CYANAMID

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

Recommended Method of Analysis - M 2379

CADRE Herbicide (CL 263,222): Capillary Electrophoresis (CE) Determinative and LC/MS Confirmatory Method for CL 263,222, CL 263,284, and CL 189,215 Residues in Peanut Hull and Peanut Nutmeat.

A. Principle

Residues of CL 263,222, CL 263,284, and CL 189,215 are extracted from the sample with acidic water-methanol. The CL 263,222, CL 263,284 and CL 189,215 are subjected to suitable cleanup involving precipitation, centrifugation and solid phase extraction. Measurement of CL 263,222, CL 263,284 and CL 189,215 residues is accomplished by capillary electrophoresis (CE) equipped with a high sensitivity flow cell and a UV detector set at 240 nm. Results are calculated as CL 263,222, CL 263,284 and CL 189,215 by the direct comparison of the peak heights in the sample to those of external standards. The validated sensitivity of the method is 0.10 ppm for each compound in each commodity.

Confirmation for total residues at the 0.3 ppm level is provided by thermospray LC/MS of the final extract.

B. Reagents

(Items from manufacturers other than those listed may be used if proved to be functionally equivalent.)

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

   a. CL 263,222 [Nicotinic acid, 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methyl-], analytical grade, known purity, American Cyanamid Company, Agricultural Research Division, Princeton, NJ. 08543-0400.

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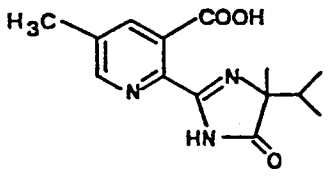
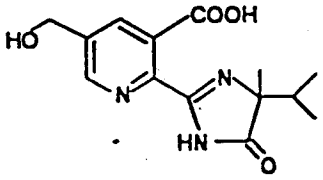


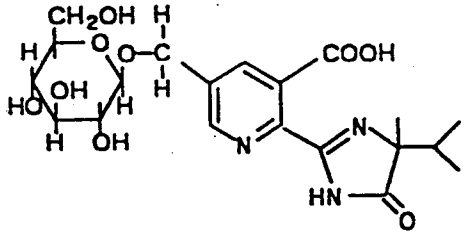
M.W. = 275

b. CL 263,284 [Nicotinic acid, 5-(hydroxymethyl)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-], analytical grade, known purity, American Cyanamid Company, Agricultural Research Division, Princeton, NJ. 08543-0400.



M.W.= 291

c. CL 189,215: [Nicotinic acid, 5-[(beta-D-glucopyranosyloxy) methyl]-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-]



M.W. = 454

2. Solvents: B & J Brand High Purity Solvents (UV grade), Baxter Burdick and Jackson.

a. Methanol
b. Tetrahydrofuran (THF)
c. Acetonitrile
d. Acetone

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3. Milli-Q Water: Water passed through Millipore's Milli-Q Plus Ultra Pure Water System. Use this water for all steps requiring water.

4. Chemicals:

   a. Fluka Chemika, Ronkonkoma, NY

      Formic acid 98% (Cat. No. 06440)

   b. "Baker Analyzed" Reagents, J. T. Baker Company.

      i. Hydrochloric Acid, Concentrated (Cat. No. 9535-01)

      ii. Potassium Chloride (Cat. No. 3040-01)

      iii. Sodium Hydroxide, Pellets, Purity 98.8% (Cat. No. 3722-01)

      iv. 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, St. Louis, MO.

      i. Sodium Phosphate, Monobasic, 98% Purity (Cat. No. S-3139)

      ii. DTAB (Dodecyltrimethylammonium bromide), 99% Purity (Cat. No. D-8638)

      iii. TRIS [tris-(hydroxymethyl)-aminomethane], 99% Purity (Cat. No. T-1378)

      iv. Sodium Phosphate, Dibasic, 99% purity (Cat. No. S-3397)

   f. Aldrich Chemical Co.

      Sodium Tetraborate Decahydrate, Electrophoresis grade 96% purity (Cat. No. 25,136-4)

   g. Bethesda Research Laboratories (BRL)

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

   h. International Products Co., Burlington, NJ.

      Micro: Laboratory Cleaner Solution (Cat. No. 6732)

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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. pH 2.5 high purity water: Adjust pH of high purity water to 2.5 with 6 N HCl using pH meter.

d. 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.

e. 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.

f. 1 M Sodium Hydroxide: Dissolve 40 g of sodium hydroxide pellets (98.8%) in approximately 600 mL of Milli-Q water and dilute to 1 liter with Milli-Q water. Filter through 0.22 um Corning Nylon Filter System (Cat. No. 25932-200). Store at room temperature.

g. 10% Lead Acetate: Dissolve 10 g of lead acetate in 100 mL of Milli-Q water. Filter through 0.22 um Corning Nylon Filter System. Store at room temperature.

h. 0.1 M Sodium Hydroxide: Dilute 10 mL of 1M sodium hydroxide to 100 mL with Milli-Q water.

i. 1% Formic acid in water: Pipette accurately 5 mL of concentrated formic acid into a 500 mL volumetric flask containing approximately 300 mL Milli-Q water. Dilute to 500 mL with Milli-Q water.

j. 0.4% Formic acid in water: Pipette accurately 2 mL of concentrated formic acid into a 500 mL volumetric flask containing approximately 300 mL Milli-Q water using a glass pipette. Dilute to 500 mL with Milli-Q water.

k. **Separation Buffer (prepare biweekly, store at room temperature) 25 mM DTAB/10 mM Tris/10 mM NaH2PO4 (pH 7.5) Current approximately 20 uA (pH of buffer solution should be approximately 7.5 without any adjustment):** Add 1.54 g of DTAB and 0.24 g of Tris and 0.24 g of sodium phosphate, monobasic to 200 mL of Milli-Q water, stir to dissolve, filter through 0.22 um Corning Nylon Filter System. Add 5% THF (215 microliters in Model 270A-HT) to separation buffer only in inlet buffer reservoir. Mix well before use. Periodically change this solution in the inlet buffer reservoir as the resolution or reproducibility become unsatisfactory. Conditions: 4 seconds load, 30 °C, polarity (-), -13 KV voltage, vacuum injection, wavelength 240 nm, attenuation 8, chart speed 1 cm/minute (See Note 2).

C. Apparatus

(Items from other manufacturers may be used if they are proven to be functionally equivalent) (See Note 1).

1. Adapter: Bond Elute 12, 20 mL adapters (Cat. No. Al-121310-03) and 1, 3, 6 mL adapters (Cat. No. Al-121310-01), Varian, Sunnyvale, CA.

2. Amber Glass Vial: 1 mL S/P Serum Vial (Cat. No. B7746-1) Baxter Healthcare Co. Edison, NJ.

3. Assorted Glassware: General laboratory.

4. Balance: Top-Loading, readability 0.10 g, weighing range 0-3100 g, Mettler Model PE 3000, Denver, CO.

5. Balance: Analytical, Sartorius Model R200D, readability 0.01/0.1 mg, weighing range 0-42 and 0-205 g, Scientific Products, Edison, NJ.

6. Capillary Electrophoresis: Applied Biosystems Model 270A-HT or Model 270A equipped with a high sensitivity optical flow cell (Cat. No. 401536), and UV detector, Foster City, California.

7. Capillary Flash Kits: Cat. No. 68-3147-022, ISCO, Inc., Lincoln, NE.

8. Cartridges:

   a. AG®1-X8 ($HCOO^-$): 200-400 mesh, bed size 0.8 x 4 cm, Bio-RAD Laboratories, Richmond, CA. Cat. No. 731-6221.

   b. C18: ISOLUTE, 500 mg/3-mL tube, unendcapped, International Sorbent Technology (IST), Cat. No. 220-0050-B, distributed by Jones Chromatography, Lakewood, CO.

   c. 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.

9. Centrifuge: Sorval Model RC-5C Centrifuge, Rotor Model SS34, Dupont, Wilmington, DE.

10. Centrifuge Tube: 50-mL Oak Ridge Centrifuge Tube, Nalgene, Cat. No. 3119-0050, Nalgene Co., Rochester, NY.

11. Filter System: Corning 150-mL Filter System, 0.22 um Nylon, Cat. No. 25934-200, Corning Incorporated, Corning, NY.

12. Filter Support Collars: Cat. No. K389730, J. & H. Berge, Inc., South Plainfield, NJ.

13. Flasks: 24/40 ȝ 50-, 100- and 300-mL pear shaped flasks, Kontes, Scientific Glassware/Instruments, Vineland, NJ.

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14. Flasks: Volumetric Flasks; 2, 5, 25, 50, 100, 200, 500, 1000 mL, Kimble Science Products, Vineland, NJ.

15. Filtering Flasks: 500-mL capacity, Corning Glass Works.

16. Filtering Funnels: Buchner, porcelain, 9-cm diameter.

17. Filter Paper: 9-cm diameter, glass-fiber filter, 934-AH, Whatman, Incorporated.

18. Frits: 15 mL, 20 um, Varian, Cat. No. 1213-1022, distributed by Jones Chromatography, Lakewood, CO.

19. Integrator: Chromjet Recording Integrator, Spectra-Physics, Fremont, CA.

20. Luer Stopcocks: Cat. No. 1213-1005, Varian, Harbor City, CA.

21. Omni Mixer Homogenizer: Model 17105, Cat. No. 17105; Stainless Steel Chamber Assembly (with 2" blade), Cat. No. 17079; Glass Chamber, 473 mL (1-quart Mason jar), Cat. No. 60245, OCI Instruments, Omni International, Gainsville, VA.

22. Pasteur Pipets: S/P Brand Dispo. Pipets 5 3/4 " and 9" Cat. No. P5201-1 and P5201-2, Baxter Diagnostics Inc., McGaw Park, IL.

23. pH Meter: Orion Model SA520, Orion Research, Boston, MA.

24. Pipettes: Volumetric Pipette 1, 2, 4, 5, 8,10, 20, 25 mL, Kimble Science Products, Vineland NJ.

25. Pipettes Tips: Pipet Tips 5 mL and 10 mL, (Cat. No. 53508-991, and 53511-678 respectively). VWR Scientific, West Chester, PA.

26. PTFE ISOLUTE Column Adaptors: IST, Cat. No. 120-1100, Jones Chromatography, Lakewood, CO.

27. Reservoirs: empty, IST, 70-mL nominal capacity, Cat. No. 120-1008-F, 25-mL capacity, Cat. No. 120-1007-E, and 15 mL capacity, Cat. No. 1213-1016 , Jones Chromatography, Lakewood, CO.

28. Rotary Evaporator: Buchler Instruments Model RE-121C equipped with a cold finger dry ice trap and a heated water bath set to approximately 32°-35°C, Westbury, NY.

29. Rubber Filter Adapter: Filter Adapter Set, (Cat. No. F7350-10), Baxter Diagnostics Inc., Edison, NJ.

30. Syringes: Luer Slip Tip, 30 mL, and 60 mL (Cat. No. S9519-30S, and S9519-60S), Scientific Products, Edison, NJ.

31. Ultrasonic Cleaner: Branson Model 3200, Branson Ultrasonics Corporation, Danbury, CT.

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33. VacMaster-20 Vacuum Manifold Sample Processing Station: IST Cat. No. 121-2016, Jones Chromatography, Lakewood, CO.

34. Vacuum Filtration Device: A 500-mL suction flask fitted with 9-cm diameter Buchner porcelain funnel by means of a rubber adapter.

35. Vortex Mixer: S/P Vortex Mixer, Cat. No. S8223-1, American Scientific Products, Edison, NJ.

36. Fritted Reservoir: Varian, 15 mL Reservoir with frit, Cat. No. 1213-1016 Varian, Harbor City, CA.

37. Syringe: Luer Slip Tip, 30 mL Cat. No. S9519-30S, Scientific Products, Edison, NJ.

D. Preparation of Standard Solutions (Prepare bimonthly, store in amber bottles with polyseal caps in refrigerator).

1. Stock Solutions

a. CL 263,222: Weigh accurately a known amount (approximately 15 mg) of CL 263,222 into an aluminum boat and place into a 50-mL volumetric flask. Dilute to the mark with methanol and mix well. Calculate and record the exact concentration of CL 263,222, correcting for the standard purity.

b. CL 263,284: Weigh accurately a known amount (approximately 15 mg) of CL 263,284 into an aluminum boat and place into a 50-mL volumetric flask. Dilute to the mark with methanol and mix well. Calculate and record the exact concentration of CL 263,284, correcting for the standard purity.

c. CL 189,215: Weigh accurately a known amount (approximately 15 mg) of CL 189,215 into an aluminum boat and place into a 50-mL volumetric flask. Dilute to the mark with methanol and mix well. Calculate and record the exact concentration of CL 189,215, correcting for the standard purity.

The concentration of CL 189,215 is expressed as CL 263,284 equivalents by converting it using the ratio of molecular weights (0.64) described by the following equation:

$$\text{mcg/mL CL 189,215} \times \frac{\text{M.W. CL 263,284 (291)}}{\text{M.W. CL 189,215 (454)}} = \text{mcg/mL CL 263,284 equivalents}$$

2. Mixed Standard Fortification Solutions (50, 10, 4, 2 mcg/mL)

a. Mixed Standard Fortification Solutions (50 mcg/mL)

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 263,222, CL 263,284 and CL 189,215 (expressed as CL 263,284 equivalents). Dilute to the mark with methanol and mix

well. This Mixed Standard Fortification Solution contains 50 mcg/mL each of CL 263,222, CL 263,284 and CL 189,215 (expressed as CL 263,284 equivalents).

b. Mixed Standard Fortification Solutions (10 mcg/mL)

Pipet into a single 100-mL volumetric flask 20-mL of the 50 mcg/mL of CL 263,222, CL 263,284, and CL 189,215 Mixed Standard Fortification Solution. Dilute to the mark with methanol and mix well. This solution contains 10 mcg/mL of each of CL 263,222, CL 263,284 and CL 189,215 (expressed as CL 263,284 equivalents).

c. Mixed Standard Fortification Solutions (4 mcg/mL)

Pipet into a single 100-mL volumetric flask 8-mL of the 50 mcg/mL of CL 263,222, CL 263,284, and CL 189,215 Mixed Standard Fortification Solution. Dilute to the mark with methanol and mix well. This solution contains 4 mcg/mL of each of CL 263,222, CL 263,284 and CL 189,215 (expressed as CL 263,284 equivalents).

d. Mixed Standard Fortification Solutions (2 mcg/mL)

Pipet into a single 100-mL volumetric flask 4-mL of the 50 mcg/mL of CL 263,222, CL 263,284, and CL 189,215 Mixed Standard Fortification Solution. Dilute to the mark with methanol and mix well. This Mixed Standard Fortification Solution contains 2 mcg/mL of each of CL 263,222, CL 263,284 and CL 189,215 (expressed as CL 263,284 equivalents).

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

a. 0.40 mcg/mL

Pipet a 1-mL aliquot of the 10 mcg/mL of CL 263,222, CL 263,284, and CL 189,215 Mixed Standard Fortification Solution into a 100-mL pear-shaped flask, evaporate to dryess using a rotary evaporator. Add accurately 25 mL Milli-Q water, using a 25-mL volumetric pipet.

b. 0.20 mcg/mL

Mix equal volumes of the 0.4 mcg/mL Mixed Calibration Standard Solution prepared in D.3.a. and Milli-Q water.

c. 0.10 mcg/mL

Mix equal volumes of the 0.2 mcg/mL Mixed Calibration Standard Solution prepared in D.3.b. and Milli-Q water.

Use the 0.40, 0.20, and 0.10 mcg/mL Mixed Calibration Standard Solutions for the linearity check (See Section F). Use the 0.20 mcg/mL Mixed Calibration Standard Solution as the bracketing standard.

E. Capillary Electrophoresis (CE) Conditions

1. Wavelength: 240 nanometers
2. Temperature: 30°C
3. Voltage: -13 KV
4. Polarity: (-)
5. Injection: Vacuum, 4 seconds (approximately 73.8 nL).
6. Capillary: Bare Fused Silica, approximately 72 cm total length, approximately 75 um i.d., 280 um o.d., (approximately 50 cm from inlet to detector); (approximately 22 cm from detector to outlet).
7. Chart Speed: 1 cm/min
8. Range: 0.010
9. Rise Time: 0.50 Sec.
10. Attenuation: 8
11. Run time: 15 min.
12. 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, 1M and 0.1M sodium hydroxide need to be filtered through Corning 0.22 um filter prior to use.

13. Equilibration: 2 minutes, 0.1N sodium hydroxide wash followed by 5 minutes with the run buffer.
14. Separation Buffer: 25 mM DTAB / 10 mM Tris / 10 mM NaH2PO4 / 5% THF (See B.5.k.).
15. Migration Time: Approximately 6.98, 7.06, and 7.22 min for CL 263,284, CL 263,222 and CL 189,215 respectively.
16. Sensitivity: Instrument sensitivity should be set so that a 4-seconds load of the 0.2 mcg/mL Mixed Calibration Standard gives an electrophoretic peak heights of approximately 30%-40% full scale for CL 263,284, CL 263,222 and CL 189,215.

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F. Linearity Check

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

1. Adjust the CE conditions for injections of 0.40, 0.20, and 0.10 mcg/mL Mixed Calibration Standard Solutions. The 0.20 mcg/mL working standard should give approximately 30%-40% full scale deflection.

2. Calculate or 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 peanut hulls 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 preparation of peanut nutmeat samples, blend the whole sample with dry ice for several minutes in a Waring Blendor to break and pulverize the sample.

4. Allow the peanut hull 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. At least one concurrent fortified control sample (but not less than 20% of the sample set, whichever is larger) must be analyzed with each set of samples. These fortifications should cover the range of expected residue values. If only a single fortified control sample is run, it should be at the validated sensitivity of this method.

1. Weigh a 20-g subsample of control into a 1-quart Mason jar.

2. Add by volumetric 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 (section I).

I. **Extraction, Precipitation, Centrifugation Preliminary Cleanup (Important: See Note 3 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 (B.5.d.) and blend at medium speed for 5 minutes using an Omni mixer. Add approximately 5 g Celite 545 AW to the mixture after blending.

3. Place a 9-cm Buchner funnel on top of a 500-mL filtration flask by using a filter support collar. Place 100 mL acetone into a 200-mL beaker and add approximately 10 g Celite 545 AW then mix with a glass rod. Dampen a double layer of 9-cm glass-fiber filter paper with acetone in the 9-cm Buchner funnel and then turn on the vacuum. Swirl the Celite/acetone slurry, then pour all at once onto the filter paper to make a Celite pad covering the filter paper. Discard the acetone in the filtration flask. Filter the extract by vacuum through the Celite pad.

4. Measure a 30-mL aliquot of the extract into a 50 mL graduated cylinder and transfer it quantitatively into a 300-mL pear-shaped flask.

5. Evaporate the aliquot to approximately 5 mL using a rotary evaporator and Vacobox. Stopper, sonicate and transfer to a 50-mL centrifuge tube.

6. Add 2 x 10 mL water to the flask, stopper, sonicate and transfer to the centrifuge tube between each addition of water. Add 3 mL of 10% lead acetate to the centrifuge tube.

7. Balance all centrifuge tubes to equal weight (using water if necessary), Centrifuge at 20,000 RPM (16,266 x g) for 15 minutes at approximately 4°C.

J. **Solid Phase Extraction Cartridges Cleanup (SPE)**

1. Transfer the contents of 3 cartridges of poly-Prep AG® 1-X8 into a 15 mL empty fritted reservoir, allow to settle, drain excess water (leave about 1 cm water at the top of the column) and do not allow the column to go to dryness. Gently, place a 20 um frit on top of the packing material and apply gentle pressure with an end of a 10-mL disposable pipette (VWR, Cat. No. 53511-678) to secure the bed (frit should barely touch the top of the sorbent bed). Condition the column with 2 column volumes of Milli-Q water. Add 2 mL Milli-Q water to the column. Assemble a 25 mL empty non-fritted filter reservoir onto the top of the AG® 1X-8 cartridge using an adapter.

2. Pass the supernatant from step I.7 through the AG® 1-X8 at the rate of 1 drop per second. Disconnect the reservoir (See Note 4).

3. Wash the AG® 1-X8 column with 2 x 5 mL of 0.4% Formic acid in water at the rate of 1 drop per second. Discard the wash. Assemble a 70 mL empty non-fritted filter reservoir onto the top of the AG® 1X-8 cartridge using an adapter.

4. Prepare an International Sorbent Technology (IST) ISOLUTE unendcapped C18 cartridge (500 mg/3 mL tube) using an IST, VacMaster Processing Station by washing the cartridge with two full column volumes (approximately 3 mL each time) of methanol followed by two full column volumes (approximately 3 mL each time) of 1% formic acid in H2O. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes. Add 1/2 column volume (approximately 2 mL) of 1% formic acid in H2O to the C18 cartridge.

5. Place the AG® 1-X8 cartridge assembly from step J.3 onto the top of the C18 cartridge, using an adapter. Dropload the sample from the AG® 1-X8 cartridge onto the C18 cartridge with 70 mL of 1% Formic acid in water at the rate of 1-2 drops per second. Discard the eluate.

6. Remove the reservoir, adapter and AG® 1-X8. Remove the adapter from top of the C 18 cartridge and wash the C18 cartridge at the rate of approximately 1 drop per second with two full column volumes (approximately 3 mL each time) of H2O. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.

7. Assemble a 25-mL, non-fritted filter reservoir onto the top of the prepared IST C18 cartridge using an adapter.

8. Elute the C18 cartridge with 15 mL of methanol at the rate of 1-2 drops per second and collect the eluate in a 100-mL pear-shaped flask containing 2 mL Milli-Q H2O, set inside the VacMaster Processing Station. Evaporate the solvent to approximately 1 mL. Do not allow eluate to go to dryness.

9. Add 10 mL of pH 2.5 water to the flask, stopper and vortex for approximately 30 seconds.

10. Prepare an IST SCX cartridge (1000 mg/6mL tube) using the VacMaster Processing Station by washing with two full column volumes (approximately 6 mL each time) of methanol and two full column volumes (approximately 6 mL each time) of pH 2.5 water. Add 1/2 column volume (approximately 4 mL) of pH 2.5 water 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.9 through the SCX cartridge at the rate of approximately 1 drop per second using VacMaster Processing Station.

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12. Rinse the flask with one 5-mL portion of pH 2.5 water, vortex for approximately 30 seconds then pass the rinse through the SCX cartridge at the rate of approximately 1 drop per second. Remove the reservoir and adapter.

13. Add 2 mL methanol to the sample flask, rinse and pass through the SCX cartridge at the rate of approximately 1 drop per second. Wash the SCX cartridge with 3 mL methanol at the rate of approximately 1 drop per second. Do not allow the liquid level to drain below the top of the sorbent bed during these washes.

14. Assemble a clean 30-mL graduated syringe onto the top of the SCX cartridge using an adapter.

15. Elute the SCX cartridge with 30 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.

16. Evaporate the eluate to near dryness. Rinse the saturated potassium chloride residue down to the flask tip with approximately 3 mL methanol then re-evaporate to dryness. Dissolve in 10 mL of 1% formic acid in water. Stopper, swirl and vortex for approximately 30 seconds.

17. 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 volumes (approximately 3 mL each time) of methanol followed by two full column volumes (approximately 3 mL each time) of 1% formic acid in H2O. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes. Add 1/2 column volume (approximately 2 mL) of 1% formic acid in H2O to the C18 cartridge.

18. Pass the sample from step J.16 through the C18 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 sample flask, reservoir and cartridge with a 5-mL portion of 1% formic acid in water at the rate of approximately 1 drop per second. 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 C18 cartridge with three full column lengths of Milli-Q water at the rate of 1 drop per second. Do not allow the liquid level to drain below the top of the sorbent bed during any of these washes.

21. Assemble a 25-mL non-fritted reservoir onto the top of the C18 cartridge using an adapter and elute the C18 cartridge with 15 mL of methanol at the rate of approximately 1-2 drops per second into a 50-mL pear-shaped flask containing 1 mL Milli-Q water, set inside the VacMaster Processing Station.

22. Using a rotary evaporator, evaporate the eluate to approximately 0.5 mL. Rinse the walls of the flask with approximately 3 mL methanol to get all the residue down to the tip of the flask. Re-evaporate to approximately 0.3 mL, add 0.5 mL Milli-Q water to the flask, sonicate for approximately 60 seconds.

23. Transfer quantitatively to a 2-mL volumetric flask, using a disposable pasteur pipette. Add 0.4 mL Milli-Q water to the flask, vortex and sonicate for approximately 30 seconds and transfer quantitatively to the 2-mL flask. Add an additional 0.4 mL Milli-Q water to the flask and repeat the process. Dilute to volume with Milli-Q water. Mix the solution properly using disposable pasteur pipette. Inject a portion of the extract into the CE.

24. Store the remaining extract in small amber glass vial with cap in refrigerator with proper identification.

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K. CE Method Parameters

Example of set up for the ABI/270A-HT Capillary Electrophoresis Unit :

Method: User-01 Report:

Cycle:	1– Detect Risetime: 0.50 Range: 0.010	Wavelength: 240 nm Autozero: Yes	Change: Yes
Cycle:	2--Flush Time: 2.0 mins Voltage: 0 KV Increment: No	Vial: 51 Vacuum:20.0 " Hg By: 1	Temperature: 30°C Wavelength: 240 nm
Cycle:	3--Flush Time: 5.0 mins Voltage: 0 KV	Vial: 53 Vacuum: 20.0 " Hg Increment: No	Temperature: 30°C Wavelength: 240 nm By: 1
Cycle:	4--Sample Time: 4.0 secs Voltage: 0 KV	Vacuum: 5.0 " Hg	Temperature: 30°C Wavelength: 240 nm
Cycle:	5--Time Time: 12.0 mins Voltage: -13 KV	Vial: 52 Vacuum: 0.0 " Hg	Temperature: 30°C Wavelength: 240 nm
Cycle:	6--End Empty cycle		
Cycle:	7--End Empty cycle		
Cycle:	8--End Empty cycle		
Cycle:	9--End Empty cycle		

NOTE 1: Vial 51: Contains 0.1 M sodium hydroxide solution.
Vial 52: Contains separation buffer.
Vial 53: Contains separation buffer.

NOTE 2: Capillary needs to be rinsed with Separation Buffer for 5 minutes daily prior to using the CE.

L. Capillary Electrophoresis Analysis

1. After obtaining a stable CE response for the Mixed Calibration Standard Solution (0.20 mcg/mL) as shown in Section E.16, 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 0.20 mcg/mL of working standard solution.

3. If the sample peaks go above the peak heights of the 0.40 mcg/mL Mixed Calibration Standard Solution, 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:

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

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 (0.20 mcg/mL)

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

V2 = Aliquot of extract taken for analysis in milliliters (30 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 peanut hull and peanut nutmeat commodities are shown in Figures 1 and 2.

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.

   c. Speedvac: Model SS1, Savant Instruments.

   d. Tube: 5 mL, 75 mm x 12 mm (Cat. No. 55.526), Sarstedt.

2. Sample Preparation for LC/MS Confirmation:

   For samples (J.23) showing total residue of greater than 0.3 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, transfer the hydrolyzed sample to a tube (N.1.d) and strip the aqueous acid using the speedvac (N.1.c). After solvent removal, reconstitute the residue in 1.0 mL LC/MS mobile phase (N.5.c).

3. LC/MS Standard Solution: With each set of samples analyzed, a 1.0-mL aliquot of the 0.10 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.	Mobile Phase	80 H_2O / 20 CH_3OH (both 0.5% in CF_3COOH)
d.	Injection Volume	50 μL
e.	LC/MS Interface Backpressure	60 bar (at 80°C)
f.	Vaporizer Temperature (Set)	80°C
g.	Aerosol Temperature (Set)	250°C
h.	Repeller Voltage	20 Volts
i.	MS High Vacuum (No LC/MS)	3E-07 t
j.	MS High Vacuum (LC/MS)	2E-05 t

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k.	Conversion Dynode	-15 kV
l.	Electron Multiplier	-1300 Volts
m.	Preamplifier Range (Full Scan)	1E-08 amps/volt
n.	Preamplifier Range (SIM)	1E-09 amps/volt
o.	Ions Monitored	m/z 276 $^{+}$, m/z 292^{+}
p.	Retention Time of Analytes	CL 263,284 3.0 min.
		CL 263,222 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:

a. Using parameters detailed in N.5, a 100 ng on-column injection of the analytes [10 μL of D.2.b.(10 ng/μL)] is used to determine the mass centroids of the ions at m/z 276^{+} and 292^{+}. 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.

b. Inject 50 μL aliquots of the working standard (N.3) until a reasonably constant response is obtained (Figure 3).

c. Follow the injection sequence: working standard, Sample Number 1, Sample Number 2, working standard, Sample Number 3, Sample Number 4, working standard,...

d. 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 >0.3 ppm total CL 263,222 related residues when:

a. 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.

Typical chromatograms for LC/MS Confirmatory Analysis are shown in figures 3 through 7.

Notes to Method M 2379

1. All glassware, should be soaked overnight in 10 % NaOH solution or in Micro solution (B.4.h), washed, rinsed with Milli-Q water and then methanol and air dried before use.

2. If impurity peaks are a problem, standards and samples can be injected using the following **Alternate Separation Buffers:**

   a. 37 mM DTAB/10 mM Tris/10 mM NaH2PO4 (approximately pH=7.5): Current approximately 28 uA: Add 2.30 g of DTAB and 0.24 g of Tris and 0.24 g of sodium phosphate, monobasic to 200 mL of Milli-Q water, stir to dissolve, filter through 0.22 um Corning Nylon Filter System. **Conditions:** 4 second load, Temperature: 33 °C, polarity (-), Voltage: -13 KV, vacuum injection, wavelength: 240 nm, attenuation: 16, chart speed: 1cm/min. (migration time approximately 6.51, 6.60, and 6.68 min. for CL 263,284, CL 263,222 and CL 189,215 respectively).

   b. 50 mM sodium dodecyl sulfate (SDS)/8.5 mM sodium tetraborate decahydrate/8.5 mM sodium phosphate dibasic/15% acetonitrile (pH=8.5): Current approximately 40 uA: Add 2.90 g of sodium dodecyl sulfate (SDS), 0.65 g of sodium tetraborate decahydrate, and 0.24 g sodium phosphate dibasic to 200 mL of Milli-Q water, stir for approximately 5 minutes, filter through 0.22 um Corning Nylon Filter System. Store at room temperature (prepare every other week). Add 15% acetonitrile only to the inlet buffer just before use (mix throughly). **Conditions:** 4 seconds load, 45°C, polarity (+), 18 KV, vacuum injection, wavelength 240 nm, attenuation 8, chart speed 1 cm/min (migration time approximately 12.9, 14.2, and 14.4 min for CL 189,215, CL 263,284 and CL 263,222 respectively).

3. Stopping Points in the method: The following are the steps in the method in which the sample workup may be halted if necessary and the samples stored in a refrigerator:

   a. Steps J.8: After eluting the C18 cartridge with methanol into the 100-mL pear-shaped flask, stopper the flask and store in refrigerator.

   b. Steps J.21: After eluting the C18 cartridge with methanol into the 50-mL pear-shaped flask, stopper the flask and store in refrigerator.

4. 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. Also, collect the supernatant after passing through the AG®1X-8 and dispose of this solution as a chemical waste.

APPROVALS:

Authors:

H. Nejad — Date 3/31/94

M. Safarpour — Date 3/31/94

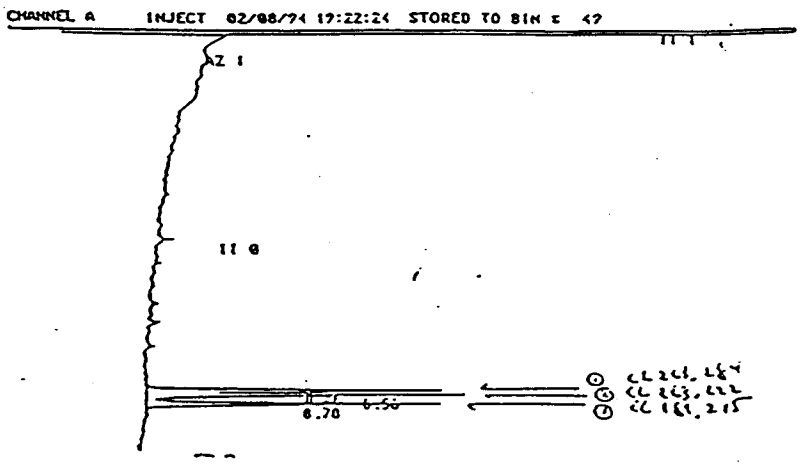
S. Chiu — Date 3/31/94

J. S. Fletcher — Date 3/31/94

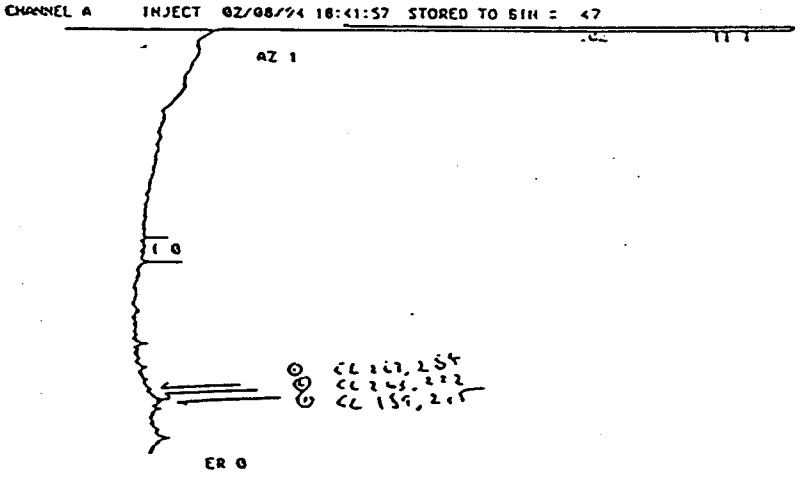
Group Leader:

G. L. Picard — Date 3/31/94

Figure 1: Typical Electropherograms for the Analysis of CL 263,284, CL 263,222 and CL 189,215 Residues in Peanut Hull

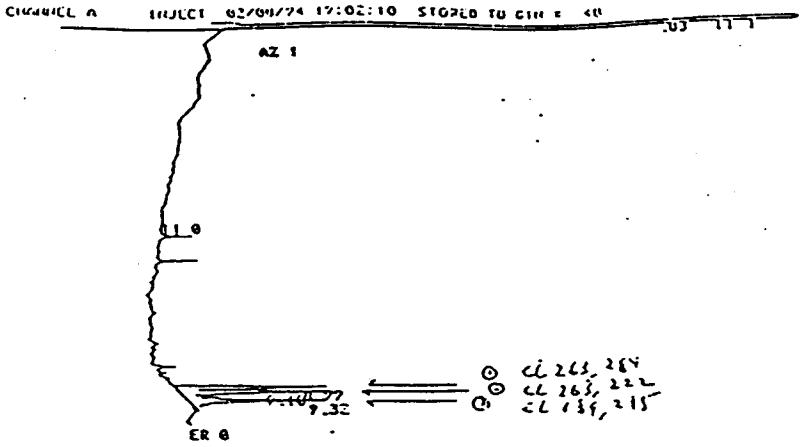


CL 263,284, CL 263,222 and CL 189,215 Standards, 15 pg of each Injected

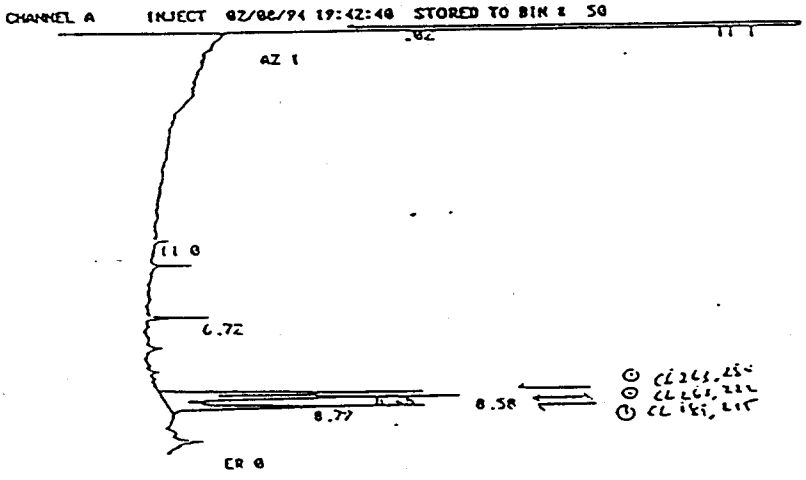


Control Peanut Hull, 74 mcg Injected, 0.006 ppm CL 263,284, 0.005 ppm CL 263,222 and 0.009 ppm CL 189,215 Found

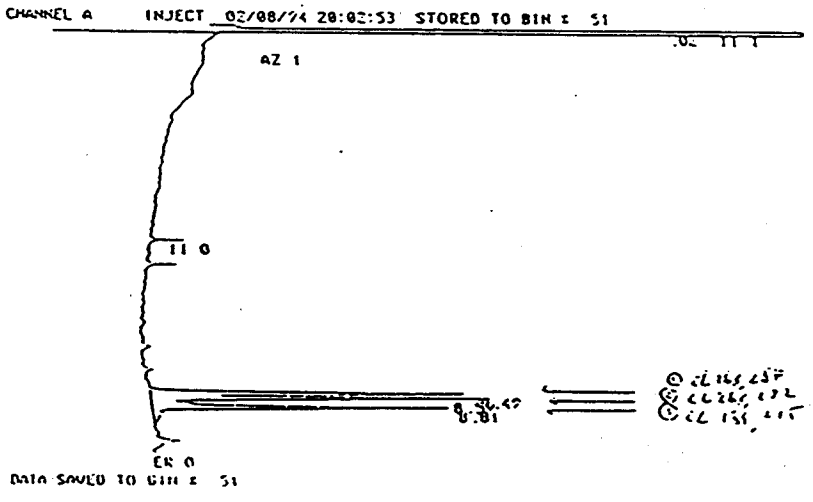
Figure 1: Continued



Control Peanut Hull, Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.1 ppm,74 mcg Injected, 102% CL 263,284, 94% CL 263,222 and 96% CL 189,215 Recovered

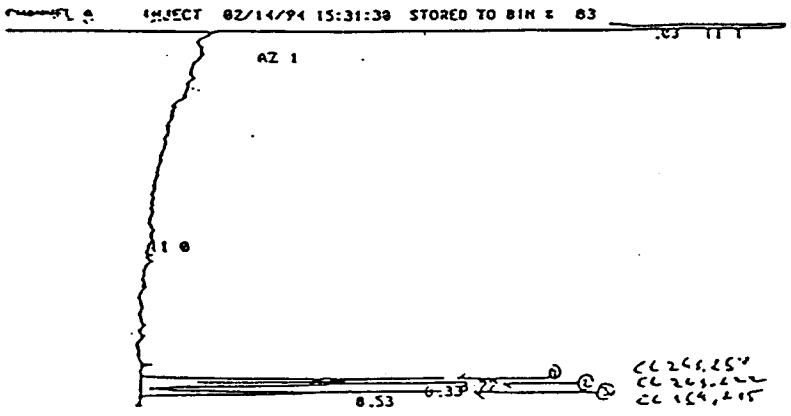


Control Peanut Hull, Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.2 ppm, 74 mcg Injected, 87% CL 263,284, 92% CL 263,222 and 86% CL 189,215 Recovered

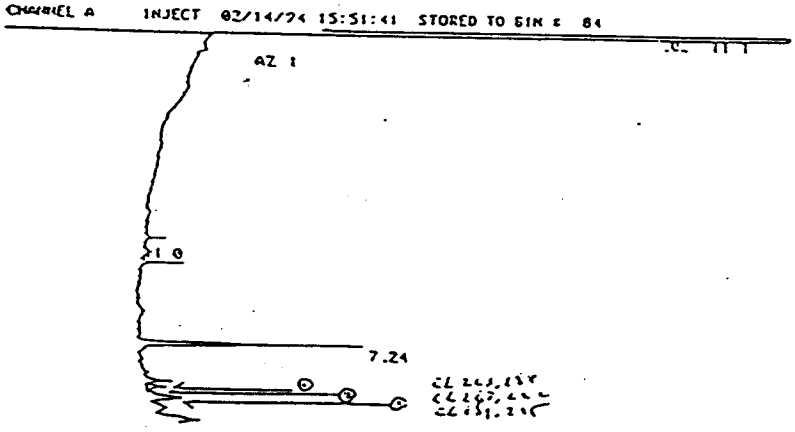


Control Peanut Hull, Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.5 ppm, 37 mcg Injected, 83% CL 263,284, 83% CL 263,222 and 79% CL 189,215 Recovered

Figure 2: Typical Electropherograms for the Analysis of CL 263,284, CL 263,222 and CL 189,215 Residues in Peanut Nutmeat

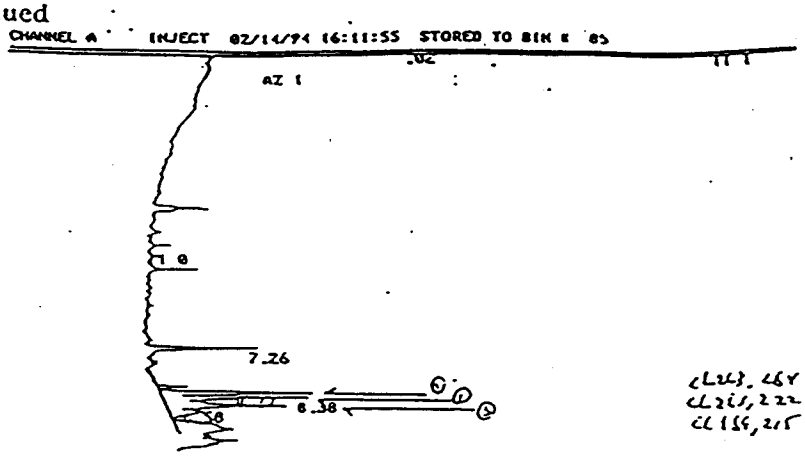


CL 263,284, CL 263,222 and CL 189,215 Standards, 15 pg of each Injected

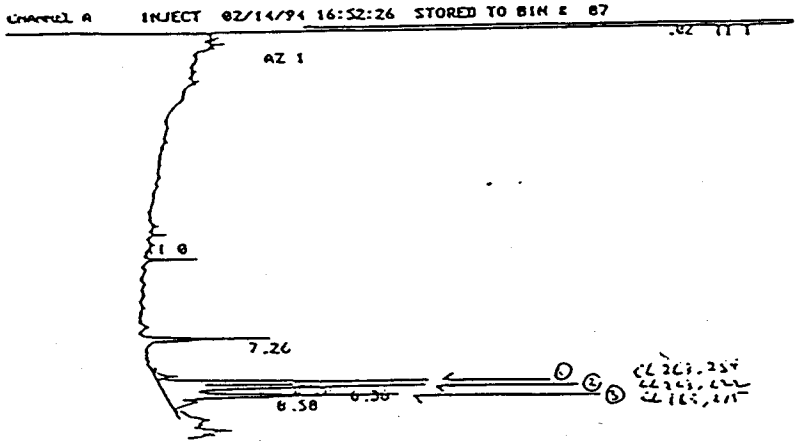


Control Peanut Nutmeat, 74 mcg Injected, 0.01 ppm CL 263,284, 0.005 ppm CL 263,222 and 0.006 ppm CL 189,215 Found

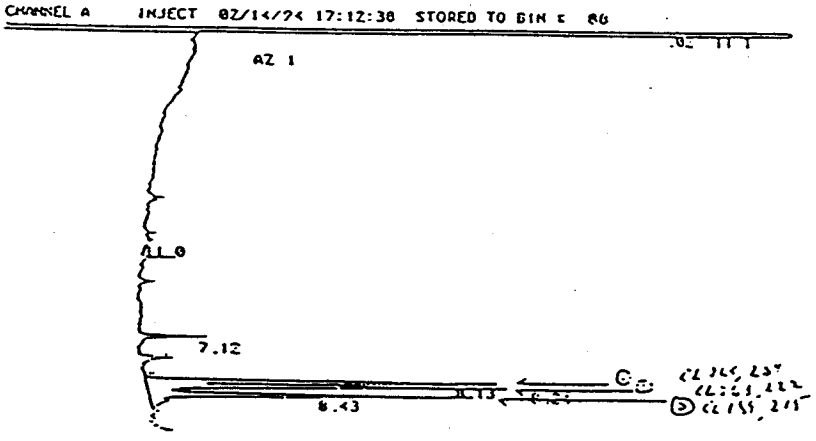
Figure 2: Continued



Control Peanut Nutmeat , Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.1 ppm, 74 mcg Injected, 96% CL 263,284, 85% CL 263,222 and 74% CL 189,215 Recovered



Control Peanut Nutmeat , Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.2 ppm, 74 mcg Injected, 86% CL 263,284, 78% CL 263,222 and 74% CL 189,215 Recovered



Control Peanut Nutmeat , Fortified with CL 263,284, CL 263,222 and CL 189,215 at 0.5 ppm, 37 mcg Injected, 89% CL 263,284, 86% CL 263,222 and 82% CL 189,215 Recovered

Figure 3: **Typical Mass Chromatograms from the Injection of 50 uL of the Hydrolyzed LC/MS Standard Solution Equivalent to 0.1 ppm of Each Analyte.**

```
CHRO:  94t005a                              14-FEB-94   Elapse:  00:00:10.6     1
Samp:  SNG STD MIX C020494-3                            Start :  13:33:20     101
Mode:  TSP +Q3MS LMR UP LR                              Study :  CD94PT02
Oper:  A. DACUNHA     Client:    H NEJAD                Inlet :
Peak:  1000.00  mmu     Label wndw:    1 > 101          Masses:  276 > 292
Area:  0, 4.00          Baseline  :    0, 3             Label :  3, 300.00
```

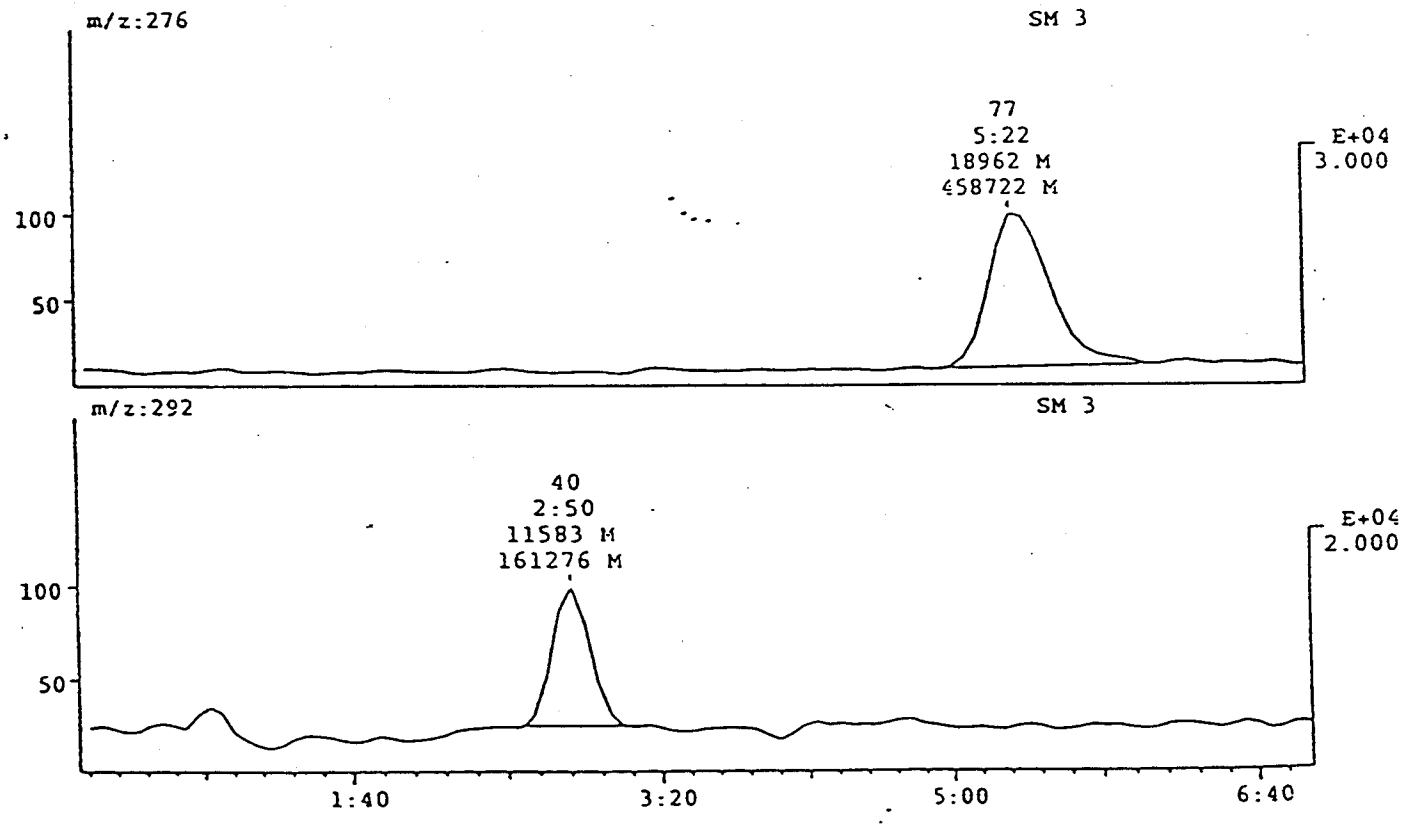


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Figure 4: **Typical Mass Chromatograms from the Injection of 50 uL of the Hydrolyzed Control Peanut Hull Sample.**

```
CHRO:  94t006a                              14-FEB-94   Elapse:  00:00:14.5     1
Samp:  ACGL0416-1A CTRL PNUT HULL                       Start :  13:44:00     101
Mode:  TSP +Q3MS LMR UP LR                              Study :  CD94PT02
Oper:  A. DACUNHA    Client:    H NEJAD                 Inlet :
Peak:  1000.00  mmu    Label wndw:    1 > 101          Masses:  276 > 292
Area:  0, 4.00         Baseline  :    0, 3             Label :  3, 300.00
```

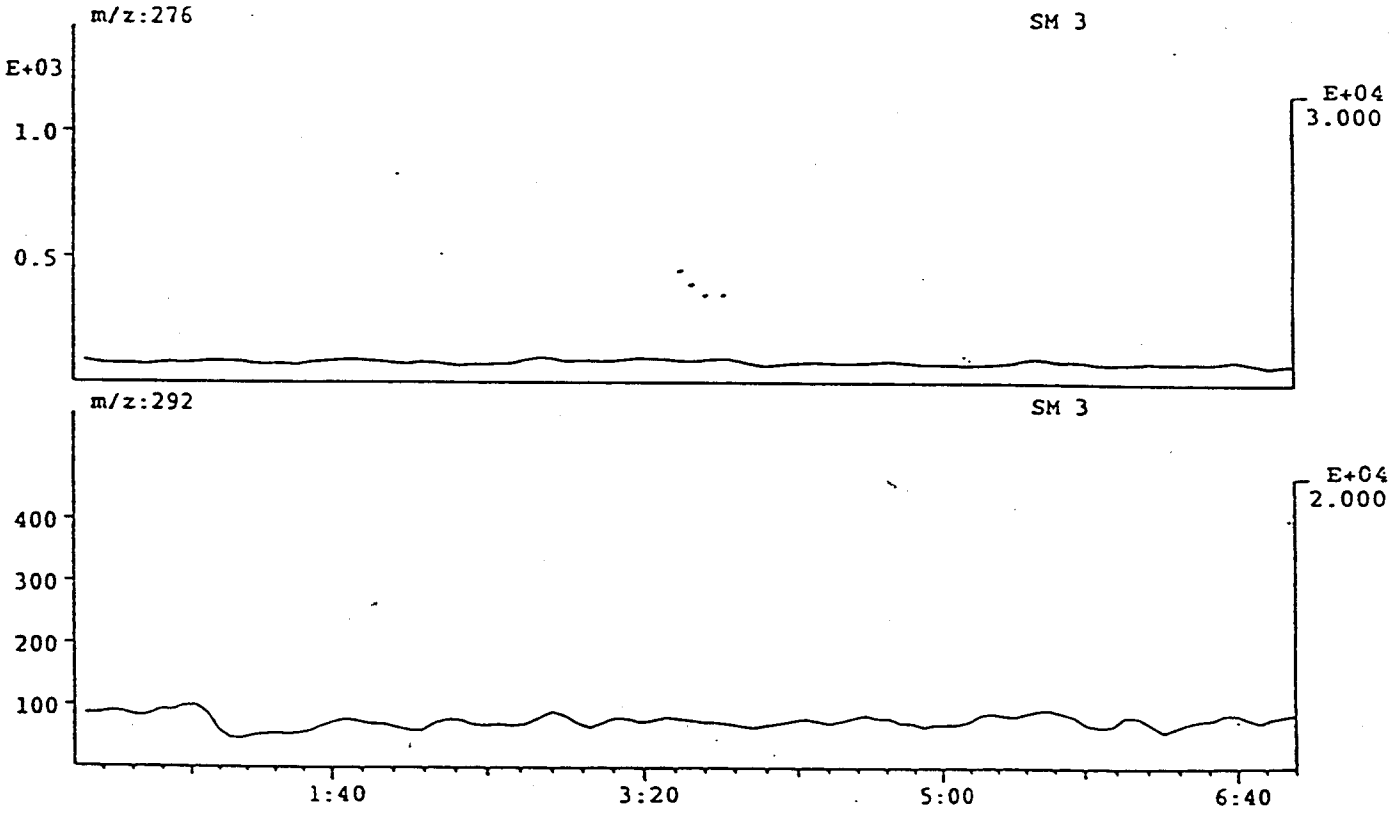

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Figure 5: **Typical Mass Chromatograms from the Injection of 50 uL of the Hydrolyzed Peanut Hull Sample Fortified with 0.1 ppm of Each Analyte.**

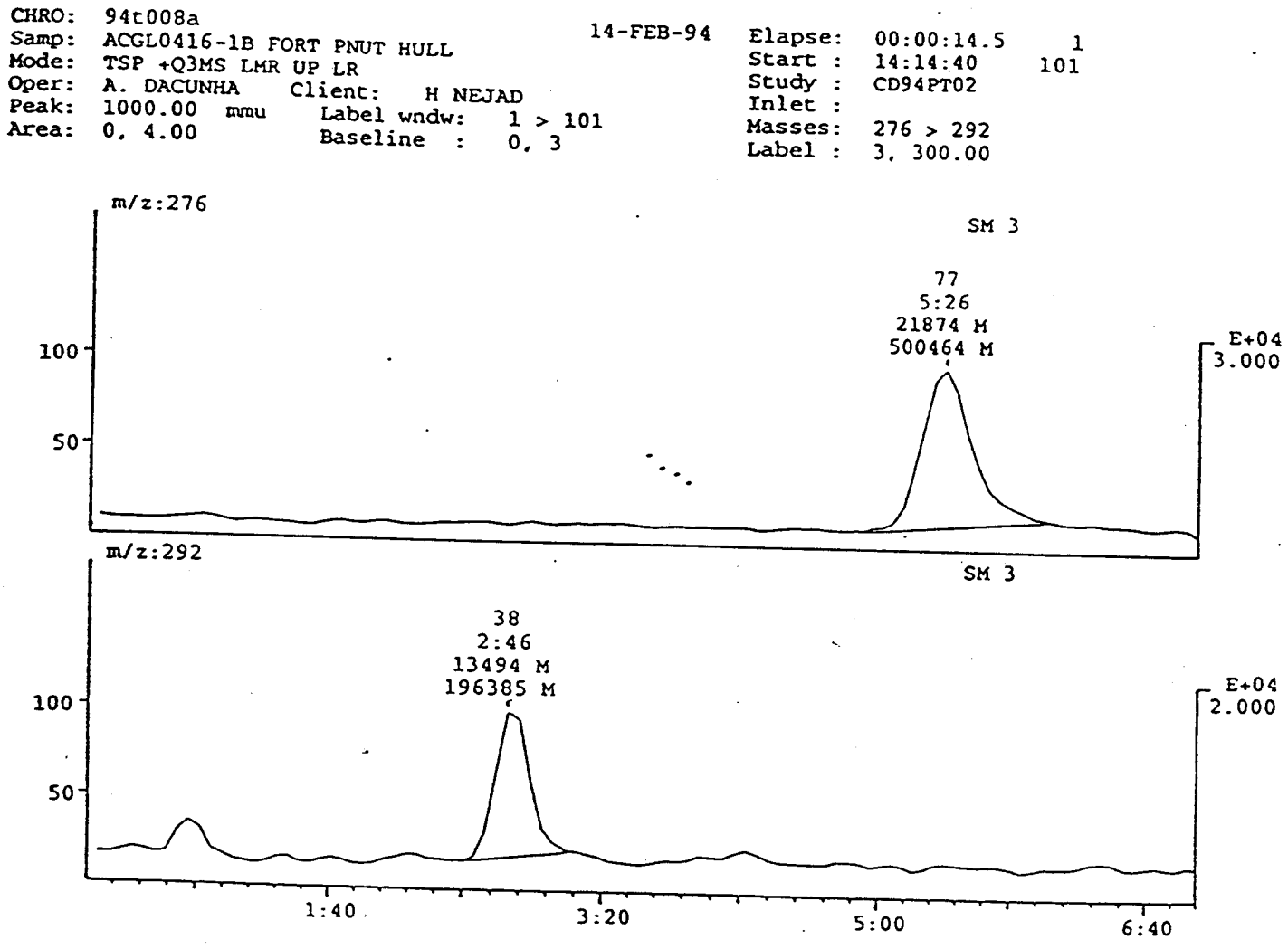

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Figure 6: **Typical Mass Chromatograms from the Injection of 50 uL of the Hydrolyzed Control Peanut Nutmeat Sample.**

CHRO: 94t007a 14-FEB-94 Elapse: 00:00:10.8 1
Samp: ACGL0416-1C CTRL PNUT NUTMEAT Start : 13:53:49 102
Mode: TSP +Q3MS LMR UP LR Study : CD94PT02
Oper: A. DACUNHA Client: H NEJAD Inlet :
Peak: 1000.00 mmu Label wndw: 1 > 102 Masses: 276 > 292
Area: 0, 4.00 Baseline : 0, 3 Label : 3, 300.00

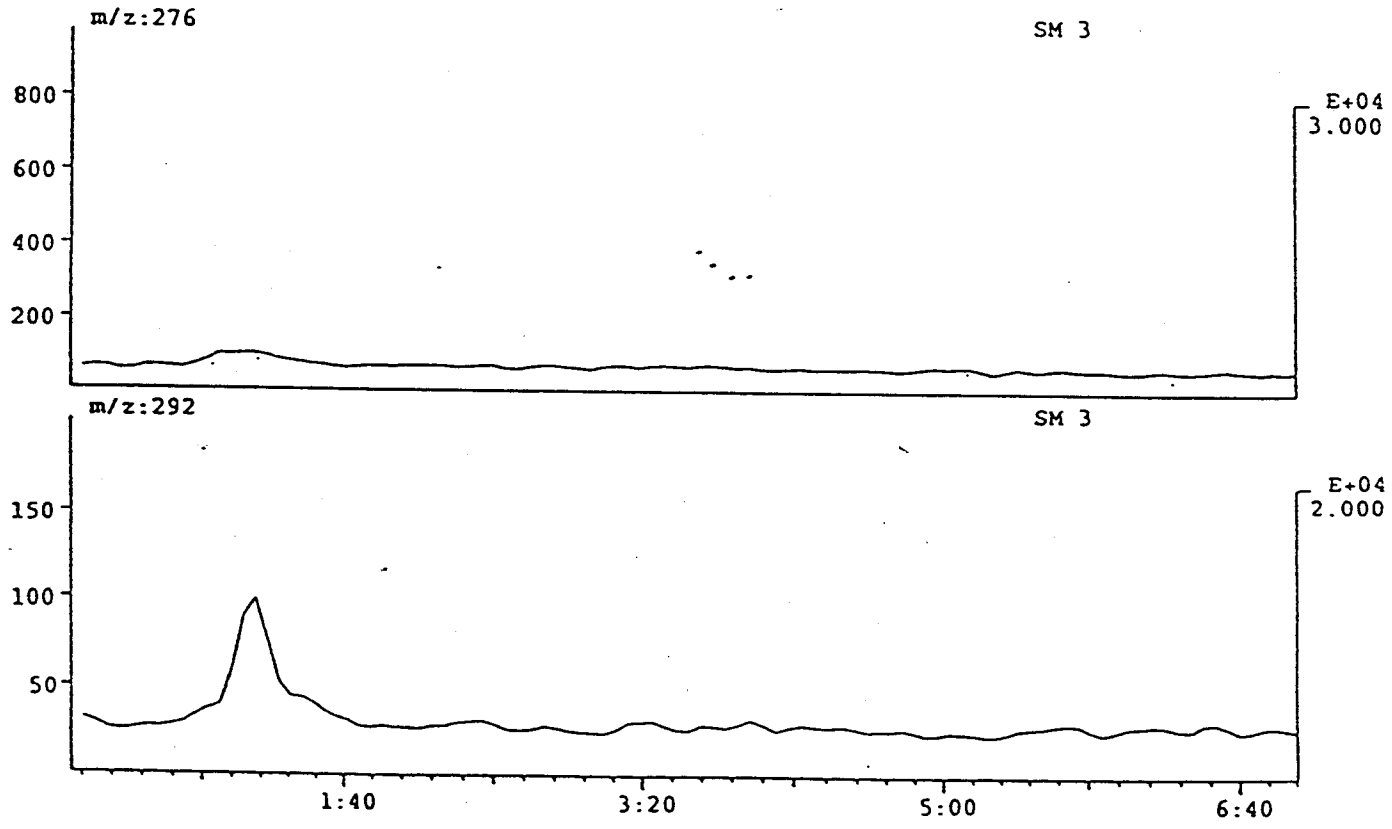
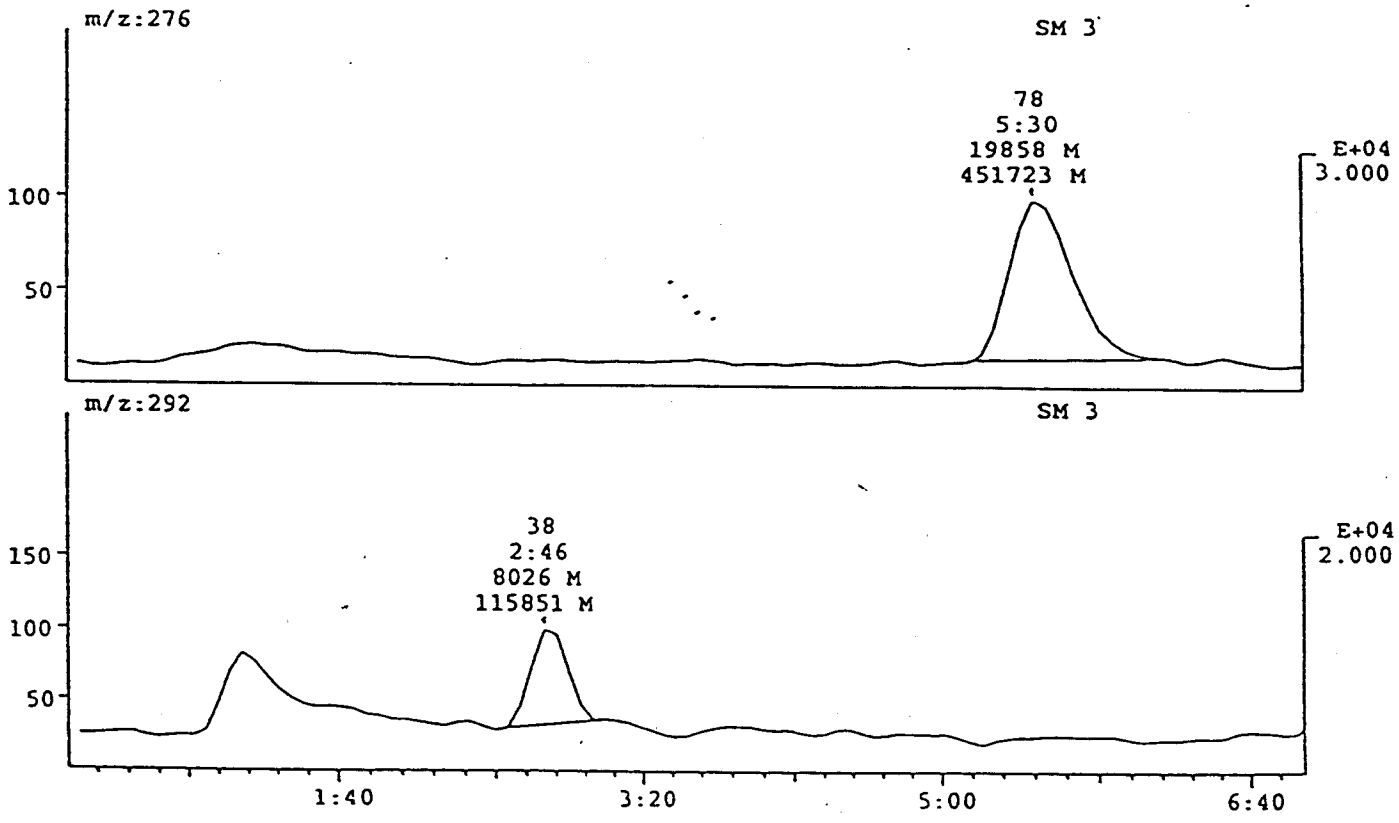


Figure 7: **Typical Mass Chromatograms from the Injection of 50 uL of the Hydrolyzed Peanut Nutmeat Sample Fortified with 0.1 ppm of Each Analyte.**

```
CHRO:  94t009a                              14-FEB-94   Elapse:  00:00:14.6     1
Samp:  ACGL0416-1D FORT PNUT NUTMEAT                    Start :  14:25:38     100
Mode:  TSP +Q3MS LMR UP LR                              Study :  CD94PT02
Oper:  A. DACUNHA     Client:    H NEJAD                Inlet :
Peak:  1000.00  mmu     Label wndw:   1 > 100          Masses:  276 > 292
Area:  0, 4.00          Baseline  :   0, 3              Label :  3, 300.00
```



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