AMERICAN CYANAMID COMPANY
AGRICULTURAL PRODUCTS RESEARCH DIVISION
ENVIRONMENTAL SCIENCES
P. O. BOX 400
PRINCETON, NEW JERSEY 08543-0400

Method of Analysis - M 2657

Imazapyr (CL 243,997): Capillary Electrophoresis (CE) Method for the Determination of CL 243,997 Residues in Corn Grain, Forage and Fodder.

A. Principle

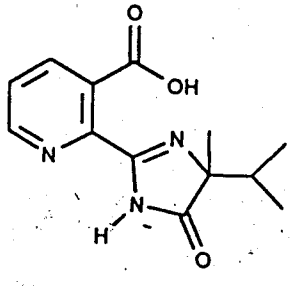
Residues of CL 243,997 are extracted from the sample with acidic acetone-water. The CL 243,997 residues are subjected to suitable cleanup involving solvent partitioning and solid phase extraction. Measurement of the CL 243,997 is accomplished by capillary electrophoresis and results are calculated as CL 243,997 by the direct comparison of peak heights to those of external standards. The validated sensitivity (LOQ, Limit of Quantitation) of the method is 50 ppb.

B. Reagents

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

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

CL 243,997: [nicotinic acid, 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl-]



M.W.=261

2. Water, Deionized: Water passed through a Millipore Milli-Q Plus Ultra Pure Water System. Use this water for all steps.

3. Solvents: B & J Brand High Purity Solvents, Baxter, Burdick and Jackson.

   a. Acetone, Cat. No. 010-4L
   b. Methanol, Cat. No. 230-4L
   c. Methylene Chloride, Cat. No. 300-4L

4. Celite 545 AW: Acid-washed, Cat. No. 2-0199, Supelco, Bellefonte, PA.

5. Chemicals:

   a. Hydrochloric Acid, Concentrated, Cat. No. 9535-01, "Baker Analyzed" Reagent, J.T. Baker Company.
   b. Formic Acid, Concentrated, ~98% purity, Cat. No. 06440, Fluka Chemika, Ronkonkoma, NY.
   c. Potassium Chloride, Crystal, Cat. No. 3040-01, "Baker Analyzed" Reagent, J.T. Baker Company.
   d. DTAB (Dodecyltrimethylammonium bromide), 99% purity, Cat. No. D-8638, Sigma Chemical Company, St. Louis, MO.
   e. Tris [tris-(hydroxymethyl)-aminomethane], 99% purity, Cat. No.T-1378, Sigma Chemical Company, St. Louis, MO.
   f. Sodium Phosphate, Monobasic (NaH_2PO_4), 98% purity, Cat. No.S-3139, Sigma Chemical Company, St. Louis, MO.
   g. Sodium Hydroxide, 1.0 M Solution, Cat. No.S-8526, Sigma Chemical Company, St. Louis, MO.

6. Solutions:

   a. 1% Formic Acid: Add 5 mL of concentrated formic acid (~98% purity) to 250 mL of Milli-Q water in a 500-mL volumetric flask and dilute to 500 mL with Milli-Q water. Mix well.

   b. Saturated KCl/Methanol: Add 50 g of potassium chloride to 1 liter of methanol. Mix well with a stir bar on a stirring platform. Allow the excess KCl to settle to the bottom of the flask.

   c. Extraction Solvent: Measure 500 mL of acetone into a 2000-mL graduated mixing cylinder. Add 20 mL of concentrated hydrochloric acid and bring the volume up to 2000 mL with Milli-Q water. Mix well.

d. 100 mM DTAB: Measure 200 mL of Milli-Q water into a 400-mL beaker. Place the beaker on a stirring platform with a stir bar. Weigh 6.17 g of DTAB (Reagent B.5.d.) and add to the water. Mix well until fully dissolved. Filter the solution through a Corning 150-mL Filter System with a 0.22 µm cellulose acetate membrane. Refrigerate this solution when not in use.

e. 100 mM Tris: Measure 200 mL of Milli-Q water into a 400-mL beaker. Place the beaker on a stirring platform with a stir bar. Weigh 2.42 g of Tris (Reagent B.5.e.) and add to the water. Mix well until fully dissolved. Filter the solution through a Corning 150-mL Filter System with a 0.22 µm cellulose acetate membrane. Refrigerate this solution when not in use.

f. 100 mM NaH_2PO_4: Measure 200 mL of Milli-Q water into a 400-mL beaker. Place the beaker on a stirring platform with a stir bar. Weigh 2.76 g of NaH_2PO_4 (Reagent B.5.f.) and add to the water. Mix well until fully dissolved. Filter the solution through a Corning 150-mL Filter System with a 0.22 µm cellulose acetate membrane. Refrigerate this solution when not in use.

g. CE Separation Buffer: 35 mM DTAB / 10 mM Tris / 10 mM NaH_2PO_4: Measure 70 mL of the 100 mM DTAB solution (B.6.d.), 20 mL of the 100 mM Tris solution (B.6.e.) and 20 mL of the 100 mM NaH_2PO_4 solution (B.6.f.) into a clean 200-mL volumetric flask. Dilute up to the 200-mL mark with Milli-Q water, stopper and mix well. Transfer the solution to a 200-mL Corning polystyrene bottle for storage. This solution should be made fresh bi-weekly and be refrigerated when not in use.

h. 0.1 M Sodium Hydroxide: Measure 10 mL of the 1.0 M sodium hydroxide solution (Reagent B.5.g.) into a clean 100-mL volumetric flask and dilute to 100 mL with Milli-Q water. Stopper and mix well. Transfer the solution to a clean glass bottle for storage. Store at room temperature.

i. 1 mM Tris/ 1 mM NaH_2PO_4: Measure 1 mL of the 100 mM Tris solution (B.6.e.) and 1 mL of the 100 mM NaH_2PO_4solution (B.6.f.) into a clean 100-mL volumetric flask and dilute to 100 mL with Milli-Q water. Stopper and mix well. Transfer the solution to a 200-mL Corning polystyrene bottle for storage. Prepare this solution weekly and refrigerate when not in use..

C. Apparatus

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

1. Capillary Electrophoresis System: Applied Biosystems (ABI) Model 270A-HT equipped with a high sensitivity optical flow cell and a UV detector, Applied Biosystems/Perkin Elmer Company.

2. Balance, Pan: Sartorius, Model 610, precision ± 5.0 mg.

3. Balance, Analytical: Sartorius, Model R200D, precision ± 0.05 mg.

4. Assorted Glassware: General laboratory.

5. Evaporation Flasks: ~~T~~24/40 joint, 250-mL capacity, pear-shaped and ~~T~~14/20 joint, 25-mL capacity, pear-shaped.

6. Filtering Flasks: 500-mL capacity.

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

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

9. Rotary Evaporator: Buchi Instruments Model R-114C, equipped with a cold finger dry ice trap and a heated water bath set at approximately 35°C, Brinkmann Instruments, Inc., Westbury, NY.

10. Omni Mixer: Model 17105, Omni Corporation International.

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

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

13. Vacuum Processing Station: IST VacMaster-20, Cat. No. 121-2016, equipped with PTFE stopcocks, Cat. No.121-0009, International Sorbent Technology, Mid Glamorgan, U.K., Distributed by Jones Chromatography, Lakewood, CO.

14. Buchi Vacobox Vacuum Pump: Model B-171, Cat. No. 15-50-705-5, Brinkmann Instruments, Inc., Westbury, NY.

15. Solid Phase Extraction Cartridges:

    a. ISOLUTE SCX cartridge, 1000 mg, 6-mL capacity tube, Cat. No.530-0100-C, International Sorbent Technology, Mid Glamorgan, U.K., Distributed by Jones Chromatography, Lakewood, CO.

    b Spe-ed RP102 cartridge, 500 mg, 6-mL capacity tube, Cat. No. 4210, Applied Separations, Allentown, PA.

16. Reservoirs: Non-fritted, 70-mL capacity, Cat. No. 120-1008-F, and non-fritted, 25-mL capacity, Cat. No. 120-1007-E, International Sorbent Technology, Mid Glamorgan, U.K., Distributed by Jones Chromatography, Lakewood, CO.

17. PTFE Cartridge Adapters: Cat. No. 120-1100, International Sorbent Technology, Mid Glamorgan, U.K., Distributed by Jones Chromatography, Lakewood, CO.

18. Corning 150-mL Filtration System: 0.22 µm cellulose acetate membrane, sterile, Cat. No. 25932-200, Corning Incorporated, Corning, New York.

19. Corning 200 mL Polystyrene Storage Bottles: Cat. No. 25625-200, Corning Incorporated, Corning, New York.

D. Preparation of Standard Solutions

1. Stock Solution (Store in amber bottles in refrigerator. May be prepared and used for up to six months.)

   CL 243,997: Weigh accurately a known amount (approximately 10 mg) of CL 243,997 and transfer into a 100-mL volumetric flask. Dilute to the mark with acetone and mix well. Calculate and record the exact concentration of CL 243,997, correcting for the standard purity.

2. Standard Fortification Solutions (Store in amber bottles in refrigerator. May be prepared and used for up to six months.)

   a. Pipet into a 100-mL volumetric flask an appropriate amount of stock solution D.1. to deliver 1000 mcg of CL 243,997. Dilute to the mark with acetone and mix well. This Standard Fortification Solution contains 10 mcg/mL CL 243,997.

   b. Pipet into a 100-mL volumetric flask, a 10-mL aliquot Standard Fortification Solution D.2.a. Dilute to the mark with acetone and mix well. This Standard Fortification Solution contain 1.0 mcg/mL CL 243,997.

3. Capillary Electrophoresis (CE) Calibration Standard Solutions (Prepare these solutions weekly. Store in the refrigerator when not in use.)

   a. Pipet into a 25-mL pear-shaped evaporation flask a 2-mL aliquot of the 1.0 mcg/mL Standard Fortification Solution D.2.b. Evaporate to dryness then rinse the flask with a small amount of methanol and re-evaporate to dryness. Redissolve the standard with 10 mL of the 1 mM Tris/ 1 mM NaH_2PO_4 solution (Reagent Solution B.6.i.) and vortex to mix. This CE Calibration Standard contains 0.2 mcg/mL CL 243,997.

b. Pipet into a 25-mL pear-shaped evaporation flask a 1-mL aliquot of the 1.0 mcg/mL Standard Fortification Solution D.2.b. Evaporate to dryness then rinse the flask with a small amount of methanol and re-evaporate to dryness. Redissolve the standard with 10 mL of the 1 mM Tris/ 1 mM NaH_2PO_4solution (Reagent Solution B.6.i.) and vortex to mix. This CE Calibration Standard contains 0.1 mcg/mL CL 243,997 and is the working standard.

c. Pipet into a 25-mL pear-shaped evaporation flask a 1-mL aliquot of the 1.0 mcg/mL Standard Fortification Solution D.2.b. Evaporate to dryness then rinse the flask with a small amount of methanol and re-evaporate to dryness. Redissolve the standard with 20 mL of the 1 mM Tris/ 1 mM NaH_2PO_4 solution (Reagent Solution B.6.i.) and vortex to mix. This CE Calibration Standard contains 0.05 mcg/mL CL 243,997.

E. Capillary Electrophoresis (CE) Conditions

1.	Temperature:	30°C
2.	Wavelength:	240 nm
3.	Voltage:	-13 KV
4.	Polarity:	(-)
5.	Injection:	Vacuum, 12 seconds (approx. 160 nL)
6.	Capillary:	Bare fused silica, 75 µm i.d., 280 µm o.d., approx.120 cm total length (approx. 98 cm from inlet to detector and approx. 22 cm from detector to outlet)
7.	Rise Time:	1.00 second
8.	Range:	0.010
9.	Chart Speed:	1 cm/minute
10.	Attenuation:	8
11.	Run Time:	approx. 30 minutes
12.	Separation Buffer:	35 mM DTAB / 10 mM Tris / 10 mM NaH_2PO_4 (Reagent Solution B.6.g.)
13.	Migration Time:	Approximately 26.5 minutes
14.	Sensitivity: standard	Instrument sensitivity should be set so that a 12-sec. injection of the 0.1 mcg/mL CL 243,997 working gives a response of approximately 30-40% full scale.

F. Linearity Check

The linearity of response of the capillary electrophoresis system should confirmed whenever a new column or instrument is used, following any instrument modification or if there is a significant alteration of the chromatographic conditions. The linearity of response should also be confirmed by injecting all three CE Calibration Standards prior to injection of each set of samples analyzed.

1. Adjust the CE conditions for injections of the 0.2-, 0.1- and 0.05 mcg/mL CE Calibration Standards. The injection of the 0.1 mcg/mL CL 243,997 working standard should give a response of approximately 30-40% full scale.

2. Determine the response factor (ratio) for all injections by dividing the peak response by the amount (nanograms) injected. Calculate the average response ratio. A deviation by any standard response factor by more than 15% from the average factor indicates instrumental difficulties, or incorrect standard preparations, which should be corrected before proceeding.

G. Sample Preparation

1. For corn grain: Pulverize sufficient dry ice in a Waring Blendor to thoroughly chill the bowl and blade. For corn forage and fodder: Pulverize sufficient dry ice in a Hobart Model 84185-D food chopper to thoroughly chill the bowl and blade.

   Add the prefrozen corn grain, forage or fodder samples in portions to enable reduction to fine particle size. Blend or chop the whole sample with dry ice for several minutes to pulverize the sample. It may be necessary to add small portions of dry ice during the blending/chopping procedure to ensure that the sample remains in a frozen state.

2. Transfer the sample into a labelled container and allow the sample to stand in a freezer overnight for the dry ice to dissipate completely.

3. Keep all samples frozen until ready for analysis.

H. Recovery Test

The validity of the procedure should always be demonstrated by recovery tests before analysis of unknown samples is attempted. At least one concurrent fortified sample must be analyzed along with every six samples analyzed. These fortifications should cover the range of expected residue values. If only a single fortified control is run, it should be at the validated LOQ of the method.

1. Weigh a 20-g subsample of control corn grain, forage or fodder 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 solid phase extraction cleanup steps as follows.

I. Extraction and Preliminary Cleanup

1. Weigh 20 g of corn grain, forage or fodder into a 1-quart Mason jar.

2. Add 200 mL of extraction solvent (Reagent B.6.c.) to the corn sample and allow the sample to soak in the extraction solution for approximately 15 minutes. Blend at medium speed for approximately 5 minutes using an Omni mixer. After mixing, add approximately 10 g of Celite 545 AW to the extract in the Mason jar and swirl to mix.

3. Pre-wet a double layer of 9-cm glass fiber filter paper, positioned on a 9-cm Buchner funnel, with acetone. Using vacuum, filter the extract and Celite mixture through the wetted filter paper into a 500-mL filtration flask.

4. Measure a 20-mL aliquot of the extract into a 125-mL separatory funnel and discard the remaining extract.

5. Add 50 mL of methylene chloride to the separatory funnel, stopper and shake for approximately 15 seconds. Drain the lower, methylene chloride layer into a 100-mL beaker being careful to not allow any of the aqueous fraction to drain into the beaker.

6. Partition the aqueous fraction with another 25 mL of methylene chloride and add this to the methylene chloride in the 100-mL beaker.

J. Solid Phase Extraction Cleanup

1. Attach an ISOLUTE SCX cartridge (1000 mg/6 mL tube) onto a PTFE stopcock on an IST VacMaster vacuum processing station. Close the stopcock. This cartridge does not require pre-conditioning.

2. Fill about half the SCX cartridge barrel with the extract from Step I.6. then attach a clean 70-mL reservoir onto the top of the cartridge using a PTFE adapter. Fill the reservoir with the remaining extract. Open the stopcock and allow the sample to pass through the SCX cartridge by gravity, collecting the methylene chloride for proper disposal. Remove the reservoir after all the sample has passed through.

3. Wash the cartridge with 5 mL of acetone followed by 5 mL of methanol, allowing each wash to pass through by gravity. Close the stopcock after the methanol passes just below the frit above the sorbent bed.

4. Remove the cartridge and open the stopcock to empty the remaining methanol from the stopcock needle. Place a 50-mL beaker inside the

vacuum processing station under the stopcock and replace the lid of the processing station.

5. Replace the cartridge onto the stopcock of the vacuum processing station. Measure 30 mL of saturated KCl/methanol (Reagent Solution B.6.b.) and fill the cartridge barrel half full then attach a clean 25-mL reservoir onto the top of the cartridge using a PTFE adapter. Add the rest of the KCl/methanol to the reservoir, open the stopcock and elutedhe cartridge, by gravity, into the 50-mL beaker.

6. Transfer the KCL/methanol eluate into a 250-mL pear-shaped evaporation flask. Rinse the beaker with a small amount of methanol and add to the sample. Evaporate the KCl/methanol to dryness using a rotary evaporator, being careful not to let the sample be drawn up into the evaporator while under vacuum. The vacuum setting on the Vacobox should be approximately 150 mbar and increased slowly.

7. Redissolve the sample in 10 mL of 1% formic acid (Reagent Solution B.6.a.). Vortex the flask to dissolve and mix the sample.

8. Prepare an Applied Separations Spe-ed RP102 cartridge (500 mg/6 mL tube) using an IST VacMaster vacuum processing station by washing the cartridge with 5 mL of methanol followed by 2 x 5 mL of water (by gravity or low vacuum). Allow the liquid level to drain just below the top of the frit above the sorbent bed between additions and after the final addition.

9. Pass the redissolved sample from Step J.7. through the RP102 cartridge, allowing it to pass through by gravity.

10. Rinse the 250-mL flask with 5 mL of Milli-Q water then pass the rinse through the cartridge. Wash the cartridge with an additional 5 mL of Milli-Q water, allowing each to pass through the cartridge by gravity. Close the stopcock.

11. Remove the cartridge and open the stopcock to empty the remaining water from the stopcock needle.

12. Replace the cartridge onto the stopcock on the vacuum processing station. By gravity, elute the cartridge, into a 50-mL pear-shaped evaporation flask placed inside the vacuum processing station, with two column volumes of methanol.

13. Evaporate the methanol to dryness using a rotary evaporator, possibly requiring the addition of more methanol.

14. Redissolve the sample in 1 mL of the 1 mM Tris/ 1 mM NaH_2PO_4 solution (Reagent Solution B.6.i.) and sonicate for approximately 15 seconds. Transfer approximately 300 mcL to a sample vial in preparation for the CE analysis.

K. CE Method Parameters

Example of the operating parameters used with the ABI 270A-HT Capillary Electrophoresis System:

Cycle 1:	Detect		
	Rise time: 1.00	Wavelength: 240 nm	
	Range: 0.010	Autozero: Yes	Change: Yes
Cycle 2:	Flush		
	Time: 3.0 minutes	Vial: 51	Temperature: 30.0°C
	Voltage: 0 KV	Vacuum: 20.0" Hg	Wavelength: 240 nm
		Increment: No	By: 1
Cycle 3:	Flush		
	Time: 3.0 minutes	Vial: 52	Temperature: 30.0°C
	Voltage: 0 KV	Vacuum: 20.0" Hg	Wavelength: 240 nm
		Increment: No	By: 1
Cycle 4:	Sample		
	Time: 12.0 seconds		Temperature: 30.0°C
	Voltage: 0 KV	Vacuum: 5.0" Hg	Wavelength: 240 nm
Cycle 5:	Time		
	Time: 30.0 minutes	Vial: 53	Temperature: 30.0°C
	Voltage: -13 KV	Vacuum: 0.0" Hg	Wavelength: 240 nm
Cycle 6:	End		
	Empty cycle		

Note: Vial 51 contains 0.1 M sodium hydroxide solution (Reagent Solution B.6.h.)
Vial 52 contains CE separation buffer (Reagent Solution B.6.g.)
Vial 53 contains CE separation buffer (Reagent Solution B.6.g.)

L. Capillary Electrophoresis Analysis

1. After obtaining a stable CE response for the injection of the 0.1 mcg/mL working standard as specified in section F., inject a 12-second load of sample.

2. Compare the peak heights of the sample with those of 0.1 mcg/mL standards injected before and after the sample (bracketing standards). An injection of the 0.1 mcg/mL working standard should be made after a maximum of every two sample injections.

3. The variation between the responses of bracketing standards must not exceed 15%. If the variation exceeds 15%, instrumental parameters should be adjusted to restore instrument performance.

4. If a sample peak goes out of the linearity range established with the calibration standards, dilute an aliquot of the sample to an appropriate volume with the 1 mM Tris/ 1 mM NaH_2PO_4 solution and re-inject the sample.

M. Calculations

For each sample calculation, use the sample peak height and the average peak height of the external (bracketing) standards injected before and after the sample injections as follows:

$$\text{ppb} = \frac{R(SAMP) \times (V1) \times (V3) \times C(STD) \times (V5) \times D.F.}{R(STD) \times W \times (V2) \times (V4)} \times 1000 \text{ ng/mcg}$$

$$\% \text{ Recovery} = \frac{\text{ppb Found}}{FV \times FC \times 1000 / W} \times 100$$

Where:

R(SAMP) = Peak height of sample

R(STD) = Average peak height of bracketing standards

W = Weight of sample taken for analysis in grams (20 g)

V1 = Volume of extraction solvent in milliliters (200 mL)

V2 = Aliquot of extract taken for analysis in milliliters (20 mL)

V3 = Final volume of sample solution for CE analysis in milliliters (1.0 mL)

V4 = Volume of sample solution injected in nanoliters (160 nL)

V5 = Volume of working standard solution injected in nanoliters (160 nL)

C(STD) = Concentration of working standard solution injected in micrograms per milliliter (0.1 mcg/mL)

D.F. = Dilution factor

FV = Fortification volume in milliliters

FC = Fortification concentration (of standard solution added) in mcg/mL

Typical electropherograms are shown in Figures 1-3.

APPROVALS:

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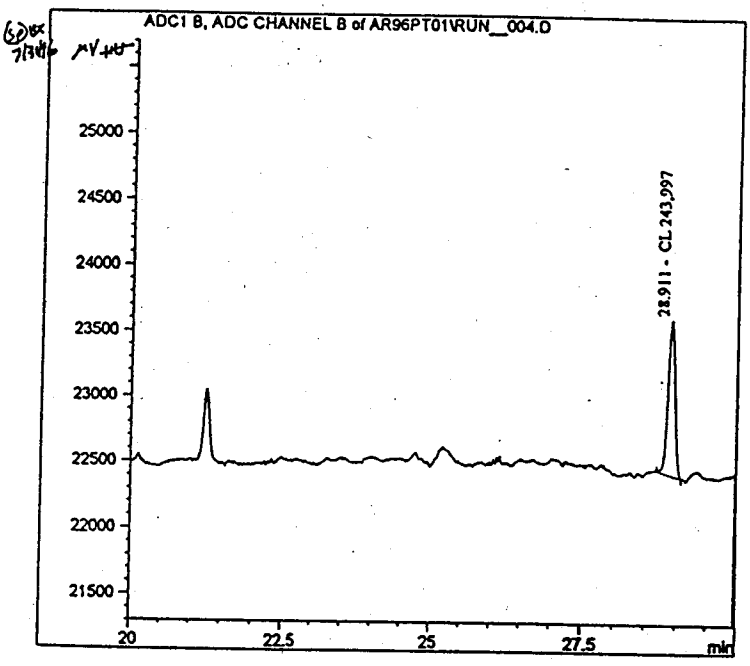
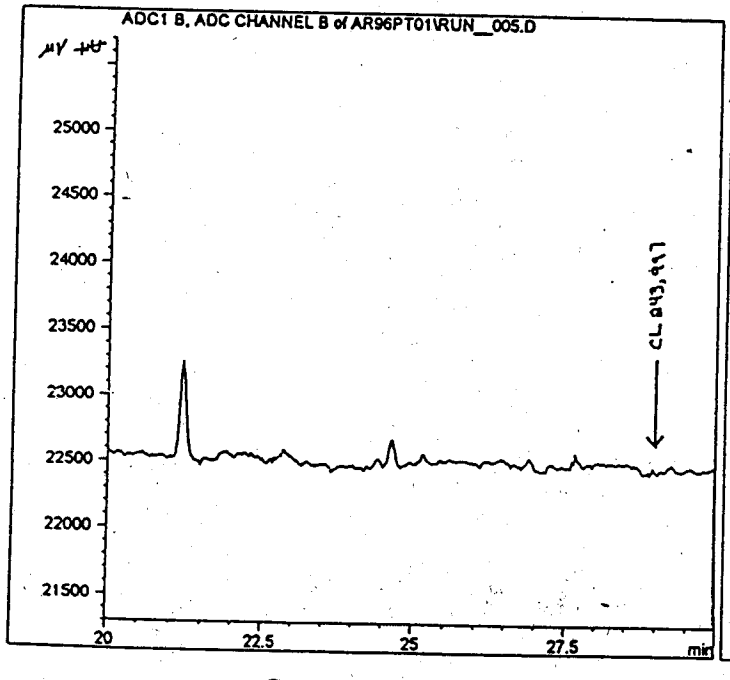
J. Shahn Fletcher Date
Author

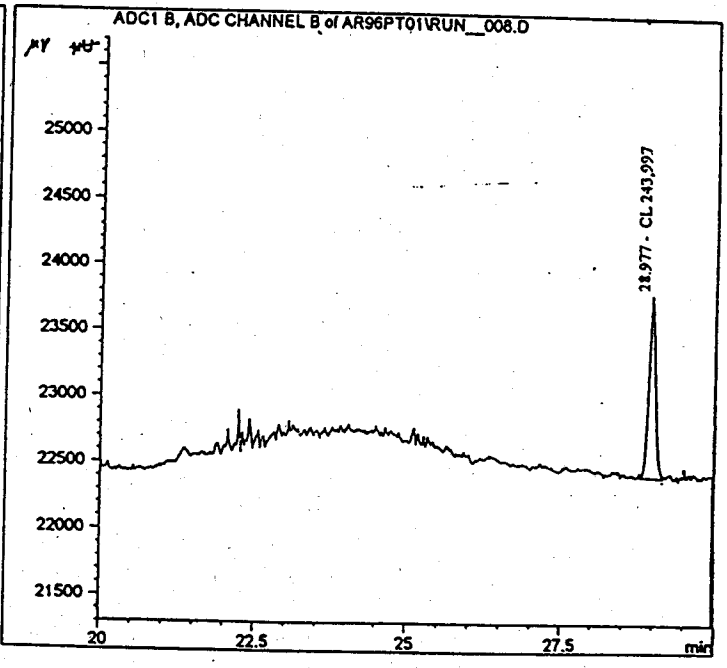
Figure 1: Typical Electropherograms from the Determination of CL 243,997 Residues in Corn Grain.



CL 243,997 standard, 0.1 mcg/mL, 16 pg injected.

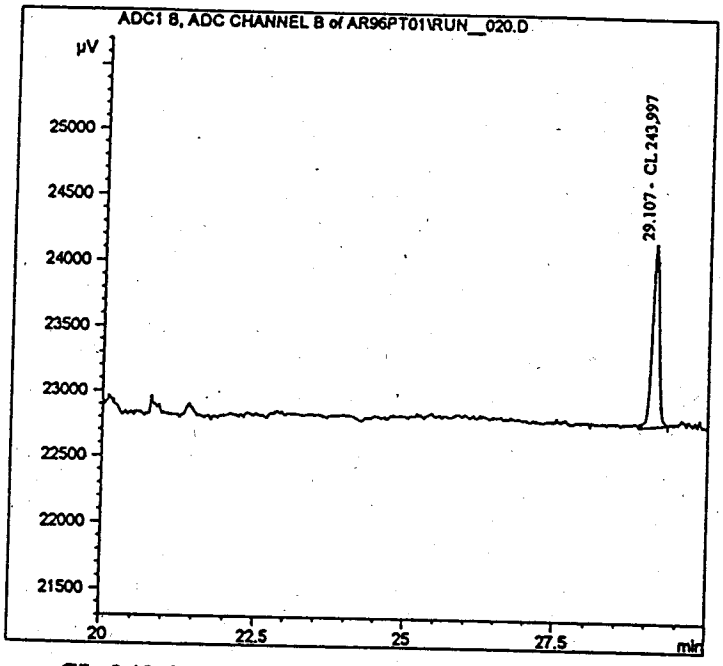


Control corn grain, 0.32 mg equivalents of sample injected, < 3.89 ppb CL 243,997 found.

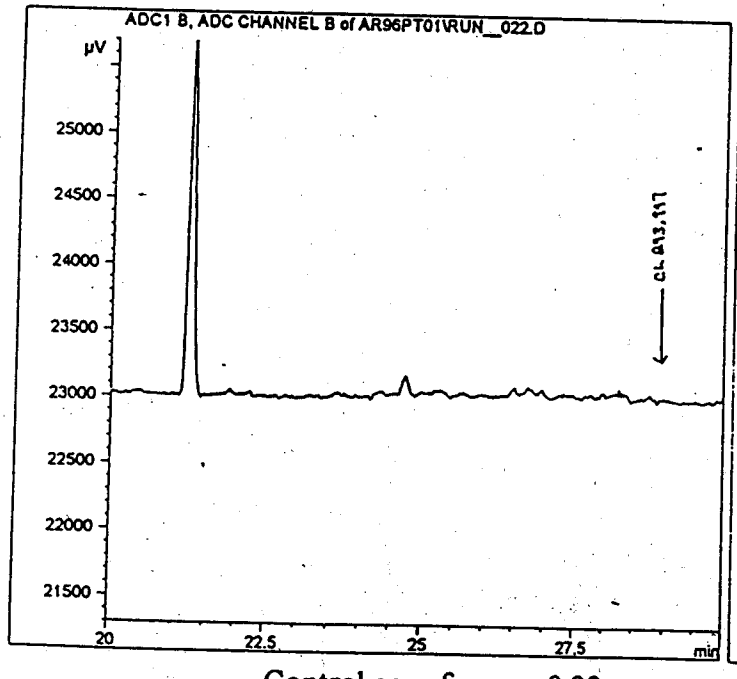


Corn grain fortified at 50 ppb, 0.32 mg equivalents of sample injected, 51 ppb (102%) CL 243,997 recovered.

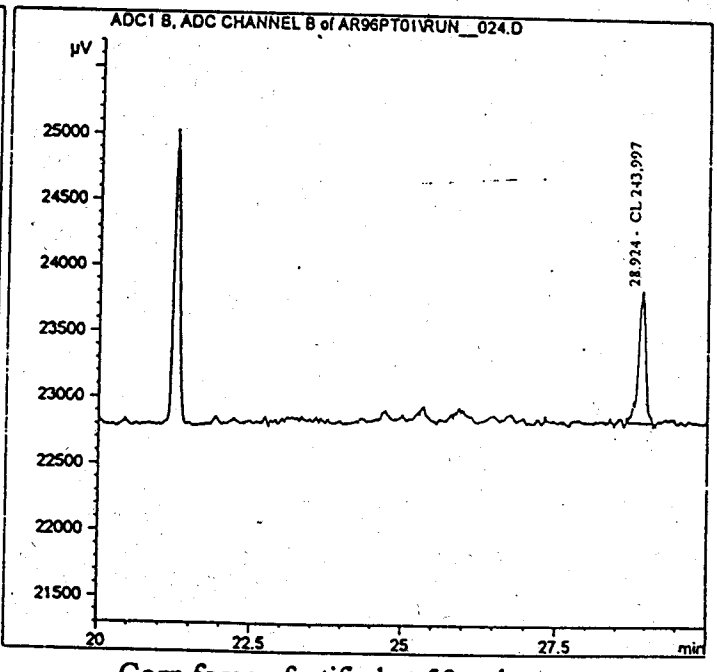
Figure 2: Typical Electropherograms from the Determination of CL 243,997 Residues in Corn Forage.



CL 243,997 standard, 0.1 mcg/mL, 16 pg injected.

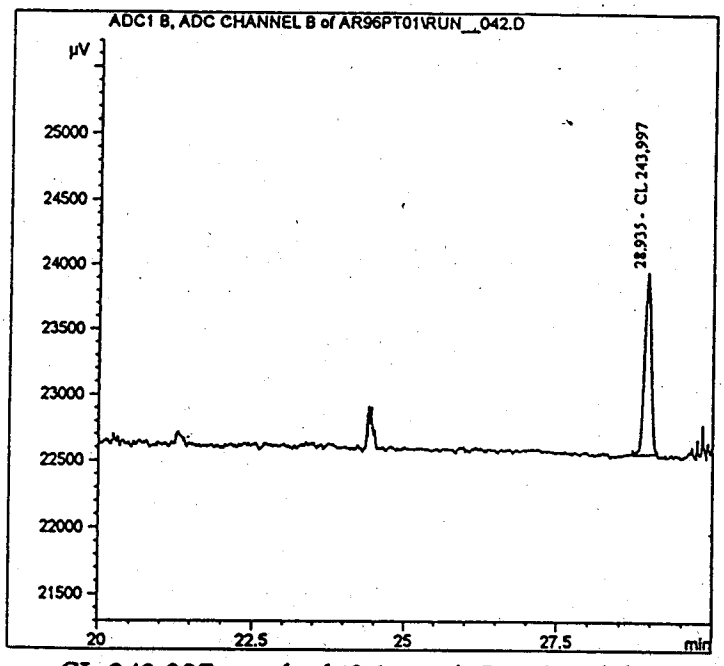


Control corn forage, 0.32 mg equivalents of sample injected, < 3.62 ppb CL 243,997 found.

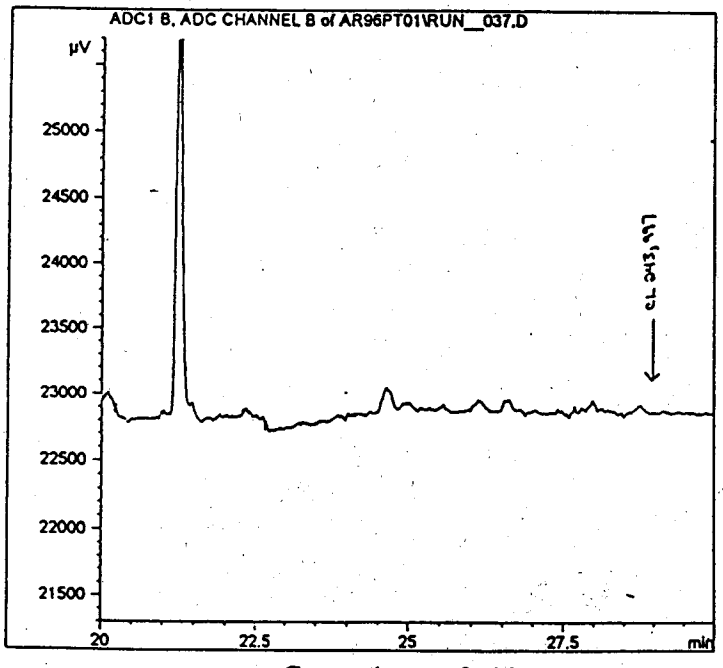


Corn forage fortified at 50 ppb, 0.32 mg equivalents of sample injected, 36.7 ppb (73%) CL 243,997 recovered.

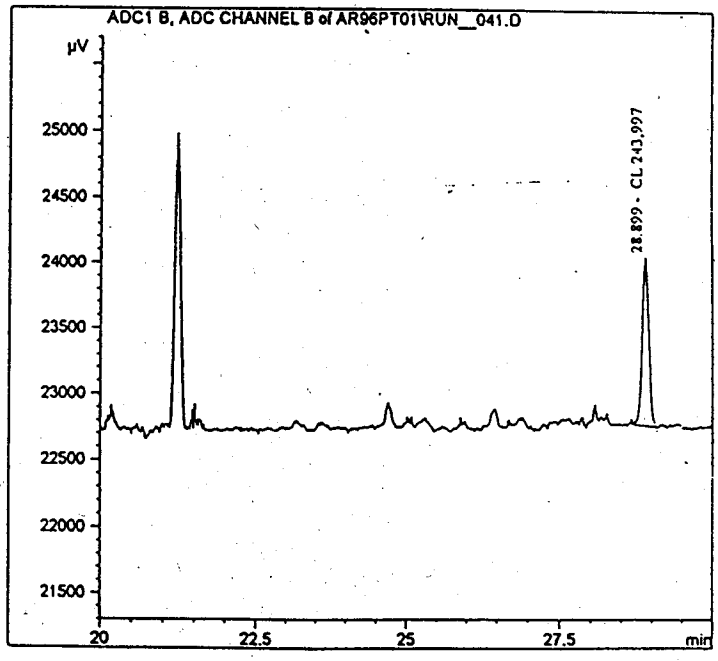
Figure 3: Typical Electropherograms from the Determination of CL 243,997 Residues in Corn Fodder.



CL 243,997 standard, 0.1 mcg/mL, 16 pg injected.



Control corn fodder, 0.32 mg equivalents of sample injected, < 3.62 ppb CL 243,997 found.



Corn fodder fortified at 50 ppb, 0.32 mg equivalents of sample injected, 45.6 ppb (91%) CL 243,997 recovered.