoir onto the top of the C-18 cartridge using an adapter and elute the C-18 with 25 mL of methanol, at the rate of approximately 2 drops per second, into a 100-mL pear-shaped flask set inside the IST VacMaster Processing Station. (See Note 4).
7. Using a rotary evaporator, evaporate the eluate to dryness in the 100-mL pear-shaped flask.
8. Dissolve the residue in 10 mL of 0.005 N hydrochloric acid. Stopper, swirl and vortex.
9. Prepare an IST ISOLUTE SCX cartridge (1000 mg/6-ml tube) using the VacMaster Processing Station by washing with two full column lengths of hexane, two full column lengths of methanol followed by two full column lengths of 0.005 N hydrochloric acid. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.
10. Add 1/2 column length (approximately 4 mL) of 0.005 N hydrochloric acid to the SCX cartridge then assemble a clean 25-mL, non-fritted reservoir onto the top of the SCX cartridge using an adapter.
11. Pass the sample from Step J. 8 through the SCX cartridge at the rate of approximately 1 drop per second using the VacMaster Processing Station. Rinse the flask, reservoir and cartridge with one 5-mL portion of 0.005 N hydrochloric acid. Do not allow the liquid level to drain below the top of the sorbent bed during sample load or rinse.
12. Remove the reservoir and adapter and wash the SCX cartridge with one full column length of methanol at the rate of 1 drop per second using the VacMaster Processing Station. Discard the wash. Do not allow the liquid level to drain below the top of the sorbent bed during the wash.
13. Assemble a 25-mL non-fritted reservoir onto the top of the SCX cartridge using an adapter and elute the SCX with 35 mL of saturated potassium chloride in methanol at the rate of 1 drop per second into a 100-mL pear-shaped flask set inside the VacMaster Processing Station. Discard the SCX cartridge.
14. Evaporate the saturated potassium chloride-methanol eluate to dryness using a rotary evaporator.
15. Dissolve the residue in 10 mL of 0.05 N hydrochloric acid. Stopper, swirl and vortex.
16. Prepare an IST ISOLUTE C-18 cartridge (500 mg/3 ml tube) using an IST VacMaster Processing Station by washing the cartridge with two full column lengths of methanol followed by two full column lengths of 0.05 N hydrochloric

- acid. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.
17. Add 1/2 column length (approximately 2 mL) of 0.05 N hydrochloric acid to the C-18 cartridge and then assemble a 25-mL non-fritted reservoir onto the top of the prepared C-18 cartridge using an adapter.
 18. Pass the sample from step J.15 through the C-18 cartridge using the VacMaster Processing Station at the rate of approximately 1 drop per second and discard the eluate. Do not allow the liquid level to drain below the top of the sorbent bed during sample loading.
 19. Rinse the beaker, reservoir and cartridge with one 5-mL portion of 0.05 N hydrochloric acid. Do not allow the liquid level to drain below the top of the sorbent bed during this rinse.
 20. Remove the reservoir and adapter and wash the C-18 cartridge with three full column lengths of Milli-Q water. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.
 21. Assemble a 20-mL non-fritted reservoir onto the top of the C-18 cartridge using an adapter and elute the C-18 cartridge with 25 mL of methanol at the rate of approximately 1-2 drops per second into a 100-mL pear-shaped flask set inside the VacMaster Processing Station. (See Note 4).
 22. Using a rotary evaporator, evaporate the eluate to dryness. Add 5 mL of acetonitrile to the flask and re-evaporate to complete dryness.
 23. Dissolve the residue in 2.0 mL of Milli-Q water in preparation for Capillary Electrophoresis Analysis.

K. CE Method Parameters

Set up the ABI/270A-HT Capillary Electrophoresis Unit with the following conditions:

Method: User-01 Report:

Cycle: 1-- Detect

Range: 0.010	Risetime: 0.50	Wavelength: 240 nm
Cycle: 2--Flush	Autozero: Yes	Change: Yes
Time: 2.0 mins	Vial: 51	Temperature: 29.0 °C
Voltage: 0 KV	Vacuum: 20.0 " Hg	Wavelength: 240 nm
Increment: No	By: 1	
Cycle: 3--Flush	Vial: 53	Temperature: 29.0 °C
Time: 5.0 mins	Vacuum: 20.0 " Hg	Wavelength: 240 nm
Voltage: 0 KV	By: 1	
Increment: No		
Cycle: 4--Sample		Temperature: 29.0 °C
Time: 4.0 secs	Vacuum: 5.0 " Hg	Wavelength: 240 nm
Voltage: 0 KV		
Cycle: 5--Time		

Time: 14.0 mins Vial: 52 Temperature: 29.0 ° C
Voltage: 15 KV Vacuum: 0.0 " Hg Wavelength: 240 nm
Cycle: 6--End
Empty cycle
Cycle: 7--End
Empty cycle
Cycle: 8--End
Empty cycle
Cycle: 9--End
Empty cycle

NOTE: Vial 51: Contains 100 mM sodium hydroxide solution.
Vial 52: Contains separation buffer.
Vial 53: Contains separation buffer.

L. Capillary Electrophoresis Analysis

1. After obtaining a stable CE response for the (0.50 mcg/mL Mixed Calibration Standard Solution) as shown in Section E.15, load a 4 second aliquot of sample into the CE.
2. Compare the peak heights with those obtained from a 4 second load of the 0.50 mcg/mL of working standard solution as prepared in Section D.3.c.
3. If the sample peaks go off scale, dilute a 1 mL aliquot to an appropriate volume with Milli-Q water.
4. Make a working standard injection after every one or two samples and use the average peak height of the standard injection before and after sample injections for the calculation.

M. Calculations

For each sample calculation, use the sample peak height and the average peak height measurement of the working standard obtained before and after the sample injections as follows:

$$\text{PPM} = \frac{\text{R(SAMP)} \times (\text{V1}) \times (\text{V3}) \times (\text{V5}) \times \text{C(STD)} \times (\text{DF})}{\text{R(STD)} \times (\text{W}) \times (\text{V2}) \times (\text{V4})}$$

Where:

R(SAMP) = Peak height of sample in millimeters

R(STD) = Average peak height of bracketing, working standard in millimeters

C(STD) = Concentration of working standard (0.50 mcg/mL)

V1 = Volume of extraction solvent in milliliters (300 mL)

- V2 = Aliquot of extract taken for analysis in milliliters (15 mL)
- V3 = Final volume of sample solution for CE analysis (2 mL)
- V4 = Volume of sample solution injected (73.8 nanoliters)
- V5 = Volume of standard solution injected (73.8 nanoliters)
- W = Sample weight (20 g)
- DF = Dilution Factor

Typical electropherograms for alfalfa and clover commodities are shown in Figures 1 through 8.

N. LC/MS Confirmatory Analysis

1. Apparatus for Sample and Standard Preparation:

a. Heating Block: MULTI-BLOK[®] Heater (Model 2050) Lab-Line Instruments, Inc., Melrose Park, IL

b. Micro Sample Vials: 3 mL (Cat. No. B7797-3), American Scientific Products, Inc.

2. Sample Preparation for LC/MS Confirmation: For samples from J. 23 showing total residue of greater than 3 ppm by CE and requiring mass spectrometric confirmation, transfer 1.0 mL from the 2 mL final volume to a micro sample vial (N.1.b) and add 150 uL of concentrated hydrochloric acid. Heat the vial for one hour at approximately 100 °C on a heating block (N.1.a). After cooling to room temperature, the hydrolyzed samples are immediately amenable to LC/MS analysis. (See Note 7).

3. LC/MS Standard Solution: With each set of samples analyzed, a 1.0-mL aliquot of the 0.5 mcg/mL mixed CE standard solution (D.3.c) is also hydrolyzed following the procedure described in N.2.

4. LC/MS Instrumentation:

a. Mass Spectrometer: Finnigan-MAT TSQ70

b. LC/MS Interface: Finnigan-MAT thermospray accessory

c. Liquid Chromatograph: ABI Kratos Spectroflow Model 400

d. LC Column: Whatman RAC II Partisil 5 ODS-3, 4.6 mm X 10 cm (Cat. No. 4222-225)

5. LC/MS Conditions: (a)

a. LC Column Temperature

Ambient

b. LC Flow Rate

1.5 mL/min. (approx. 160 bar)

c. <u>Mobile Phase</u>	70 H ₂ O / 30 CH ₃ OH (both 0.5% in CF ₃ COOH)
d. <u>Injection Volume</u>	20 μ L
e. <u>LC/MS Interface Backpressure</u>	60 bar (at 80 °C)
f. <u>Vaporizer Temperature (Set)</u>	80 °C
g. <u>Aerosol Temperature (Set)</u>	250 °C
h. <u>Repeller Voltage</u>	20 Volts
i. <u>MS High Vacuum (No LC/MS)</u>	3E-07 t
j. <u>MS High Vacuum (LC/MS)</u>	2E-05 t
k. <u>Conversion Dynode</u>	-20 kV
l. <u>Electron Multiplier</u>	-1300 Volts
m. <u>Preamplifier Range (Full Scan)</u>	1E-08 amps/volt
n. <u>Preamplifier Range (SIM)</u>	1E-09 amps/volt
o. <u>Ions Monitored</u>	m/z 290 ⁺ for CL 263,499 , m/z 306 ⁺ for CL 288,511
p. <u>Retention Time of Analytes</u>	CL 288,511 3.0 min. CL 263,499 6.0 min.

^a The conditions above are specific for the instruments on which they were determined. Conditions will vary from instrument to instrument and should be adjusted to give sensitivity and adequate resolution of well defined peaks at approximately the retention times listed in N.5.p. Prior to analysis, the mass spectrometer should be tuned to give proper resolution and peak shape on an appropriate reference material and the data system should be calibrated.

6. LC/MS Confirmatory Analysis:

- Using parameters detailed in N.5, a 100 ng on-column injection of the analytes (100 μ L of D.3.b) is used to determine the mass centroids of the ions at m/z 290⁺ and 306⁺. Set the mass spectrometer for selected ion monitoring of these ions with a +/- 0.2 dalton scan window and a dwell time of 1 second/ion.
- Inject 20 μ L aliquots of the working standard (N.3) until a reasonably constant response is obtained (Figure 9).
- Follow the injection sequence: working standard, Sample Number 1, Sample Number 2, working standard, Sample Number 3, Sample Number 4, working standard,...
- If the response of the working standard decreases to an unacceptable level during the analysis, instrumental parameters should be adjusted to restore adequate sensitivity. If such adjustments are made, inject duplicate aliquots of the working standard to determine the new response values of the standard.

7. Data Treatment: The sample is confirmed as containing > 3 ppm total CL 263,499 related residues when:

- The retention times of the presumed analytes in the sample are within 5 scans (10 seconds) of the averaged retention times of the analyte peaks in the bracketing standards.

- b. **The summed response for the analyte peaks in the sample exceeds the average response of the summed analyte peaks in the bracketing standards.**

Notes to Method M 2261

1. If impurity peaks are a problem, standards and samples can be injected at an alternate wavelength of 272 nm.
2. If impurity peaks are a problem, standards and samples can be injected with Separation Buffer, (B.5.p) adjusted to pH=8.0 using 6 N hydrochloric acid.
3. If impurity peaks are a problem, standards and samples can be injected using the following Alternate Separation Buffers:
 - a. Separation Buffer (pH=9.2). Current approximately 70 μ A: 96 mM Cholic Acid/8 mM Sodium Tetraborate Decahydrate: Add 8.27 g of Cholic Acid and 0.61 g of Sodium Tetraborate Decahydrate to 200 mL of Milli-Q water, stir for approximately 5 minutes, filter through 0.22 μ m Corning Nylon Filter System. Store in a refrigerator. Conditions: 4 second load, 39 ° C, polarity (+), 15 KV, vacuum injection, wavelength 240 nm, attenuation 16, chart speed 0.5 cm/min.
 - b. Separation Buffer (pH=7.6). Current approximately 29 μ A: 25 mM DTAB/25 mM Tris/100 mM Tricine/34% Isopropyl Alcohol: Add 1.54 g of DTAB and 0.60 g of Tris and 1.79 g of Tricine to 200 mL of Milli-Q water, stir for approximately 5 minutes, filter through 0.22 μ m Corning Nylon Filter System. Store in a refrigerator. Add 34% Isopropyl Alcohol to Separation Buffer only in inlet buffer reservoir. Conditions: 4 second load, 45 ° C, polarity (-), -30 KV, vacuum injection, wavelength 240 nm, attenuation 16, chart speed 1 cm/min.
 - c. Separation Buffer (pH=8.9). Current approximately 58 μ A: 15 mM Sodium Tetraborate Decahydrate/10 mM Tetrabutylammonium Dihydrogen Phosphate/60 mM 1-Hexanesulfonic Acid: Add 1.14 g of Sodium Tetraborate Decahydrate and 0.68 g of Tetrabutylammonium Dihydrogen Phosphate and 2.26 g of 1-Hexanesulfonic Acid to 200 mL of Milli-Q water, stir for approximately 5 minutes, filter through 0.22 μ m Corning Nylon Filter System. Store in a refrigerator. Add 23% Acetonitrile to Separation Buffer only in inlet buffer reservoir. Conditions: 4 second load, 45 ° C, polarity (+); 15 KV, vacuum injection, wavelength 240 nm, attenuation 16, chart speed 0.5 cm/min.
4. Stopping Points in the method: The following are the steps in the method in which the sample workup may be halted and the samples stored in a refrigerator:
 - a. Step I.9: After transferring the acetonitrile solution into the 500-mL pear-shaped flask, stopper the flask and store in refrigerator.
 - b. Step J.6: After eluting the C-18 cartridge with methanol into the 100-mL pear-shaped flask, stopper the flask and store in refrigerator.
 - c. Step J.21: After eluting the C-18 cartridge with methanol in the 100-mL pear shaped flask, stopper the flask and store in refrigerator.

5. The MAE-CL 182,704 under these conditions is converted to CL 182,704. (Reference PD-M Volume 28-1/23,29-30,47, 1991 American Cyanamid report).
6. Disposal of lead acetate after use: Allow the lead acetate to dry in the bottom of the centrifuge tubes (approx. 1-2 hours). Using a spatula, scrape the dried lead acetate into a sealable container. The lead acetate should be disposed of properly, as a hazardous chemical waste.
7. CL 182,704 is converted to CL 288,511 and detected as total CL 288,511.

APPROVALS:**Author:**

M. Safarpour 7/22/93

M. Safarpour

Date

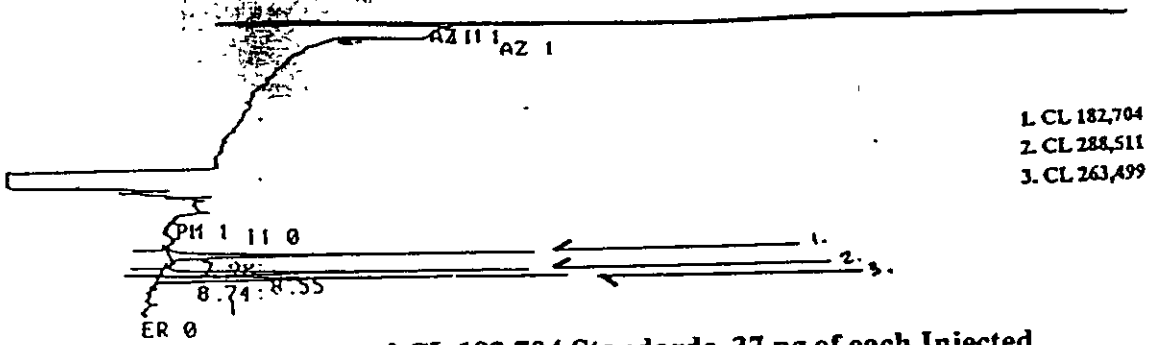
Group Leader:

G. L. Picard 7/22/93

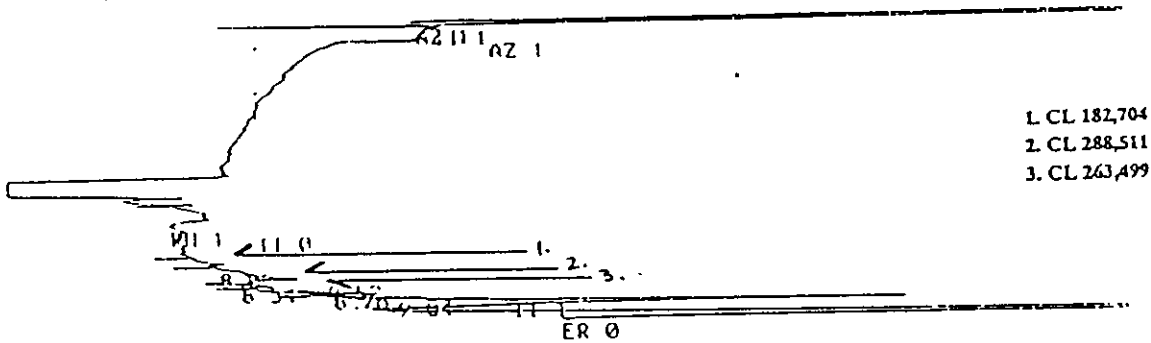
G. L. Picard

Date

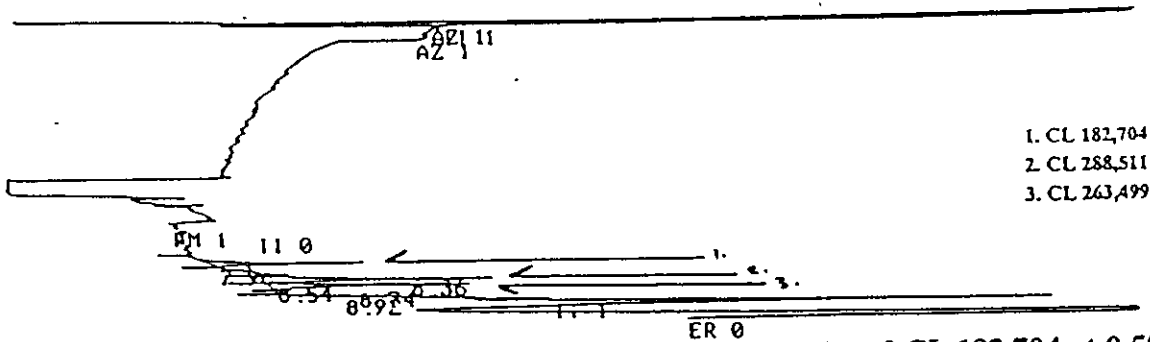
Figure 1: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Alfalfa Seed



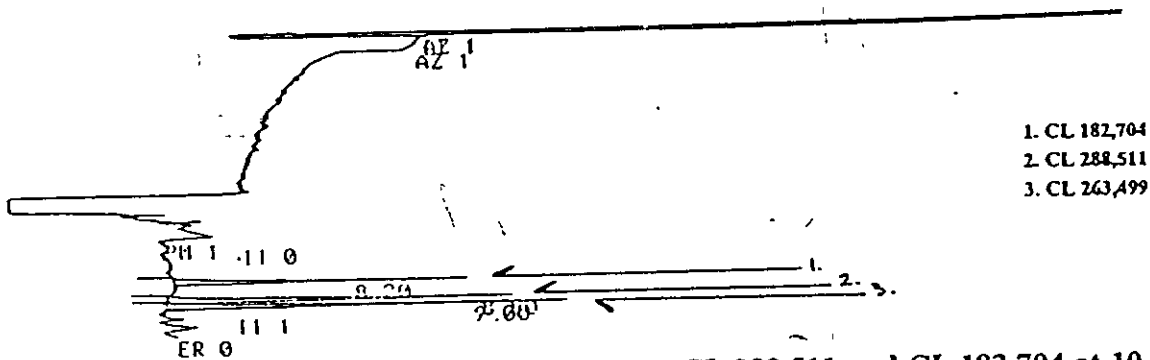
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Alfalfa Seed, 37 mcg Injected

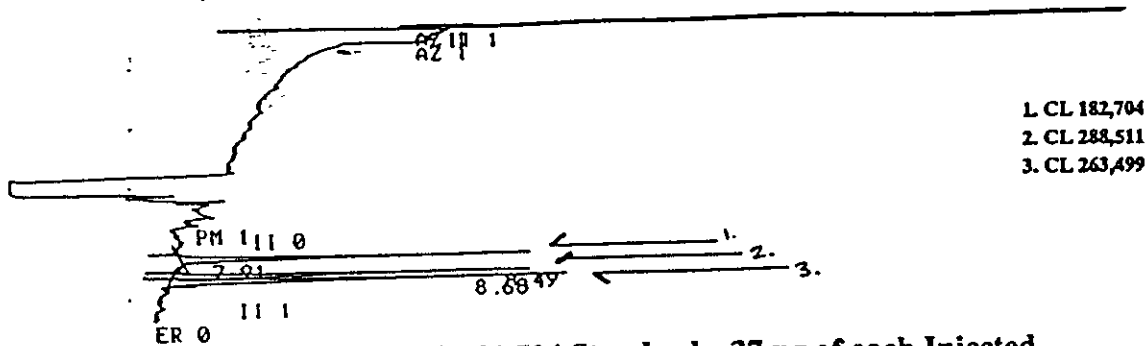


Control Alfalfa Seed, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 37 mcg Injected, 96% CL 263,499, 125% CL 288,511 and 89% CL 182,704 Recovered

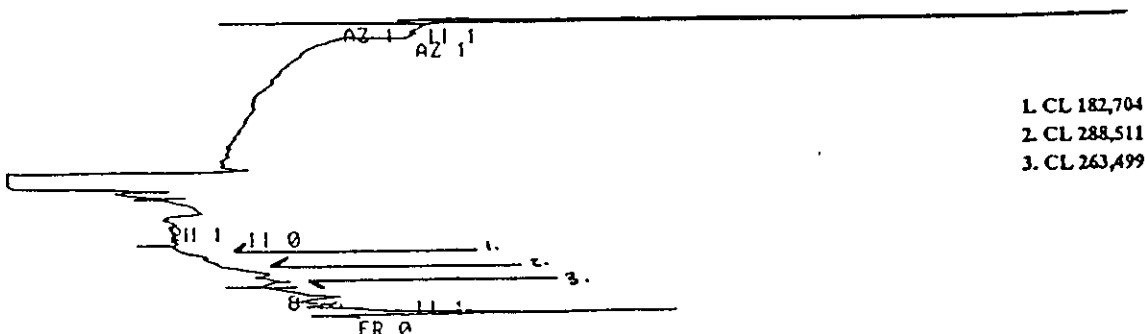


Control Alfalfa Seed, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 97% CL 263,499, 94% CL 288,511 and 82% CL 182,704 Recovered

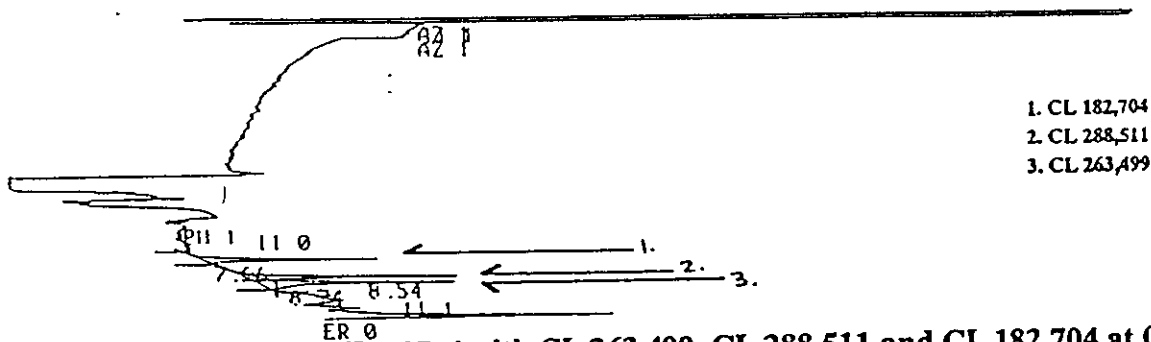
Figure 2: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Alfalfa Forage



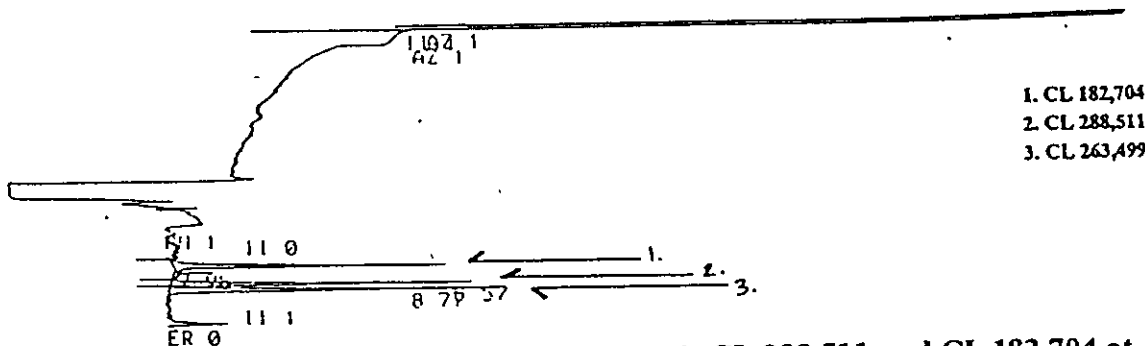
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Alfalfa Forage, 36.9 mcg Injected

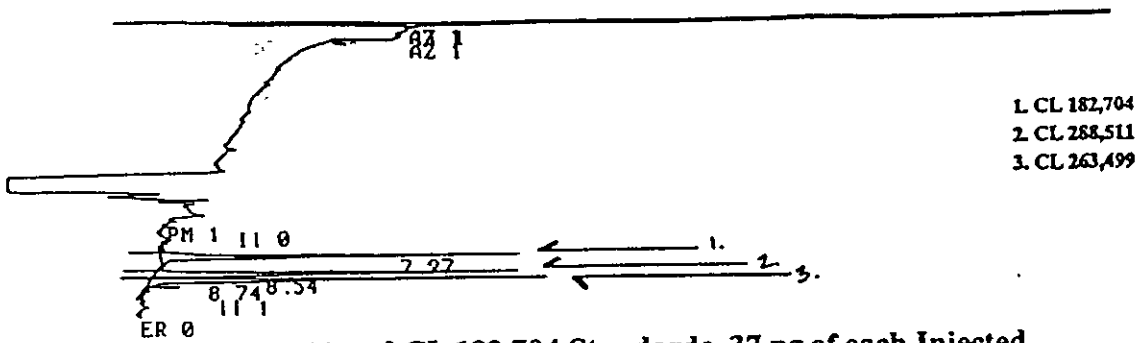


Control Alfalfa Forage, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50ppm, 37 mcg Injected, 90% CL 263,499, 110% CL 288,511 and 102% CL 182,704 Recovered

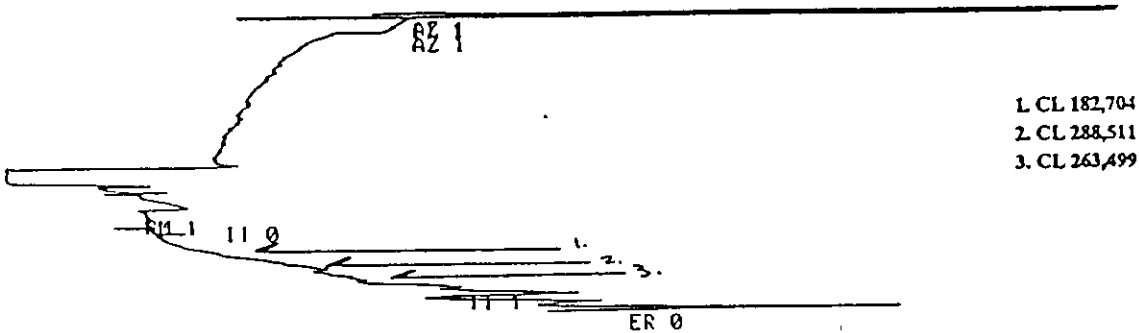


Control Alfalfa Forage, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 67% CL 263,499, 85% CL 288,511 and 79% CL 182,704 Recovered

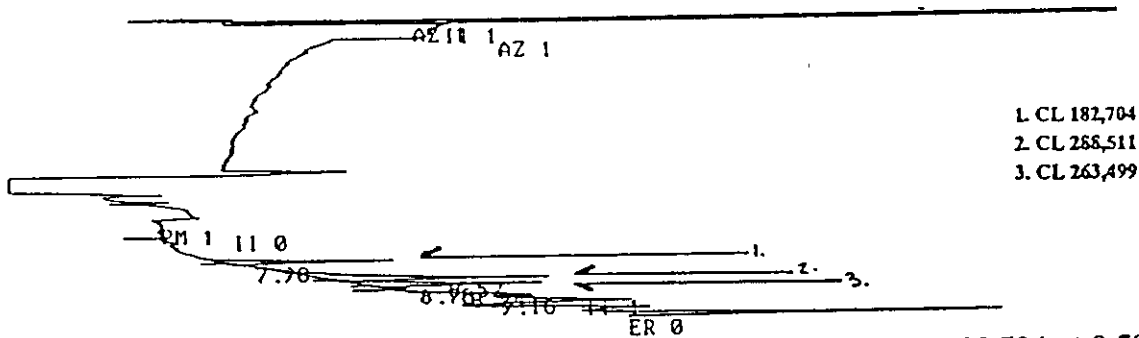
Figure 3: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Alfalfa Hay



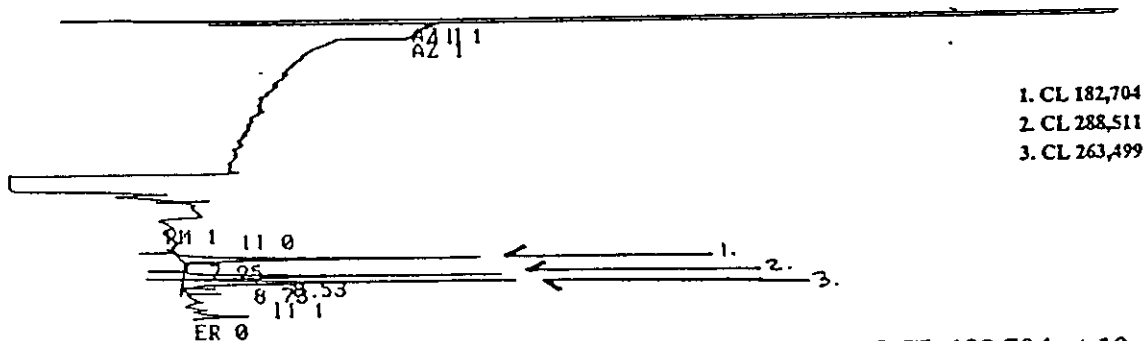
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Alfalfa Hay, 37 mcg Injected

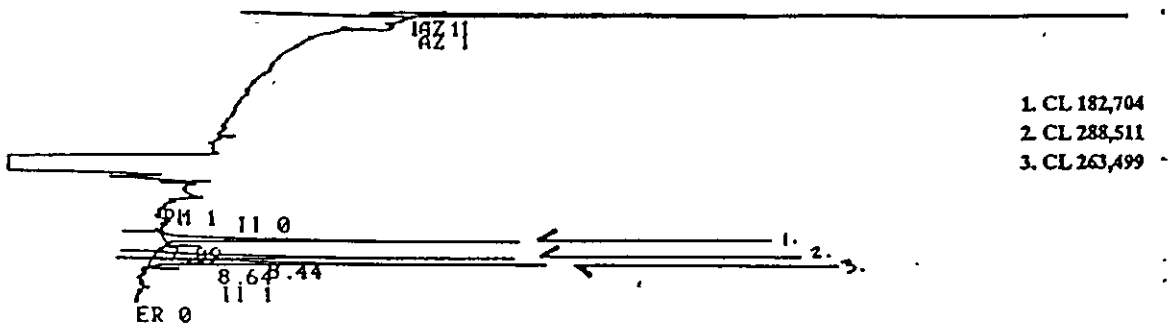


Control Alfalfa Hay, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 37 mcg Injected, 88% CL 263,499, 120% CL 288,511 and 93% CL 182,704 Recovered

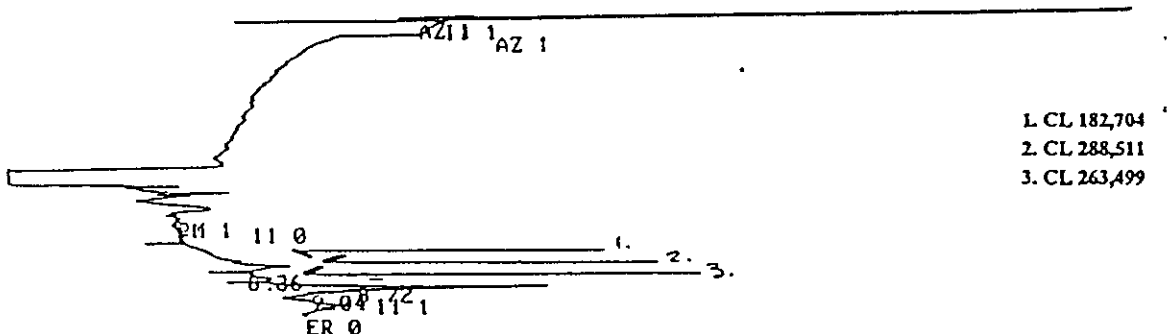


Control Alfalfa Hay, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 84% CL 263,499, 87% CL 288,511 and 83% CL 182,704 Recovered

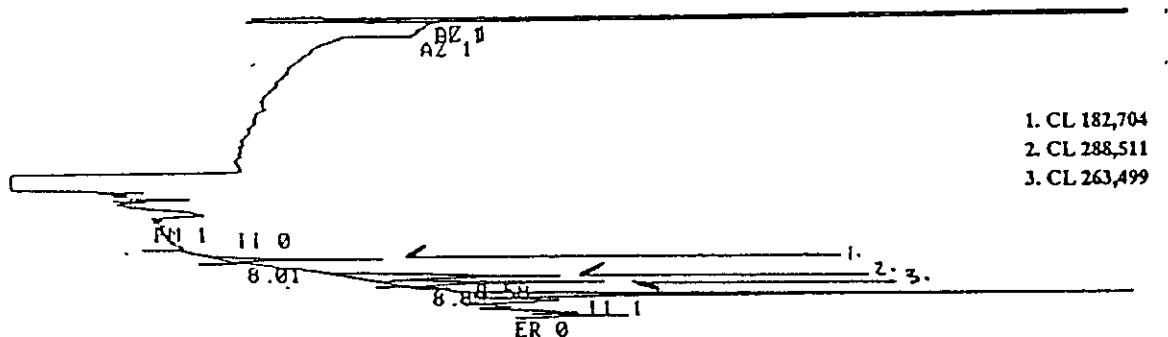
Figure 4: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Alfalfa Process Meal



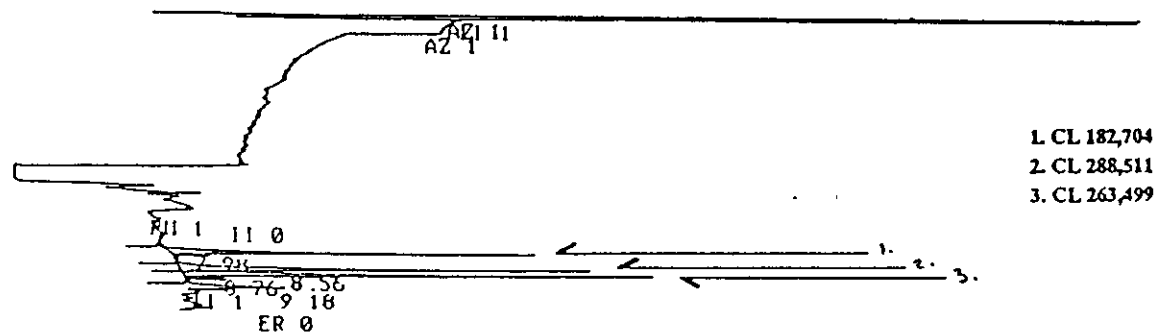
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Alfalfa Process Meal, 37 mcg Injected

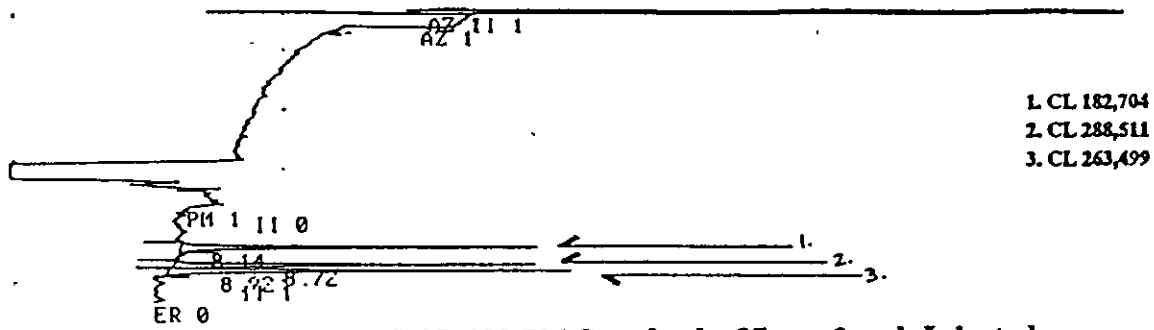


Control Alfalfa Process Meal, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 37 mcg Injected, 91% CL 263,499, 101% CL 288,511 and 84% CL 182,704 Recovered

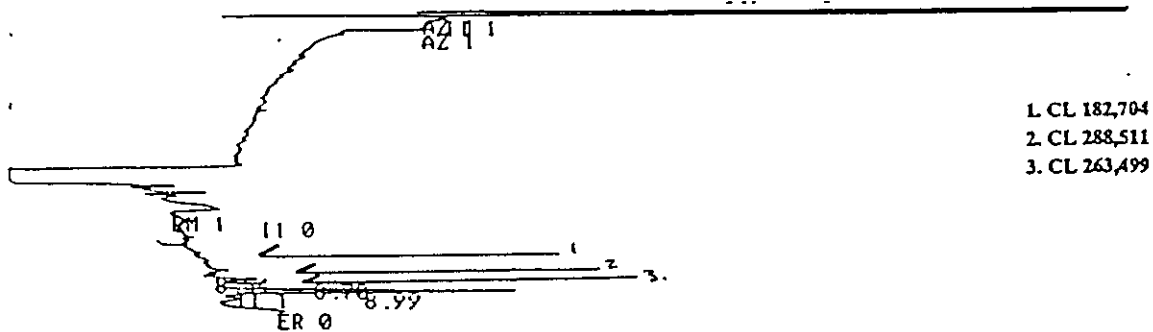


Control Alfalfa Process Meal, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 103% CL 263,499, 103% CL 288,511 and 94% CL 182,704 Recovered

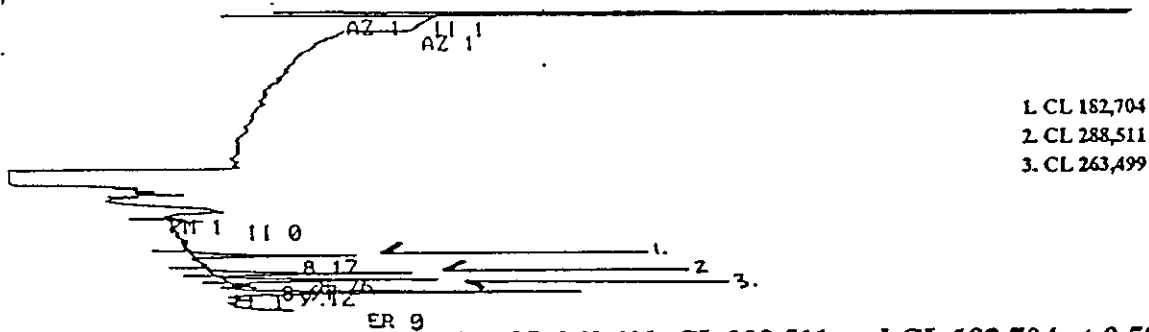
Figure 5: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Clover Seed



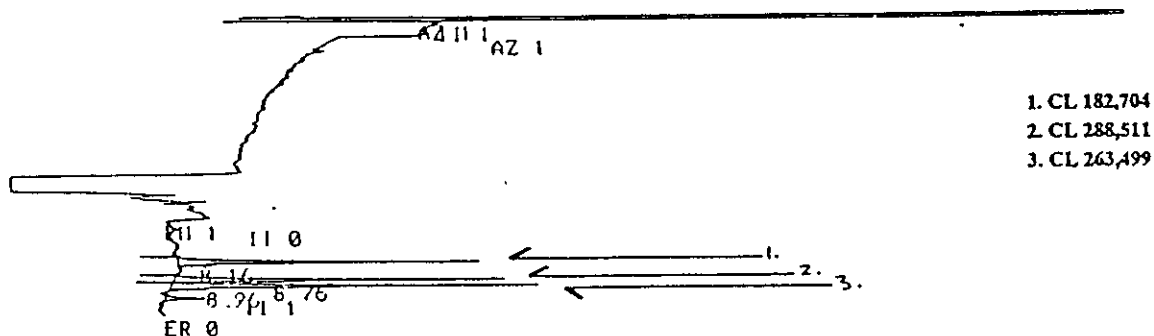
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Clover Seed, 37 mcg Injected

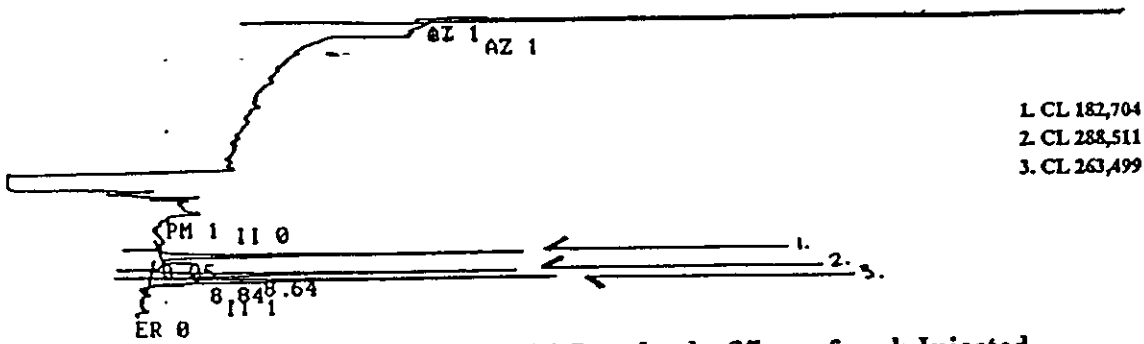


Control Clover Seed, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 37 mcg Injected, 100% CL 263,499, 106% CL 288,511 and 89% CL 182,704 Recovered

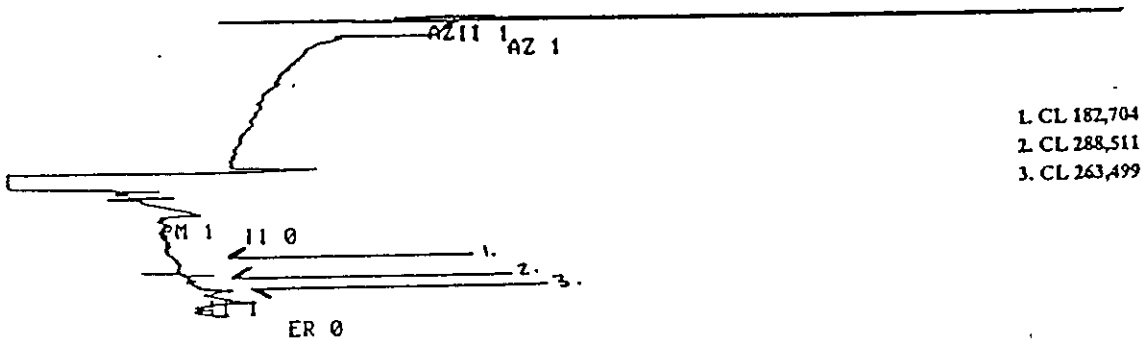


Control Clover Seed, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 90% CL 263,499, 91% CL 288,511 and 83% CL 182,704 Recovered

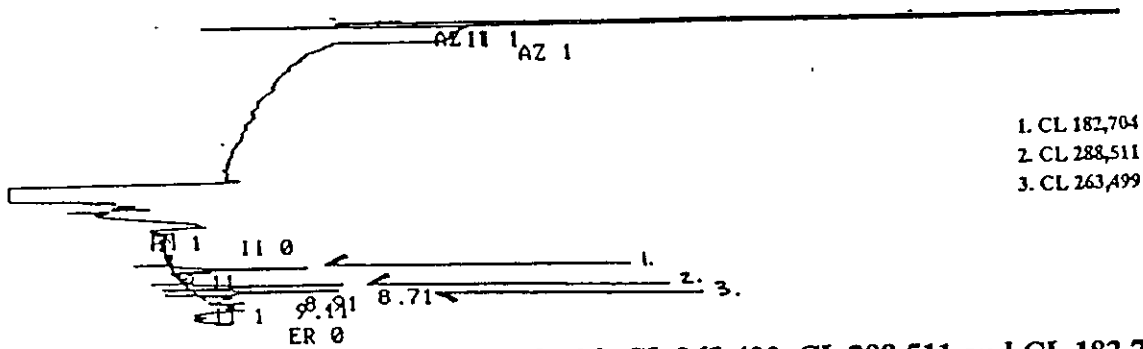
Figure 6: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Clover Green Forage



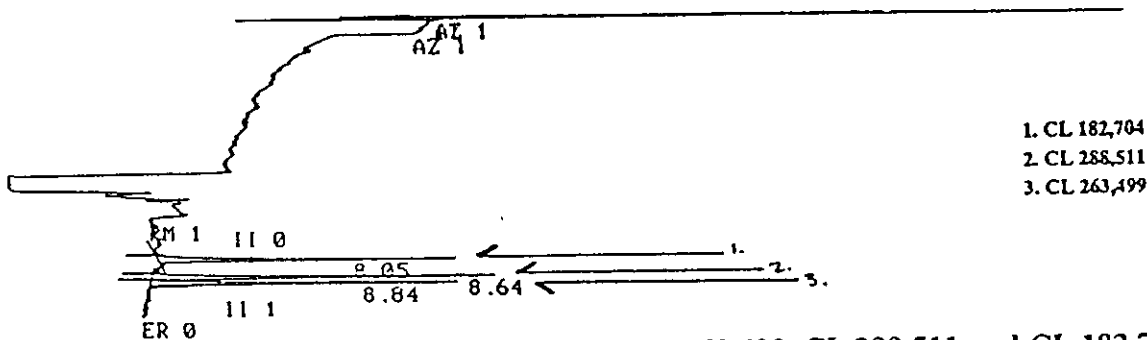
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Clover Green Forage, 37 mcg Injected

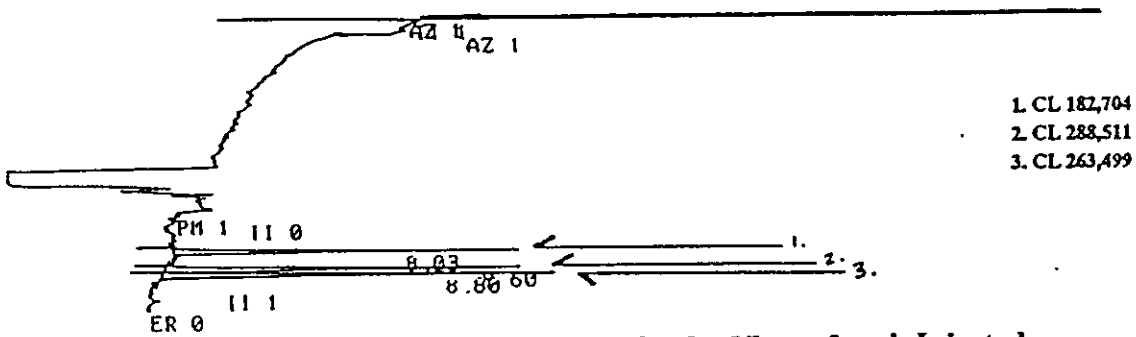


Control Clover Green Forage, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 36.9 mcg Injected, 74% CL 263,499, 85% CL 288,511 and 75% CL 182,704 Recovered

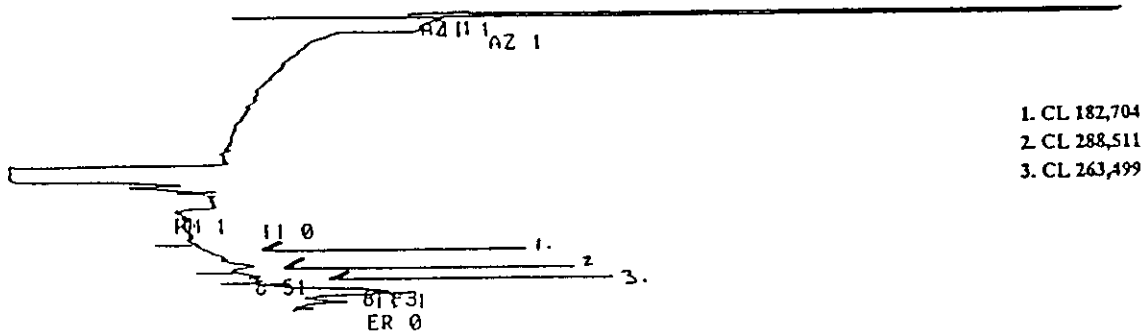


Control Clover Green Forage, Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 75% CL 263,499, 93% CL 288,511 and 81% CL 182,704 Recovered

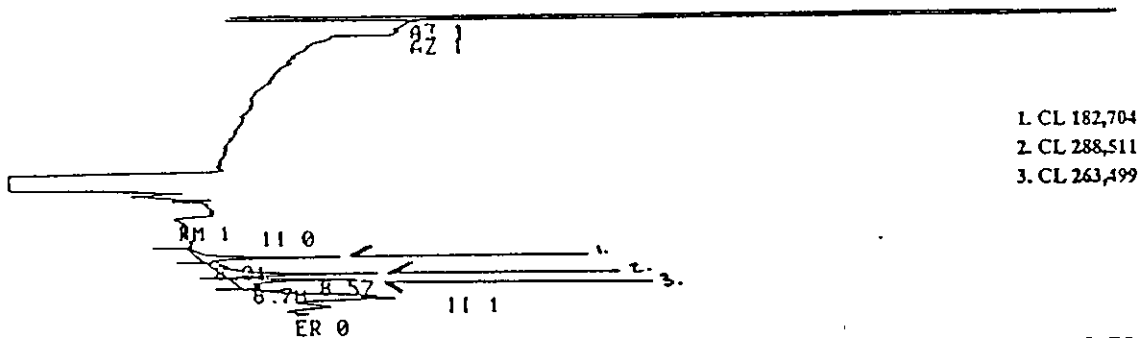
Figure 7: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Clover Hay



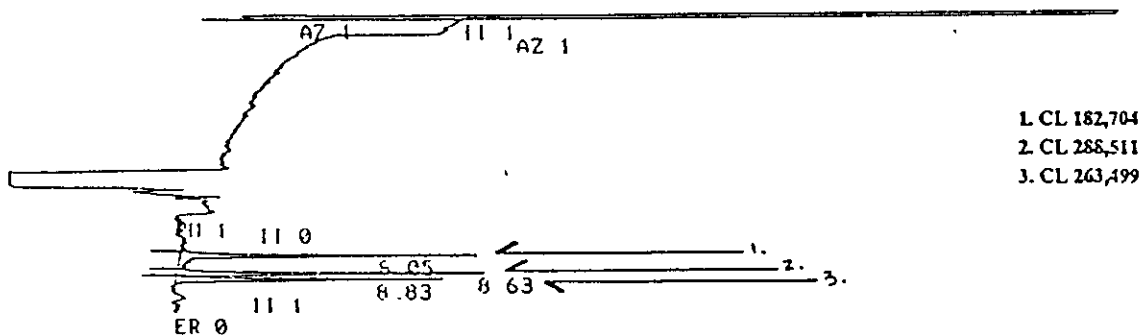
CL 263,499, CL 288,511 and CL 182,704 Standards, 37 pg of each Injected



Control Clover Hay, 37 mcg Injected



Control Clover Hay Fortified with CL 263,499, CL 288,511 and CL 182,704 at 0.50 ppm, 37 mcg Injected, 64% CL 263,499, 90% CL 288,511 and 81% CL 182,704 Recovered



Control Clover Hay Fortified with CL 263,499, CL 288,511 and CL 182,704 at 10 ppm, 3.7 mcg Injected, 68% CL 263,499, 86% CL 288,511 and 83% CL 182,704 Recovered

Figure 8: Typical Electropherograms for the Analysis of CL 263,499, CL 288,511 and CL 182,704 Residues in Clover Seed Screenings

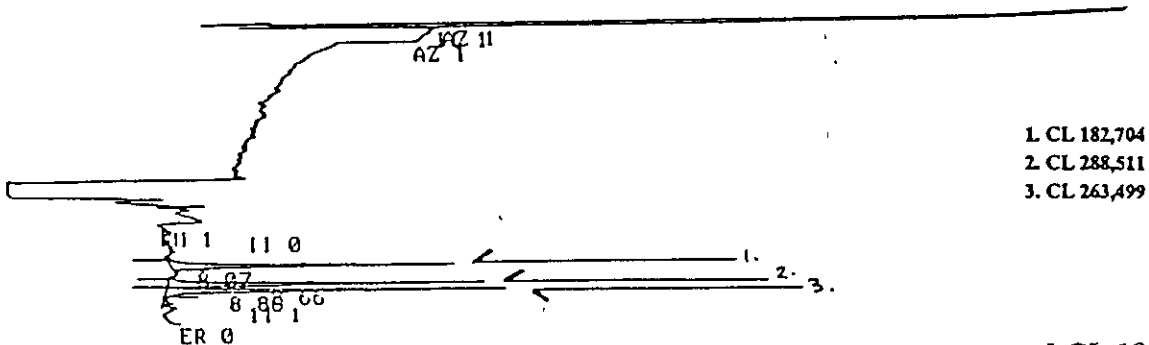
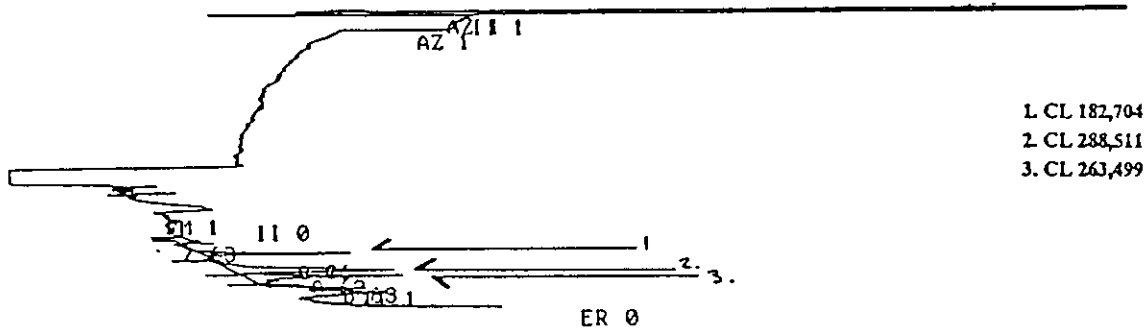
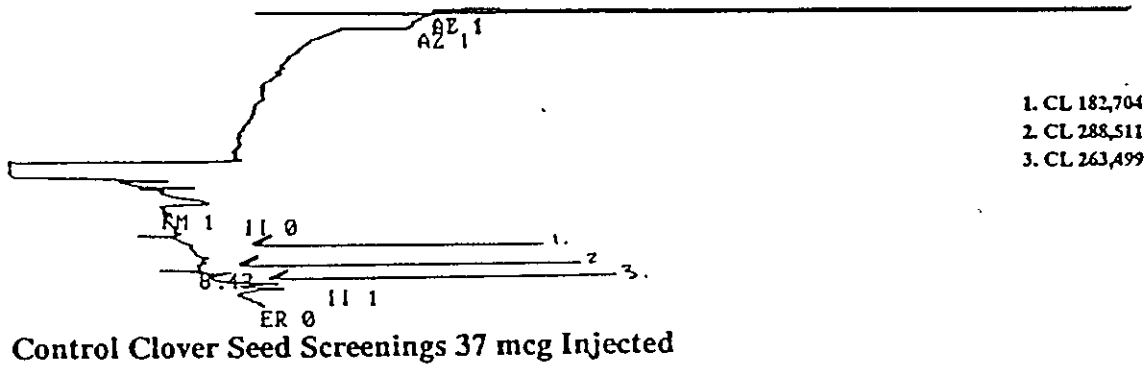
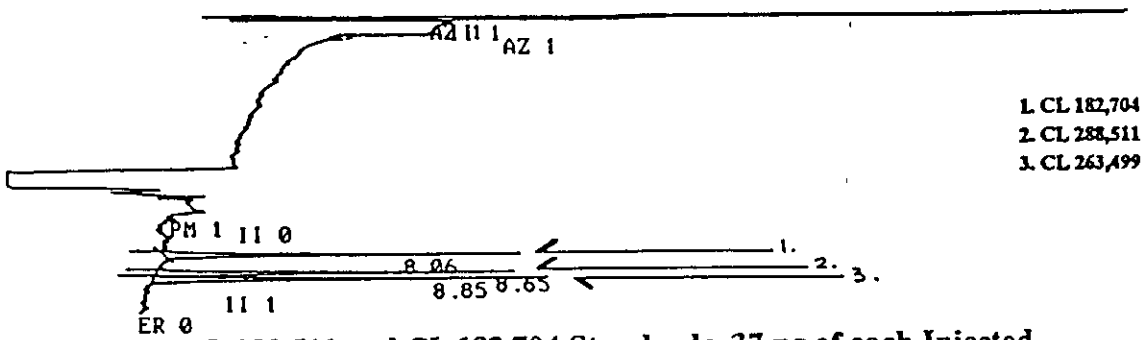


Figure 9: Typical Mass Chromatograms from the Injection of LC/MS Standard Solution.

CHRO: 93t105d
 Samp: C060993-3 HYDROLYZED
 Comm: VAP=80 AERO=250
 Mode: TSP +Q3MS LMR UP LR
 Oper: DACUNHA/STOUT Client: M. SAFARPOUR
 Peak: 1000.00 mmu Label wndw: 1 > 174
 Area: 0, 0 50 Baseline 0, 3

22-JUN-93 Elapse: 00:02:04.5 1
 Start : 11:44:36 174
 Study : PU93PT01
 Inlet :
 Masses: 290 > 306
 Label : 3, 30.00

