M 2124
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Approved By:

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Date

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

**Recommended Method of Analysis**

Imazaquin (CL 252,214): HPLC Method for the Determination of CL 252,214 Residues in Corn Grain, Whole Corn Plant, and Corn Fodder

## A. Principle

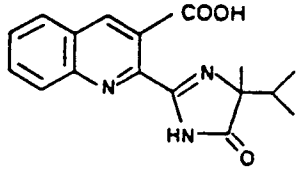
Residues of CL 252,214 are extracted from finely chopped corn tissues with an acidic aqueous methanol solution. After filtration, the extract is partitioned with methylene chloride. The methylene chloride is evaporated; the residue is then dissolved in acetonitrile and washed three times with hexane. The acetonitrile is evaporated and the residue is dissolved in methanol-water. Cleanup is achieved by a combination of solid phase extraction (SPE) cartridges. Quantitation of CL 252,214 residues is accomplished by high pressure liquid chromatography (HPLC) utilizing UV detection at 240 nm. Results are calculated by the direct comparison of peak heights to those of external CL 252,214 standards. The validated sensitivity of the method is 0.05 ppm for each substrate.

## B. Reagents

Items from manufacturers other than those listed may be used provided that they have been shown to be functionally equivalent.

1. **Analytical Standard**: Obtained from American Cyanamid Company, Agricultural Research Division, P.O. Box 400, Princeton, New Jersey 08543-0400.

   CL 252,214: [3-quinolinecarboxylic acid, 2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl-].



2. **Water, Deionized:** Millipore's Milli-Q water. Use deionized water for all steps.

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

   a. Methylene Chloride
   b. Acetonitrile (UV Grade)
   c. Hexane
   d. Methanol

4. **Solutions:**

   a. Mobile Phase: Add 500 mL of methanol (B.3.d) to 500 mL of Milli-Q water (B.2) and mix well. Add 4 mL of phosphoric acid (by pipette) and mix well. Filter, under gentle vacuum, through a Rainin Nylon-66 (0.45μm) filter or equivalent.

   b. Extraction Solvent (20% Water in Methanol): Add 200 mL of Milli-Q water (B.2) to a volumetric flask and dilute to the mark with methanol (B.3.d).

   c. Phosphate Buffer: Dissolve 6.8 g of potassium phosphate monobasic and 7.1 g of potassium phosphate dibasic in 900 mL of Milli-Q water (B.2) in a 1L volumetric flask. Dilute to the mark with Milli-Q water (B.2) and mix well. Adjust pH to 9.5 using an aqueous solution of sodium hydroxide and a pH meter.

   d. 1N Hydrochloric Acid: Dilute 83 mL of concentrated hydrochloric acid to 1L of Milli-Q water (B.2). Add the acid in small portions to the water, and mix well.

   e. 20% Methanol in Phosphate Buffer: Dilute 20 mL of methanol (B.2) to 100 mL with phosphate buffer solution (B.4.c).

   f. 50% Methanol in Water: Add 500 mL of Milli-Q water (B.2) to 1000 mL volumetric and dilute to the mark with methanol (B.3.d).

C. Apparatus

1. **HPLC:**

   Items from manufacturers other than those listed may be used provided that they have been shown to be functionally equivalent.

Applied Biosystems Model 400 equipped with:

a. Pump: Applied Biosystems Model 400 or equivalent.

b. Detector: Applied Biosystems model 783A UV detector.

c. Sample Loop: 100 mcL capacity.

d. Injector: Applied Biosystems Model 491.

e. Column: Supelco, LC-8-DB (octyldimethylsilyl, deactivated for basic compounds); 15 cm x 4.6 mm ID [Catalog Number 5-8347].

f. HPLC Guard Column: Supelco, Supelguard Kit with 2 cm x 4.6 mm Supelcosil LC-8-DB cartridge [Catalog Number 5-9553].

2. **Integrator:** Spectra-Physics Model 4290 recording integrator.

3. **Balance:** Analytical, Mettler or equivalent; precision ± 0.05 mg.

4. **Balance:** Pan, Sartorius, Model L610 or equivalent; precision ± 0.05 mg.

5. **Assorted Glassware:** General laboratory.

6. **Flasks:** 24/40 ȣ 100, 250, 500, and 1,000 mL round bottom.

7. **Filtering Funnels:** Glass powder funnels.

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

9. **Flash Evaporator:** Buchler Instruments Model PF-10DN or equivalent equipped with heated water bath (approximately 35°C) in which evaporator flasks can be partially submerged.

10. **Glass Wool:** PYREX fiber glass sliver 8 micron.

11. **Plastic Syringe, Disposable:** Luer-Lok, 10 and 30 mL capacity; Becton Dickinson.

12. **Solid Phase Extraction (SPE) Cartridges:**

a. Analytichem Benzensulfonic Acid (SCX) Bond-Elut Cartridge (500 mg) [Catalog Number 617406-1225-6011].

b. Analytichem C-18 Bond-Elut Cartridge (500 mg) [Catalog Number 607306/1210-2028].

13. **Vac-Elut Processing Station or Equivalent:** Analytichem, International [Catalog Number AI 6000].

14. **Separatory Funnels:** Squibb-type with Teflon stopcocks, 250 and 500 mL capacity; Kontes Glass Company.

15. **Bond Elut Adapters:** Analytichem International [Catalog Number 636001]

16. **Reciprocating Shaker**

17. **pH Meter:** Orion Research Model 501 or equivalent.

D. Preparation of Standard Solutions

1. **Stock Solution:**

(Prepare monthly, store in amber bottles in refrigerator, clearly labelled).

Weigh accurately a known amount (approximately 50 mg) of CL 252,214 into a 100 mL volumetric flask. Dilute to the mark with methanol. Calculate and record the exact concentration of CL 252,214. Label appropriately (approximately 0.5 mg CL 252,214/mL).

2. **Standard Fortification Solutions:**

a. Pipet into a single 100 mL volumetric flask an appropriate amount of the stock solution to deliver 1,000 mcg of CL 252,214. Dilute to the mark with Milli-Q water and mix well. This solution contains 10.0 mcg CL 252,214/mL.

b. Pipet 10.0 mL of the 10.0 mcg/mL CL 252,214 fortification standard into a 100 mL volumetric flask. Dilute to the mark with Milli-Q water and mix well. This solution contains 1.0 mcg CL 252,214/mL.

3. **Standard HPLC Solutions:**

Pipet 10.0, 5.0, and 2.5 mL aliquots of the 1.0 mcg CL 252,214/mL (Standard Fortification Solution D.2.b) into 100 mL volumetric flasks. Dilute to the mark with 20% methanol in phosphate buffer (B.4.e.) and mix well. These solutions contain 0.10, 0.05 and 0.025 mcg CL 252,214/mL and are to be used for the linearity check.

NOTES:

1. The 0.05 mcg CL 252,214/mL chromatographic standard solution is prepared each day and used as the analytical working HPLC standard for quantitation of CL 252,214 residues.

2. Freshly prepared standard solutions should be compared to the existing standard solutions. If an existing standard solution is not available, a comparison is to be made against an identically prepared solution from a separate weighing of CL 252,214 (B.1.).

E. HPLC Conditions

1. **Instrument:** Applied Biosystems Model 400.

2. **Column:** Supelco LC-8-DB; 15 cm x 4.6 mm with a 2 cm x 4.6 mm Supelcosil LC-8-DB guard column.

3. **Instrument Conditions:**

| | |
|---|---|
| a. Flow Rate | 1.0 mL/minute |
| b. Wavelength | 240 nm |
| c. Range (detector) | 0.001 AFS |
| d. Loop Injector | 100 mcL |
| e. Mobile Phase | Water: Methanol: Phosphoric Acid (500:500:4) |
| f. Integrator | 0.5 cm/minute chart speed, 10mv |

4. **Sensitivity:** Attenuation on the recording integrator is set so that 5.0 ng of CL 252,214 (100 mcL x 0.05 mcg CL 252,214/mL) gives a peak height of approximately 50% full scale deflection (FSD).

5. **Retention Time:** Approximately four (4) minutes.

F. Linearity Check

The HPLC should be checked daily for linearity of response as well as when a new column or instrument is used.

1. Adjust the HPLC conditions to attain a peak height of approximately 50% full-scale deflection for a 5.0 ng injection of CL 252, 214 (100 mcL x 0.05 mcg CL 252,214/mL). The HPLC response can be stabilized with several injections of sample extracts.

2. Inject 100 mcL aliquots of solutions prepared in Section D.3.

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

G. Sample Preparation

1. Chill a Hobart food chopper (Model 84181D or equivalent) by placing pulverized dry ice in the bowl and running for several minutes.

2. Remove the sample from the freezer and, if necessary, chop the corn grain, whole corn plant, or fodder sample into smaller pieces with a large knife or hatchet.

3. Add the sample in small increments to the dry ice in the bowl and grind the entire sample. Additional dry ice should be added as necessary to keep the samples frozen as well as to aid in the grinding/chopping process.

4. Mix the ground, frozen sample well with a large spatula or spoon to obtain a thoroughly homogenous mix.

5. Allow the samples to stand in a freezer overnight in order for the dry ice to sublime completely.

6. All samples are to remain frozen until analysis.

H. Recovery Test

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

1. Weigh a 20 g subsample of the appropriate control into a 1 quart wide-mouth glass bottle.

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 either I.1.b or I.2.b depending on the substrate.

I. Extraction

1. Corn Grain and Whole Corn Plant

a. Weigh 20 g of finely ground corn grain or whole corn plant sample into a 1 quart wide-mouth glass bottle.

b. Add 200 mL of extraction solvent (B.4.b) to the sample and 2 mL of 1N hydrochloric acid solution (B.4.d). Cap the bottle tightly and shake on a horizontal reciprocating shaker for thirty (30) minutes at high speed.

c. Proceed as in step I.2.d.

2. Corn Fodder

a. Weigh 20 g of finely ground corn fodder into a one quart wide-mouth glass bottle.

b. Add 80 mL of Milli-Q water and allow the mixture to stand for thirty (30) minutes.

c. Add 320 mL of methanol and 4 mL of 1N hydrochloric acid solution. Cap the bottle tightly and shake on a horizontal reciprocating shaker for thirty (30) minutes at high speed.

d. Remove the extracted sample from the shaker and filter the extracted sample, with the aid of vacuum, through a 600 mL Buchner funnel fitted with glass-fiber filter paper.

e. Rinse the bottle and sample with 100 mL of extraction solvent.

f. Transfer the filtered sample extract to a 500 mL volumetric flask, dilute to the mark with extraction solvent, and mix well.

J. Partitioning

1. a. For corn grain and whole corn plants, transfer a 100 mL aliquot of the sample extract to a 500 mL separatory funnel; add 50 mL of Milli-Q water followed by 200 mL of methylene chloride to the separatory funnel. Stopper and shake for one (1) minute; proceed with step J.2.

b. For corn fodder, transfer a 100 mL aliquot of the sample extract to a 500 mL separatory funnel; add 80 mL of Milli-Q water followed by 250 mL of methylene chloride to the separatory funnel. Stopper and shake for one (1) minute; proceed with step J.2..

2. Allow the phases to separate and draw off the lower methylene chloride layer into a 1000 mL round bottom flask.

3. Partition the aqueous layer twice more with 100 mL portions of fresh methylene chloride, shaking for one (1) minute each time.

4. Each time, allow the layers to separate and draw the lower methylene chloride layer into the 1000 mL round bottom flask combining all organic phases.

5. Evaporate the methylene chloride just to dryness using a rotary flash evaporator with a water bath set at approximately 35°C.

K. Hexane-Acetonitrile Partitioning

1. Dissolve the residue remaining in the 1000 mL round bottom flask (step J.5) in 5 mL of methanol, add 50 mL of hexane and transfer to a 250 mL separatory funnel.

2. Rinse the flask with 100 mL of acetonitrile; transfer this to the separatory funnel. Stopper and shake for 30 seconds.

3. Allow the layers to separate and draw off the lower acetonitrile layer back into the flask. Discard the hexane layer.

4. Partition the acetonitrile layer with two additional 50 mL portions of fresh hexane, shaking for 30 seconds each time.

5. Each time, allow the layers to separate and draw off the lower acetonitrile layer into a 250 mL round bottom flask, discarding all hexane washes.

6. Evaporate the acetonitrile to dryness on a rotary flash evaporator with a water bath set at approximately 35°C.

7. Dissolve the residue in 25 mL of methanol and 25 mL of Milli-Q water.

L. Solid Phase Extraction

1. Prepare a C-18 cartridge by washing it with 3 mL of methanol followed by 3 mL of Milli-Q water.

2. Prepare a SCX cartridge by washing the cartridge sequentially with 3 mL of hexane, 3 mL of methanol, and 3 mL of Milli-Q water.

3. With the aid of an adapter (C.15) connect the C-18 cartridge (step L.1) to the top of the SCX cartridge (step L.2) and fit a disposable syringe to the top of the C-18 cartridge

4. Vacuum filter the sample (from step K.7) through the tandem cartridge system at a rate of approximately two drops per second, discarding the eluate. Wash the flask and cartridge system with 10 mL of 50% methanol in water.

5. Disconnect the C-18 cartridge from the SCX cartridge and elute the CL 252,214 residue from the SCX cartridge with 10 mL of 20% methanol in phosphate buffer solution (B.4.e) directly into a scintillation vial. Mix well.

6. Proceed with HPLC Analysis (Section M).

M. HPLC Analysis

1. After obtaining the proper chromatography (including the linearity check) and response, inject in sequence a 100 mcL aliquot of the CL 252,214 working standard (0.05 mcg CL 252,214/mL) 100 mcL aliquots of two samples followed by another 100 mcL of the working standard.

2. If a sample peak goes off scale, dilute an aliquot of the sample solution to an appropriate volume with the phosphate buffer solution (B.4.e) and reinject. The dilution factor (DF) is then included in the calculations (Section N).

3. Use average peak height of the standards bracketing the samples for quantitation.

N. Calculations

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

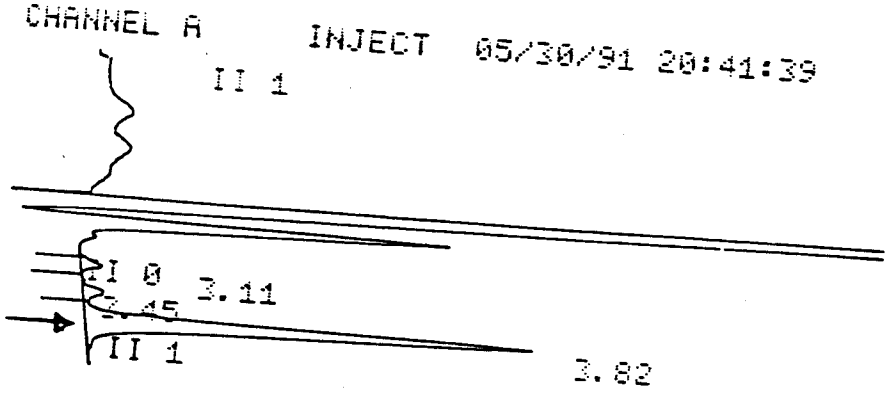
$$ppm = \frac{R(SAMP) \times V1 \times V3 \times C(STD) \times V5 \times DF}{R(STD) \times W \times V2 \times V4}$$

Where:

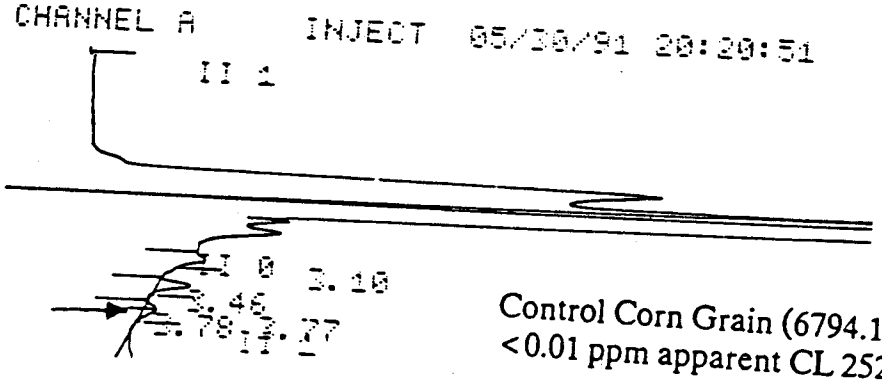
| | | |
|---|---|---|
| R(SAMP) | = | Peak height of sample. |
| R(STD) | = | Average peak height of standards. |
| W | = | Weight of sample taken for analysis, in grams. |
| V1 | = | Volume of extraction solvent added to sample, in milliliters. |
| V2 | = | Aliquot of extract taken for analysis, in milliliters. |
| V3 | = | Volume of phosphate buffer solution (B.4.e) used to elute the final residue, in milliliters. |
| V4 | = | Volume of sample solution injected, in microliters. |
| V5 | = | Volume of working standard solution injected, in microliters. |
| C(STD) | = | Concentration of working standard solution, in micrograms per milliliter. |
| DF | = | Dilution factor, if necessary. |

Typical chromatograms for the analysis of CL 252,214 residues in corn grain, whole corn plant, and corn fodder are shown in Figures 1, 2, and 3, respectively.

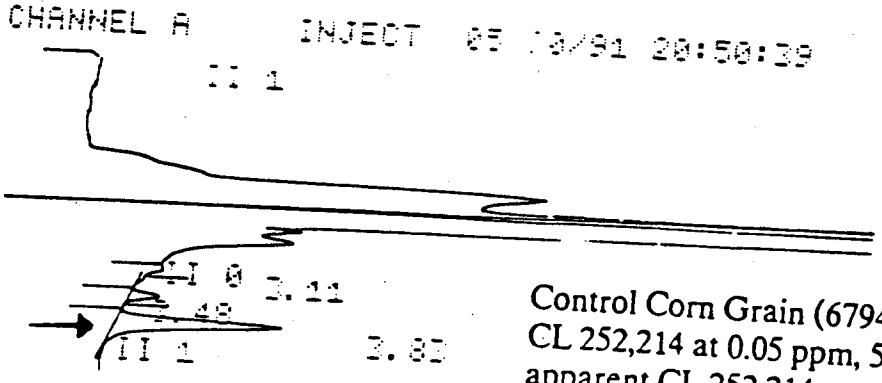
Figure 1: Typical Chromatograms for the Determination of CL 252,214 Residues in Corn Grain




CL 252,214 Standard; 5 ng injected.

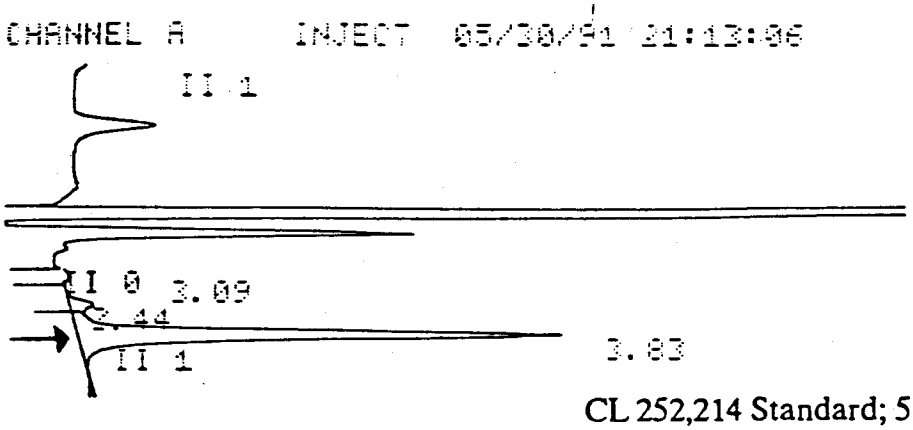



Control Corn Grain (6794.15B), 50 mg injected; <0.01 ppm apparent CL 252,214 found.

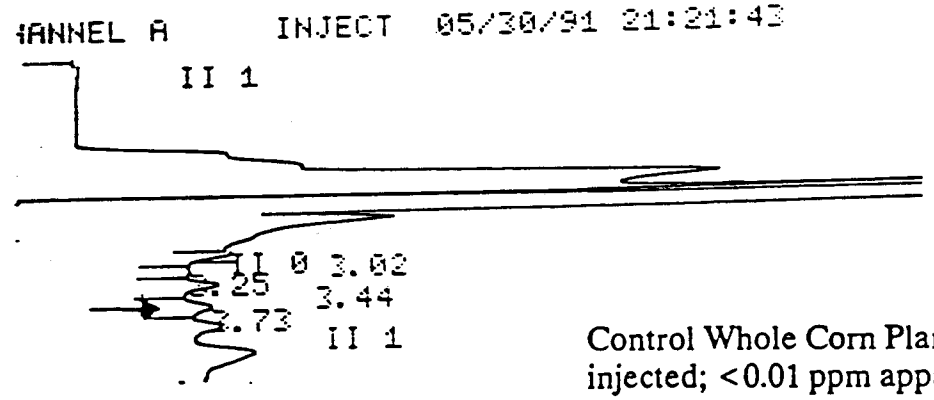



Control Corn Grain (6794.15C), fortified with CL 252,214 at 0.05 ppm, 50 mg injected; 97% apparent CL 252,214 recovered.

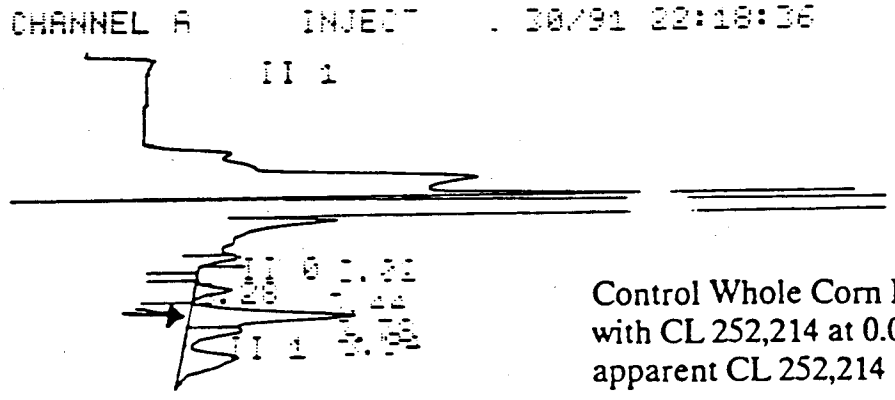
**Figure 2:** Typical Chromatograms for the Determination of CL 252,214 Residues in Whole Corn Plants




CL 252,214 Standard; 5 ng injected.

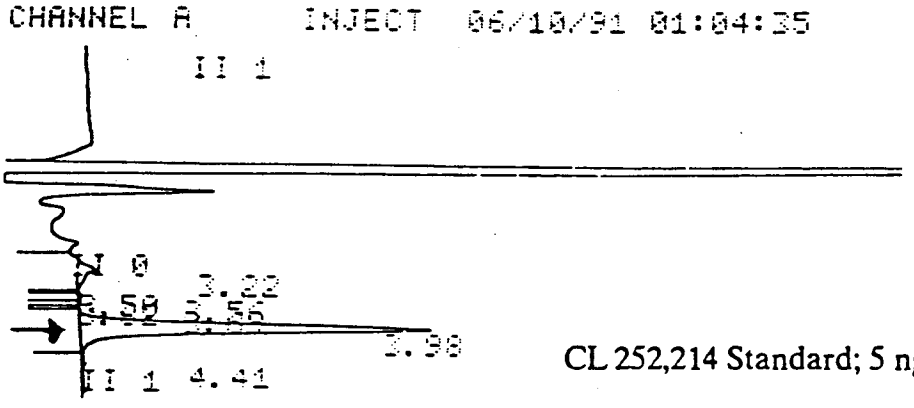



Control Whole Corn Plant (5421.148B), 50 mg injected; <0.01 ppm apparent CL 252,214 found.

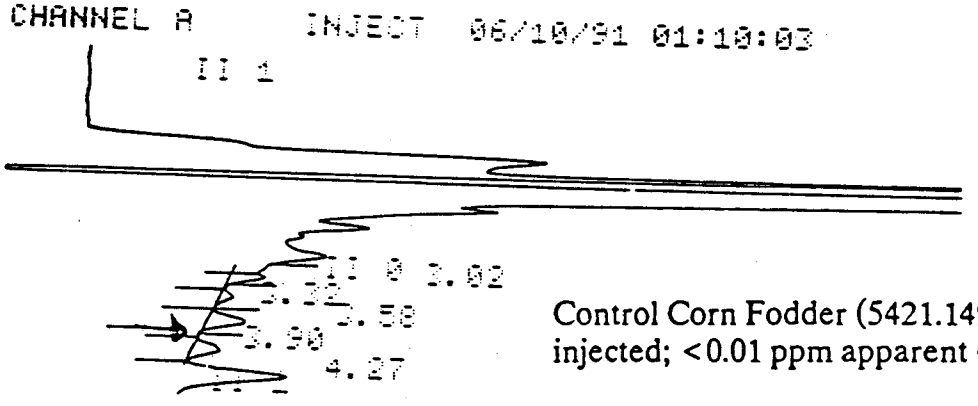



Control Whole Corn Plant (5421.148C), fortified with CL 252,214 at 0.05 ppm, 50 mg injected; 85% apparent CL 252,214 recovered.

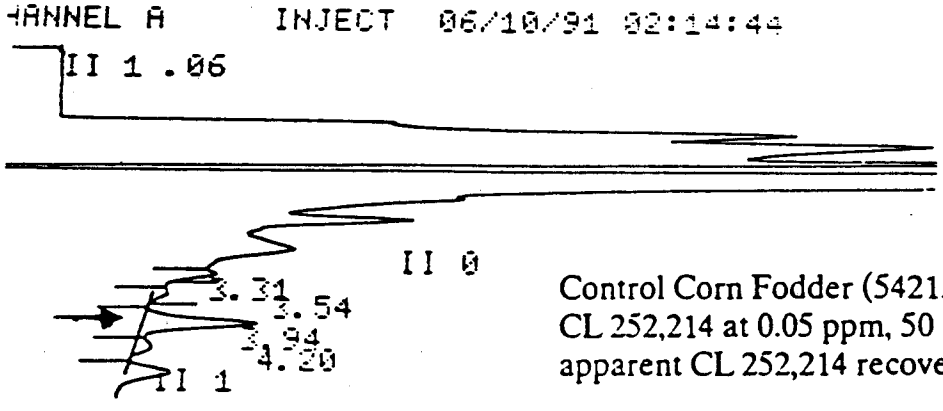
Figure 3: Typical Chromatograms for the Determination of CL 252,214 Residues in Corn Fodder




CL 252,214 Standard; 5 ng injected.




Control Corn Fodder (5421.149B), 50 mg injected; <0.01 ppm apparent CL 252,214 found.




Control Corn Fodder (5421.149D), fortified with CL 252,214 at 0.05 ppm, 50 mg injected; 93% apparent CL 252,214 recovered.