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

Recommended Method of Analysis - M 2468

Imazapyr (CL 243,997): GC/MS Method for the Determination of CL 243,997 Residues in Corn Grain, Forage and Fodder.

A. Principle

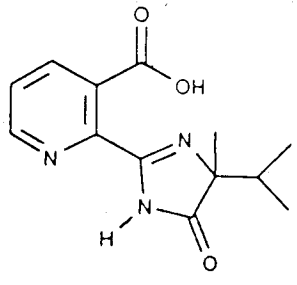
Residues of CL 243,997 are extracted from the sample with acidic acetone-water. The CL 243,997 residues are subjected to suitable cleanup involving solid phase extraction. Measurement of the CL 243,997 is accomplished by gas chromatography/negative ion chemical ionization mass spectrometry (GC/MS). Results are calculated as CL 243,997 by the direct comparison of peak areas 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
   b. Methanol
   c. Methylene Chloride
   d. Hexane (UV Grade)
   e. Acetonitrile (UV Grade)

4. Chemicals: "Baker Analyzed" Reagents, J. T. Baker Company.

   a. Hydrochloric Acid, Concentrated

5. Methylation Reagent: Trimethylphenylammonium Hydroxide (TMAH), 0.1M in methanol, Cat. No. 10943, Eastman Kodak Company.

   Alternate source: Phenyltrimethylammonium hydroxide Solution (TMAH), 0.1 M in methanol, Cat. No. 79265, Fluka Chemika.

6. Solutions:

   a. 1N 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. Mix well.

   b. 0.05N Hydrochloric Acid: Dilute 50 mL of reagent solution B.6.a. to 1 liter with Milli-Q water. Mix well.

   c. Extraction Solvent: Place 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.

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

C. Apparatus

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

1. Gas Chromatograph: Varian Model 3400 fitted with a Varian 1075 split/splitless capillary injector.

2. Splitless Liner: Varian, Cat. No. 03-949437-00.

3. GC Column: 15 m x 0.25 mm ID, 0.25 micron DB-5MS, Cat. No. 122-5512, J & W Scientific, Folsom, CA.

4. AutoInjector: Finnigan-MAT Model A200S.

5. Mass Spectrometer: Finnigan-MAT SSQ710.

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

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

8. Assorted Glassware: General laboratory.

9. Evaporation Flasks: T 14/20 joint, 25-mL capacity, pear-shaped.

10. Filtering Flasks: 500-mL capacity.

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

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

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

14. Omni Mixer: Model 17105, OCI Instruments.

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

16. Vacuum Processing Station: IST VacMaster, Cat. No. 121-2016, equipped with PTFE stopcock/needle system, Cat. No.121-0009, International Sorbent Technology, Mid Glamorgan, U.K., Distributed by Jones Chromatography, Lakewood, CO.

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

18. Solid Phase Extraction Cartridge: Varian Bond Elut C18 cartridge, 200 mg, 3-mL capacity tube, Cat. No. 1210-2025, Varian, Harbor City, CA.

D. Preparation of Standard Solutions

1. Stock Solution (Prepare monthly, store in amber bottles in refrigerator)

   a. 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 (Prepare monthly, store in amber bottles in refrigerator)

   a. Pipet into a 100-mL volumetric flask an appropriate amount of stock solution D.1.a. 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 Solutions contain 1.0 mcg/mL CL 243,997.

3. Standard Gas Chromatographic(GC) Solutions

   (Prepare the solutions described in 3.a. and 3.b. monthly, store in amber bottles in the refrigerator. Solutions described in 3.c. should be prepared daily.)

   a. Pipet into a 100-mL volumetric flask a 10-mL aliquot of the 1.0 mcg/mL Standard Fortification Solution D.2.b. Dilute to the mark with acetone and mix well. This Standard GC Solution contains 0.1 mcg/mL CL 243,997.

   b. Pipet into a 100-mL volumetric flask a 5-mL aliquot of the 0.1 mcg/mL Standard GC Solution D.3.a. Dilute to the mark with acetone and mix well. This Standard GC Solution contains 0.005 mcg/mL CL 243,997.

   c. Pipet into separate, labelled 25-mL evaporation flasks 1.0-, 2.0- and 5.0-mL aliquots of the 0.005 mcg/mL Standard GC Solution D.3.b. In a fourth 25-mL evaporation flask, pipet a 1-mL aliquot of the 0.1 mcg/mL Standard GC Solution D.3.a. Add 100 mcL of TMAH (Reagent B.5.) and evaporate each to dryness on a rotary evaporator with a heated waterbath. Redissolve each in 1 mL of methanol and transfer by pipet into labelled autoinjector vials. These Standard GC Solutions contain 0.005-, 0.01-, 0.025-, and 0.1 mcg/mL CL 243,997, respectively. The 0.005-, 0.01- and 0.025-mcg/mL solutions are used for the linearity check and the 0.1-mcg/mL solution is used for determination of the centroid for selected ion monitoring (Section K.1.). The 0.01 mcg/mL Standard GC Solution is to be used as the working standard for quantitation of CL 243,997.

   NOTE: All gas chromatographic standards for injection (D.3.c.) are to be prepared daily. Add 100 mcL TMAH to the standard aliquot before evaporation and redissolve the standard in only 1 mL of methanol after evaporation (See D.3.c.).

E. Gas Chromatographic / Mass Spectrometric Conditions

1. Gas Chromatograph: Varian Model 3400 gas chromatograph fitted with a Varian 1075 split/splitless capillary injector.

2. Splitless Liner: Insert a 1 to 1-1/2 inch plug of glass wool inside the liner.

3. Column: 15 m x 0.25 mm ID, 0.25 micron DB-5MS, J & W Scientific.

4. Gas Chromatograph Conditions:

a.	Column Temperature:	60°C for 0.5 min. then 10°C/min. to 250°C. Hold at 250°C for 5 min.
b.	Injector Temperature:	300°C
c.	Transfer Line Temperature:	250°C
d.	Carrier Gas and Flow Rate:	Helium at 15 psi/sec.
e.	Injection Volume (AutoInjector):	1 mcL
f.	Splitless Injection:	Split valve open at 0.5 min.
g.	Retention Time of Analyte:	approx. 13.15 min.

5. Mass Spectrometer Conditions:

a.	Source Temperature:	150°C
b.	CI Reagent Gas:	Methane at 9000 mT (indicated)
c.	Conversion Dynode:	+15 kV
d.	Electron Multiplier:	+1350 Volts
e.	Preamplifier:	1 E-08 amps/volt
f.	Scan Range (Full Scan):	m/z 250⁻ to 350⁻
	(SIM):	m/z 289⁻ nominal ±0.2 dalton
g.	Scan Rate:	0.5 sec./scan

F. Linearity Check

The gas chromatography should be checked for linearity of response whenever a new column or instrument is used.

1. Inject 1-mcL aliquots of solutions prepared in Section D.3.c. which are to be used for the linearity check.

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. A fortified sample should also be processed with each daily set of samples analyzed.

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 methanol. Using vacuum, filter the extract and Celite mixture through the wetted filter paper and into a 500-mL filtration flask.

4. Pipet a 2-mL aliquot of the extract into a 25-mL pear-shaped flask and discard the remaining extract.

5. Using a rotary evaporator and a Buchi Vacobox, evaporate the acetone from the extract under vacuum but do not evaporate to dryness. The vacuum pressure on the Vacobox should be changed gradually to avoid possible bumping. Start the pressure at approximately 150 mbar and increase the vacuum about 50 mbar every 5 minutes to 20 mbar. The aqueous volume after this step should be approximately 1 mL.

J. Solid Phase Extraction Cleanup

1. Prepare a Varian Bond Elut C18 cartridge (200 mg/3 mL tube) using an IST VacMaster vacuum processing station by washing the cartridge with 1 column volume (approx. 3 mL) of each of the following solvents and in the exact order listed. Allow the liquid level to drain just below the top of the frit above the sorbent bed after each wash. The washes should pass through the sorbent easily by gravity. Use vacuum only if necessary.
   Wash Solvents: hexane, methylene chloride, methanol, Milli-Q water then 0.05 N HCl (Reagent solution B.6.b.).

   Close the stopcock on the vacuum processing station after the final wash.

2. Transfer the extract from Step I.5. using a Pasteur pipet into the cartridge tube.

3. Rinse the 25-mL flask with 2 x 1 mL of Milli-Q water, using the Pasteur pipet to rinse the walls of the flask several times with each 1-mL wash. Transfer each rinse into the cartridge tube containing the extract.

4. Open the stopcock on the vacuum processing station and allow the mixture to pass through the sorbent bed by gravity. Close the stopcock when the liquid level passes just below the top of the frit above the sorbent bed. Discard the eluate.

   NOTE: Vacuum may be used to pull the sample through the sorbent, if necessary, at a rate of approximately 1 drop every 1-2 seconds.

5. Wash the cartridge with 1 column volume of Milli-Q water followed by 1 column volume of hexane at the rate of approximately 1 drop per second. Vacuum will need to be applied to pull the hexane through following the water wash. Close the stopcock when the hexane wash passes just below the top of the frit above the sorbent bed.

6. Remove the cartridge and open the stopcock to empty the remaining hexane from the stopcock needle. Apply vacuum to the processing station and wash out the stopcock needle with methanol to remove any water droplets.

7. Replace the cartridge onto the stopcock on the vacuum processing station. Using vacuum, elute the cartridge directly into a clean 25-mL pear-shaped flask with 3 column volumes of methylene chloride, at a rate of approximately 1 to 2 drops per second.

8. Evaporate the methylene chloride to dryness using a rotary evaporator (See NOTE), being careful not to let the sample bump while under vacuum. The vacuum setting on the

Vacobox should be started at approximately 500 mbar. The methylene chloride will evaporate easily at this pressure and in a heated waterbath. Increase the vacuum pressure to evaporate the final drops in the flask. If there are any water droplets in the flask after evaporation of the methylene chloride, a small amount (approximately 1 mL) of acetonitrile UV may be added to help evaporate the water.

NOTE: When processing multiple samples, the methylene chloride may be evaporated under a stream of dry nitrogen with the samples (in vials) placed in a heated waterbath. A heated waterbath must be used when using nitrogen for evaporation to help avoid the condensation of water droplets on the inside of the vial as the methylene chloride is blown to dryness. The presence of water droplets, when making the final dilution of the sample in methanol for injection, can cause problems in the GC column.

9. Redissolve the residue in 1 mL of methanol and sonicate for approximately 30 seconds. Transfer a 0.5-mL aliquot of the sample into a clean 25-mL pear-shaped flask. Stopper the evaporation flask containing the remaining sample and save this sample in case a dilution needs to be made.

10. Add 50 mcL TMAH (Reagent B.5.) to the sample aliquot in the clean 25-mL pear-shaped flask. Evaporate the sample and TMAH to dryness using a rotary evaporator. Redissolve the sample in 0.5 mL of methanol.

11. Transfer the sample, by pipet, into a labelled autoinjector vial for the GC/MS analysis. At this point, the samples and GC standards may be kept for injection until the next day if necessary.

NOTE: If a dilution of the remaining sample needs to be made, take a 250 mcL aliquot of that sample and add an amount of TMAH so that, following evaporation, the final diluted sample will have the equivalent of 50 mcL TMAH for every 0.5 mL of methanol.

K. GC/ MS Analysis

1. Using the parameters detailed in Section E, a 100-pg on-column injection of CL 243,997 (1 mcL of 0.1 mcg/mL, Standard GC Solution D.3.c.) should give a background-subtracted mass spectrum similar to that shown in Figure 1, page 11. Determine the mass centroid of the ion at m/z 289^- and set the mass spectrometer for selected ion monitoring of this ion with a ±0.2 dalton scan window and dwell time of 500 msec/ion (0.5 sec/scan).

2. Inject 1-mcL aliquots of the working standard (0.01 mcg/mL) until a reasonably constant response is obtained (Figure 2, page 12).

3. Establish the linearity of the CL 243,997 response following the procedure described in Section F.

4. An injection of the working standard is to be made after at least every two sample injections.

5. The variation in peak response between bracketing standards must not exceed 15%. If the variation exceeds 15%, instrumental parameters should be adjusted to restore instrument performance. If such adjustments are made, inject duplicate aliquots of the working standard to determine the new response values of the standard. Then, the samples bracketed when this >15% variation occurred must be reinjected.

L. Calculations

For each sample calculation, use the sample peak area and the average peak area of the external 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 area of sample

R(STD) = Average peak area 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 (2 mL)

V3 = Final volume of sample solution for GC/MS analysis in milliliters (1.0 mL)

V4 = Volume of sample solution injected in microliters (1 mcL)

V5 = Volume of working standard solution injected in microliters (1 mcL)

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

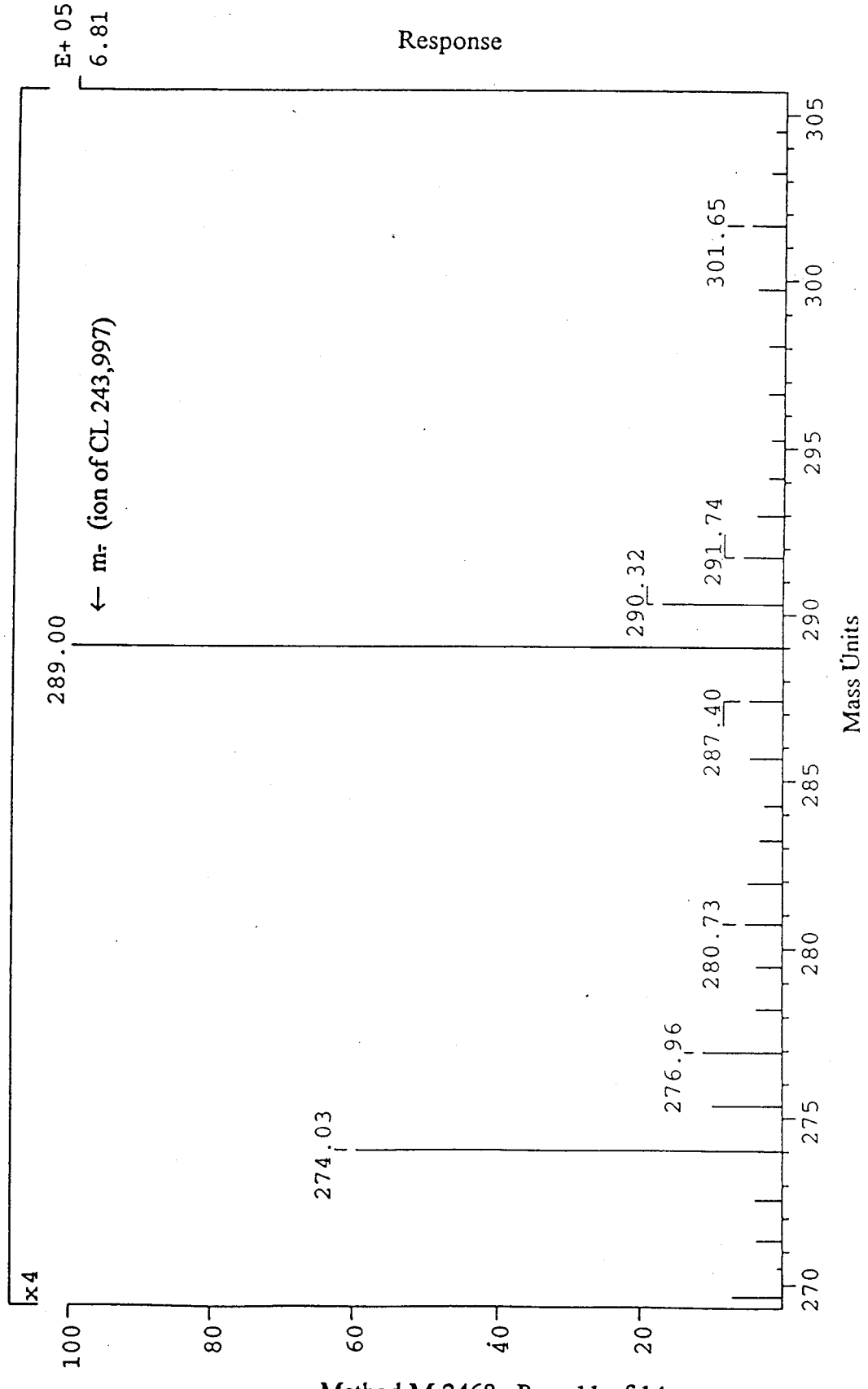
Typical chromatograms are shown in Figures 1-4.

APPROVALS:

Gerald L. Picard 7/10/95
Gerald L. Picard Date
Group Leader

jay shahn fletcher 7/10/95
J. Shahn. Fletcher Date
Author

Figure 1: Typical full scan mass spectrum from the injection of 100 pg of CL 243,997 standard.



Page 31

Figure 2: Typical mass chromatogram from the injection of 10 pg of CL 243,997 working standard.

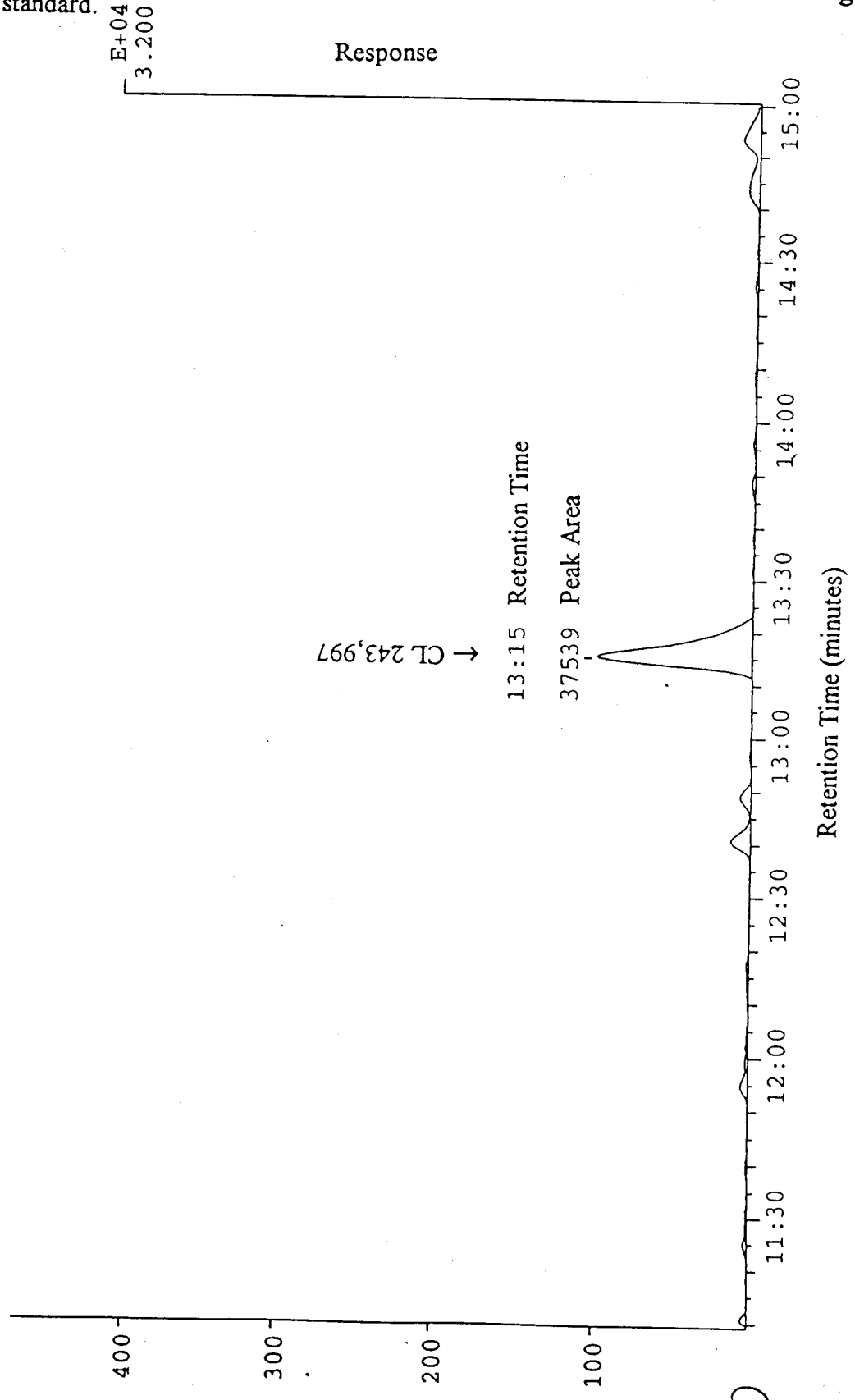


Figure 3: Typical mass chromatogram from the injection of control corn fodder, 0.2 mg equivalents of sample injected, <6.0 ppb apparent CL 243,997 found.

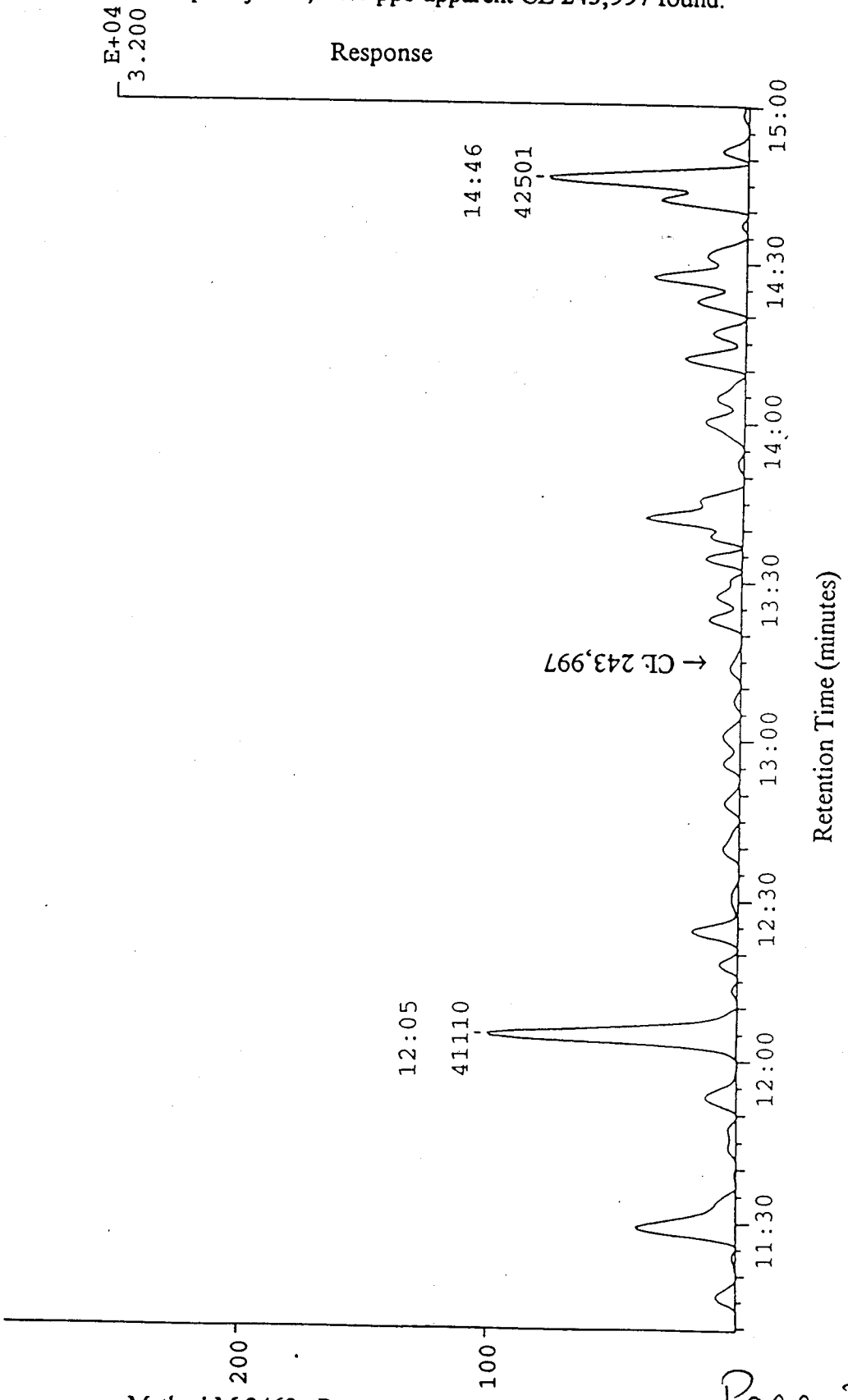
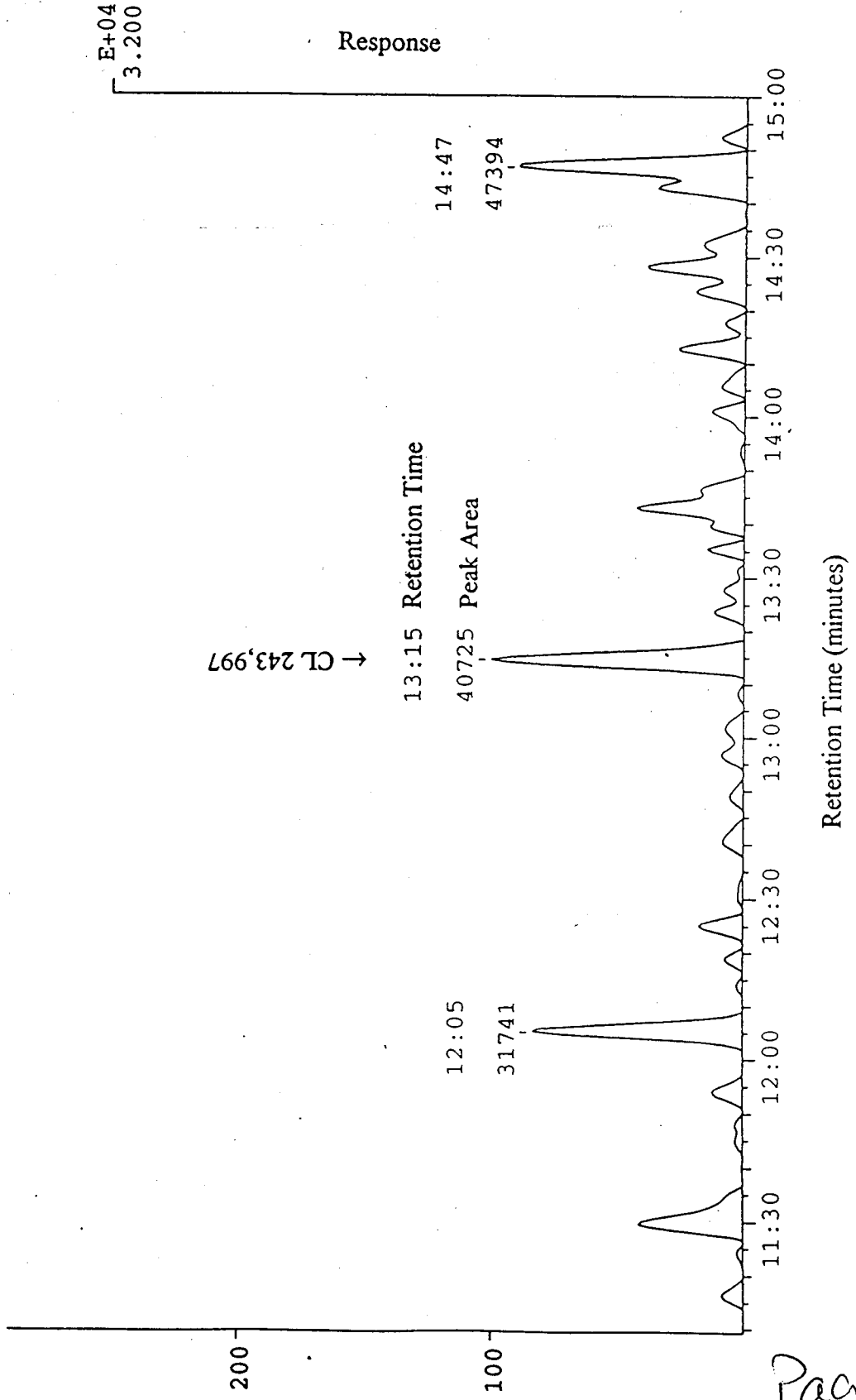


Figure 4: Typical mass chromatogram from the injection of corn fodder fortified at 50 ppb, 0.2 mg equivalents of sample injected, 56.8 ppb (114%) CL 243,997 recovered.



Page 34