APPENDIX A

American Cyanamid Method M 2248.01

AMERICAN CYANAMID COMPANY
AGRICULTURAL RESEARCH DIVISION
HUMAN AND ENVIRONMENTAL SAFETY
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PRINCETON, NEW JERSEY 08543-0400

Recommended Method of Analysis - M 2248.01

CL 299,263: HPLC Method for the Determination of CL 299,263 Residues in Soybean Seeds

A. Principle:

Residues of CL 299,263 are extracted from soybean seeds with acidic aqueous methanol. The sample extract is concentrated to remove the methanol and the CL 299,263 is partitioned into dichloromethane from this concentrated aqueous acid extract. The combined dichloromethane phases are evaporated and reconstituted in ethyl acetate in preparation for gel permeation chromatographic (GPC) column cleanup. The CL 299,263 eluate is passed through a solid phase extraction (SPE) strong cation exchange (SCX) cartridge cleanup. The CL 299,263 residue isolated from this step is determined by high performance liquid chromatography (HPLC) on a C8 reverse phase column with a UV detector operating at 254 nm. Residues of CL 299,263 in the sample are calculated by comparison of the peak response of the peak of interest with CL 299,263 analytical standard. The validated level of sensitivity (LOQ) of this method is 0.05 ppm CL 299,263.

B. Apparatus:

This is a list of suggested apparatus and supplies. Items from other suppliers, that have been shown to be functionally equivalent, may be substituted.

1. Liquid Chromatograph: Kratos Spectroflow Model 400 Pump, capable of isocratic operation up to 2 mL/minute.

2. Detector: Kratos Model 783 UV Spectrophotometer, set at 254 nanometers.

NOTE: This method supersedes M 2248 and is procedurally identical to M 2248.

3. HPLC Column: Supelcosil LC-8-DB, 5 micron, 4.6 mm x 150 mm, deactivated for bases (Supelco Catalog Number: 5-8347M).

4. Guard Column: Supelcosil LC-8-DB cartridge (Supelcoguard, Supelco Catalog Number: 5-9563).

5. Column Oven for HPLC: Spark Holland, SpH 99.

6. HPLC Injector: Rheodyne Model 7125 injection valve fitted with a 200-mcL loop.

7. Integrator: SP4400 Chromjet recording integrator, Spectra-Physics, Fremont, CA.

8. pH Meter: Jenco Microcomputer pH Vision 6071.

9. Vacuum Filtration Apparatus: A 500-mL suction flask fitted with a 600-mL Buchner porcelain funnel by means of a rubber adapter.

10. Filter Paper: Whatman Number 1, 9-cm diameter (Catalog Number: 1001-090).

11. Evaporation Flasks: Round bottom, 200-mL, 500-mL capacity.

12. General Laboratory Glassware: Assorted.

13. Rotary Evaporator: Buchler Instruments Model RE 121, equipped with a water bath maintained between 35° and 40°C.

14. Ultrasonic Extractor: Brinkmann Instruments POLYTRON® Model PT3000 Homogenizer, equipped with a PT-DA 3012/2S generator.

15. Gel Permeation Chromatography (GPC) Unit: ABC Laboratories GPC Autoprep Model 1002B, equipped with a UV detector and a Linear Instruments flat bed recorder.

    NOTE: If the ABC GPC Autoprep is not available, a manually operated system can also be used.

16. GPC Column: 2.5 cm I.D. x 60 cm glass column (ABC Laboratories Catalog Number: 624-110).

17. Millex-SR Filter: 0.5 μM (Millipore Catalog Number: SLSR025NB).

18. Solid Phase Extraction (SPE) Vacuum Manifold. Visiprep (Supelco Catalog Number: 5-7030M).

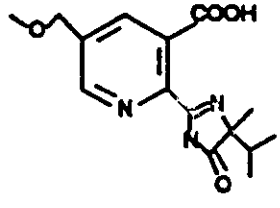
19. Strong Cationic Exchange (SCX) Cartridge: 1000 mg, Varian Mega Bond-Elut Aromatic Sulfonic Acid (Varian Catalog Number: 1225-6011).

C. Reagents:

This is a list of suggested reagents. Items from other manufacturers that have been shown to be functionally equivalent to those listed, may be substituted.

1. Analytical Standard: CL 299,263, analytical grade of known purity. American Cyanamid Company, Agricultural Research Division, P. O. Box 400, Princeton, New Jersey 08543-0400.

   CL 299,263:
   2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl)-nicotinic acid.



2. Solvents:

   B & J Brand High Purity Solvents; Baxter, Burdick and Jackson, Muskegon, Michigan.

   a. Dichloromethane (Catalog Number: 300-4).

   b. Ethyl Acetate (Catalog Number: 100-4).

   c. Methanol (Catalog Number: 230-4).

   d. Acetonitrile (Catalog Number: 015-4).

3. High Purity Deionized Water: Millipore S, Milli-Q water.

4. Chemicals:

   a. Formic acid, 96% A.C.S. Reagent Grade; Aldrich Catalog Number 25,136-4.

b. BAKER ANALYZED® Reagents; J. T. Baker Company:

1) Hydrochloric acid, concentrated, 36.5-38.0% purity (Catalog Number: 9535-01).

2) Potassium chloride, 99.3% purity (Catalog Number: 3040-01)

5. Solutions:

a. 1N HCl: Dilute 8.3 mL of concentrated HCl to exactly 100 mL with high purity deionized water in a stoppered graduated cylinder; mix thoroughly.

b. Water, pH 2.5: Adjust the pH of high purity deionized water to exactly 2.5 using 1N HCl and a pH meter.

c. 50% Methanol in pH 2.5 Water: Combine equal volumes of methanol and pH 2.5 water in an appropriate sized bottle; mix thoroughly.

d. Saturated Potassium Chloride (KCl) in Methanol: Add 50 grams of KCl to 1 liter of methanol; stir for 30 minutes with magnetic stirrer.

e. Extraction Solvent: Combine 40 mL of 1N HCl, 1560 mL high purity deionized water and 2400 mL methanol in a 4-L solvent bottle; mix thoroughly.

f. HPLC Mobile Phase: Combine 200 mL of acetonitrile, 10 mL formic acid and 790 mL high purity deionized water in a 1-L volumetric flask; mix thoroughly.

6. Bio-Beads: S-X3 (200/400 mesh); Bio-Rad Catalog Number 152-2750.

D. Preparation of Standard Solutions:

All standard solutions are stable for at least seven weeks stored in amber glass under refrigeration (<4°C).

1. Stock Standard Solution:

Accurately weigh 10.0 mg (corrected for purity) of analytical standard grade CL 299,263 into a small beaker. Dissolve the compound in a small volume of high purity deionized water. Quantitatively transfer the solution to a 10-mL volumetric flask: dilute to the mark with water. If necessary, sonicate the solution until all of the CL 299,263 dissolves. This solution contains 1000 mcg CL 299,263/mL and is labeled Standard Solution A.

2. Intermediate Standard Solution:

Pipet into a 100-mL volumetric flask a volume of the Standard Solution A to deliver 1000 mcg of CL 299,263. Dilute to the mark with high purity deionized water; mix thoroughly. This solution contains 10 mcg CL 299,263/mL and is labeled Standard Solution B.

3. Chromatographic Standard Solutions:

a. Pipet a 1-mL aliquot of Standard Solution B into a 10-mL volumetric flask. Dilute to the mark with water; mix thoroughly. This solution contains 1.0 mcg CL 299,263/mL and is labeled Standard Solution C.

b. Pipet a 5-mL aliquot of Standard Solution C into a 10-mL volumetric flask. Dilute to the mark with water; mix thoroughly. This solution contains 0.50 mcg CL 299,263/mL and is labeled Standard Solution D.

c. Pipet a 5-mL aliquot of Standard Solution D into a 10-mL volumetric flask. Dilute to the mark with water; mix thoroughly. This solution contains 0.25 mcg CL 299,263/mL and is labeled Standard Solution E.

d. Pipet a 5-mL aliquot of Standard Solution E into a 10-mL volumetric flask. Dilute to the mark with water; mix thoroughly. This solution contains 0.125 mcg CL 299,263/mL and is labeled Standard Solution F.

e. Pipet a 5-mL aliquot of Standard Solution F into a 10-mL volumetric flask. Dilute to the mark with water; mix thoroughly. This solution contains 0.0625 mcg CL 299,263/mL and is labeled Standard Solution G.

4. Fortification Standards:

Standard Solutions C through G are also to be utilized for the fortification of control samples used in concurrent recoveries. The concentration of any fortification solution should be such that no less than a 0.5-mL and no more than a 3.0-mL aliquot be added to the untreated control sample to yield the desired fortification level.

E. Packing and Calibration of the Gel Permeation Chromatography (GPC) Column:

1. Packing:

a. Weigh 50 grams of Bio-Beads S-X3 into a 250-mL Erlenmeyer flask, add 200 mL ethyl acetate, cover and soak overnight.

NOTE: **Only S-X3 Bio-Beads from Bio-Rad should be used. It has been shown that comparable beads from other suppliers have yielded unacceptable results.**

b. Set up the GPC column for upward flow (sample injector valve at the bottom end) at a rate of about 5 mL/minute.

c. Pour the Bio-Beads/ethyl acetate slurry into the column and drain the solvent; do not allow the bed to go dry at any time. Insert the upper piston into the column and adjust it so that its frit is just touching the top of the bed.

d. Pump ethyl acetate continuously through the column for approximately twenty-four (24) hours to ensure a firm bed. Solvent may be recycled during this operation.

e. If necessary, carefully adjust the lower piston so that its frit is in contact with the lower end of the bed. This step should be repeated as often as necessary to ensure that there are no gaps between the bed and the frits at both ends.

2. Calibration:

a. Transfer 25 mcL (25 mcg CL 299,263) of Standard Solution A (1000 mcg CL 299,263/mL) into a 50-mL round bottom flask. Evaporate the solvent just to dryness.

b. Dissolve the residue in 10 mL of ethyl acetate and mix well.

c. Load the ethyl acetate containing CL 299,263 in position number 1 of the GPC Autoprep Model 1002B.

d. Set dump time to 0 minutes, collect time to 30 minutes and wash time to 0 minutes.

e. Connect the column outlet to the UV detector.

f. Set the UV detector range to 0.05, rise time to 1 second, the recorder to 10 mV and chart speed to 0.5 cm/minute.

g. The CL 299,263 peak will begin to emerge at approximately 13 minutes and will take about 6 minutes to totally emerge from the column.

h. Set the dump time to end 1 minute before the peak begins to emerge. Set the collect time to end 1 minute after the peak has totally emerged.

NOTE: **Quantitative recovery of CL 299,263 analytical standard alone, without the presence of soybean matrix, is not to be expected from these calibration steps.**

Consistent chromatographic performance of the GPC column is to be established [symmetrical CL 299,263 peak shape, consistent peak response and consistent retention times] prior to any routine soybean seed residue analyses. Consistent performance is usually attained following three (3) consecutive calibrations of the GPC column performed within a two-hour period.

F. High Performance Liquid Chromatographic (HPLC) Conditions:

Operating conditions described below are provided for use as a guide in establishing actual operating conditions and should be adjusted only as necessary to obtain peak shape, peak response and resolution from background peaks that is equivalent to or better than the chromatograms shown in Figure 1.

1. Instrument:

a.	HPLC Pump:	Kratos Spectroflow Model 400
b.	Detector:	Kratos Model 783
c.	Integrator:	Spectra Physics Model SP 4400 Chromjet recording integrator
d.	Injector:	Rheodyne Model 7125; 200 mcL Loop
e.	Column Oven:	Spark Holland Model SpH 99

2. Column: Supelcosil LC-8-DB, 5 micron, 4.6 mm I.D. x 150 mm length and a Supelco guard column fitted with a Supelcosil LC-8-DB cartridge.

3. Instrumental Conditions:

a.	Mobile Phase (Isocratic)	Acetonitrile/Water/Formic Acid (200/790/10; v/v/v)
b.	Flow Rate	1.0 mL/minute
c.	Column Temperature	40°C controlled with column heater
d.	Injection Volume	200 mcL, autoinjector or manual loop injector
e.	Detector Wavelength	254 nm
f.	Integrator Parameters	
	Chart Speed	0.5 cm/minute
	Detector Output	10 mV FSD
	Attenuation	256
	Peak Width	6
	Peak Threshold	12
g.	Retention Time	Approximately 6 minutes

Instrument sensitivity should be established such that a 200 mcL injection of Standard Solution F (0.125 mcg CL 299,263/mL) yield a peak response of approximately 20 mm.

G. Linearity Check:

The HPLC must be checked for linearity of response at least once for each related group of analyses. Linearity must also be confirmed following any change of column, modification of the instrument or significant alteration of chromatographic conditions.

Establish the HPLC conditions described in Section F and obtain a stable peak response of between 30% and 40% full scale deflection (FSD) for a 50-ng injection of Standard Solution E [200 mcL 0.250 mcg CL 299,263/mL].

1. Inject and chromatograph 200 mcL of each of the Chromatographic Standard Solutions (Standard Solutions D, E, F and G) containing 0.50 mcg CL 299,263/mL, 0.25 mcg CL 299,263/mL, 0.125 mcg CL 299,263/mL and 0.0625 mcg CL 299,263/mL, respectively, each day analyses are conducted.

2. Manually measure the peak height for each standard chromatogram or use the integrated peak response provided by a data system or integrator. Calculate the response ratio for each standard injected by dividing the peak response (height or area) of the standard by the mass (nanograms) injected. Calculate the average response ratio. Significant departure from linearity as indicated by deviation of any response factor from the average response factor greater than 15% indicates instrumental difficulties or faulty standard preparation, which must be corrected before proceeding with the analysis.

H. Recovery Test:

The validity and performance of this procedure must always be demonstrated by the analyst before the analysis of unknown samples is attempted through the use of recovery tests. At least one fortified control sample must be processed with each daily set of samples analyzed. The fortification levels chosen for a study should include the validated sensitivity of the method (0.05 ppm CL 299,263) and should bracket the sample concentration range expected or found.

1. Weigh a 20-gram subsample of an untreated soybean seed control sample into a 250-mL Erlenmeyer flask. Pipet an appropriate volume of the Fortification Standard Solution that is required for the fortification level to be tested. A practical guide is provided in the following table:

Fortification Level (ppm)	Standard Solution	Standard Solution Concentration	Fortification Solution Volume
0.05	C	1.0 mcg CL 299,263/mL	1.0 mL
0.10	C	1.0 mcg CL 299,263/mL	2.0 mL
0.50	B	10.0 mcg CL 299,263/mL	1.0 mL

Fortification of control soybean seed samples is accomplished prior to the addition of extraction solvent (Section J.2).

I. Sample Preparation:

Soybean samples are to be prepared according to American Cyanamid Company Standard Operating Procedure MREE.R.0507.

J. Extraction:

1. Weigh a 20-gram subsample of the soybean sample into a 250-mL Erlenmeyer flask.

2. Add 150 mL of the extraction solvent [1N HCl: high purity deionized water: methanol (1: 39: 60, v/v/v)]; blend the mixture with a POLYTRON® ultrasonic extractor for 5 minutes.

3. Place a Whatman Number 1 filter paper in a Buchner funnel and wash with 50 mL of the extraction solvent. After discarding this wash, filter the soybean extract into a 500-mL side arm vacuum flask with the aid of vacuum. Wash the filter cake with an additional 100 mL of the extraction solvent.

4. Quantitatively transfer the combined filtrate to a 250-mL stoppered graduated cylinder. Adjust the volume to 250 mL with extracting solvent. Stopper the cylinder and shake to mix the contents thoroughly.

5. Transfer a 125-mL aliquot (equivalent to 10 grams of sample) to a 500-mL round bottom flask; concentrate this solution to approximately 50 mL using a rotary flash evaporator with a water bath at approximately 35°C.

   NOTE: Apply vacuum gradually to prevent excessive bumping; complete evaporation of the methanol is required and may take as long as 2 hours.

6. Adjust the pH of the concentrated aqueous extract to 2.5 with 1N HCl using a pH meter (approximately 2.2 mL of 1N HCl will be required).

7. Quantitatively transfer the acidified aqueous extract from the 500-mL round bottom flask to a 250-mL separatory funnel.

8. Partition this acidified aqueous extract with 50 mL dichloromethane; shaking gently for 20 seconds (this is accomplished by tumbling up and down by hand 20 times).

9. Drain the lower, dichloromethane layer into a clean 500-mL round bottom flask.

10. Partition the remaining aqueous phase three more times with 50 mL of dichloromethane each time. Transfer each dichloromethane layer to the same 500-mL round bottom flask in Step J.9.

11. Evaporate the combined dichloromethane phases just to dryness using a rotary flash evaporator with a water bath at approximately 35°C; dissolve the residue remaining in the round bottom flask in 10 mL of ethyl acetate.

12. Proceed with the Gel Permeation Chromatographic (GPC) cleanup step described in Section K.

K. Sample Cleanup:

1. Gel Permeation Chromatography (GPC):

Program the Autoprep 1002B GPC system (based on the calibration criteria described in Section E.2) to elute the samples sequentially through the S-X3 Bio-Bead® column. Typical operating conditions for the system are as follows:

Column: 2.5 mm I.D. x 600 mm glass column, packed with 50 grams of S-X3 Bio-Beads (200/400 mesh) compressed to a bed length of 260 mm with the plunger assembly.

Solvent System: ethyl acetate

Flow Rate: 5.5 mL/minute

Dump Time: 13 minutes

Collect Time: 6 minutes (CL 299,263 fraction)

Wash Time: 10 minutes

After calibrating the GPC, filter each sample through a Millipore filter (Millex®-SR for organic solvent) into the sample introduction tube of the instrument using a 10-mL syringe.

NOTE: Wash the filter with 5 mL ethyl acetate prior to use. The sample loop system retains 5 mL of each sample extract (equivalent to 5 grams of sample) and discards the remainder automatically.

After loading all the samples into the sample introduction tube, initiate the run and collect the sample eluate in individual 250-mL round bottom flasks. Evaporate the ethyl acetate just to dryness using a rotary flash evaporator with a water bath at approximately 35°C. Proceed with the solid phase extraction SCX cartridge cleanup (Section K.2).

2. Solid Phase Extraction (SPE) Strong Cation Exchange (SCX) Cartridge Cleanup:

NOTE: Use SPE vacuum manifold system to aspirate the samples at approximately 2 drops every 4 seconds.

a. Sequentially wash an SCX cartridge with 5 mL methanol and 5 mL high purity deionized water.

b. Dissolve the residue remaining from the evaporation of the GPC eluate in 10 mL of 50% methanol in pH 2.5 water; load this solution onto the SCX cartridge.

c. Wash the SCX cartridge with 5 mL methanol.

d. Elute CL 299,263 with 30 mL of saturated potassium chloride (KCl) in methanol [this solution should be stirred for approximately 10 minutes prior to use] into a 100-mL pear-shaped flask; evaporate the eluate just to dryness using a rotary flash evaporator with a water bath at approximately 35°C.

e. Add 20 mL of pH 2.5 water to the residue remaining in the pear shaped flask.

f. Quantitatively transfer the aqueous residue to a 125-mL separatory funnel.

g. Partition the aqueous phase with 20 mL of dichloromethane, shaking vigorously for 20 seconds.

h. Drain the lower, dichloromethane layer into a clean 200-mL round bottom flask.

i. Partition the remaining aqueous phase three more times with 20 mL of dichloromethane each time. Transfer each dichloromethane layer to the same 200-mL round bottom flask in Step K.2.h.

j. Evaporate the combined dichloromethane phases just to dryness using a rotary flash evaporator with a water bath at approximately 35°C; dissolve the residue remaining in the round bottom flask in 2 mL of high purity deionized water.

NOTE: Sonicate the sample for a few minutes to ensure that all residue adhering to the flask is dissolved before HPLC analysis.

k. Proceed with the High Performance Liquid Chromatographic (HPLC) Analysis described in Section L.

L. <u>High Performance Liquid Chromatographic (HPLC) Analysis:</u>

1. Set up the HPLC according to the conditions listed in Section F; obtain a satisfactory baseline and chromatographic response (between 30% and 40% FSD) for a 50 ng injection of Standard Solution E (200 mcL x 0.25 mcg CL 299,263/mL). Chromatographic conditions may only be adjusted slightly to obtain equivalent or better peak shape and response than the chromatograms shown in Figure 1.

2. Inject 200 mcL of each of the Chromatographic Standard Solutions (Standard Solutions D, E, F and G) containing 0.50 mcg CL 299,263/mL, 0.25 mcg CL 299,263/mL, 0.125 mcg CL 299,263/mL and 0 0625 mcg CL 299,263/mL for the linearity check as described in Section G.

3. Once stable response and linearity have been established, chromatographic analysis can begin. A recommended injection sequence is as follows: 200 mcL aliquots of the working standard (0.125 mcg CL 299,263/mL) bracketing 200 mcL aliquots of two soybean seed samples. Allow sufficient time for late-eluting peaks to clear between each injection.

4. Identify the CL 299,263 peak in each chromatogram and measure the peak response. Calculate the apparent CL 299,263 residue using the formula described in Section M (Calculations).

NOTE: If the peak response of any sample exceeds the peak response for the highest on-scale HPLC Chromatographic Standard Solution, that sample must be diluted and reinjected.

M. Calculations:

Calculate the apparent CL 299,263 residue in the injected samples (ppm CL 299,263) from the sample peak response and the average peak response for the working standard immediately preceding and immediately following that sample as follows:

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

Where:

R(SAMP) = Sample Response (chromatographic response of the sample peak of interest; in mm or integrator units).

R(STD) = Average Standard Response (average chromatographic response for the peak of interest in the working standard chromatograms before and after the sample chromatograms; in mm or integrator units).

C(STD) = Concentration of working standard solution injected, in micrograms per milliliter (0.125 mcg CL 299,263/mL).

V1 = Volume of the extraction solvent used, in milliliters (250 mL).

V2 = Aliquot of extract taken for analysis, in milliliters (62.5 mL).

V3 = Volume of solution used to dissolve the final residues for HPLC analysis, in milliliters (2.0 mL).

V4 = Volume of sample solution injected for HPLC analysis, in microliters (200 mcL).

V5 = Volume of working standard solution injected for HPLC analysis, in microliters (200 mcL).

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

DF = Dilution Factor.

NOTE: Values in parentheses are nominal values if the procedure is carried out exactly as described.

Typical chromatograms for the determination of CL 299,263 residues in soybean seeds are shown in Figure 1.

N. Analytical Notes for Method M 2248.01:

1. Do not allow any CL 299,263 residue to sit overnight without the presence of solvent.

2. If necessary, the composition of the mobile phase may be modified slightly to improve separation by varying the amount of acetonitrile.

3. Since CL 299,263 is not readily soluble in water, the stock solution should be sonicated in an ultrasonic bath for at least 30 minutes before use for further dilution.

4. Sonication should be utilized when residue is being re-constituted in water or organic solvents.

APPROVALS:

Sigrid Witkonton — 2/23/94

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Author — Date

W. E. Horton
Group Leader
Residue Chemistry II — February 23, 1994
Date

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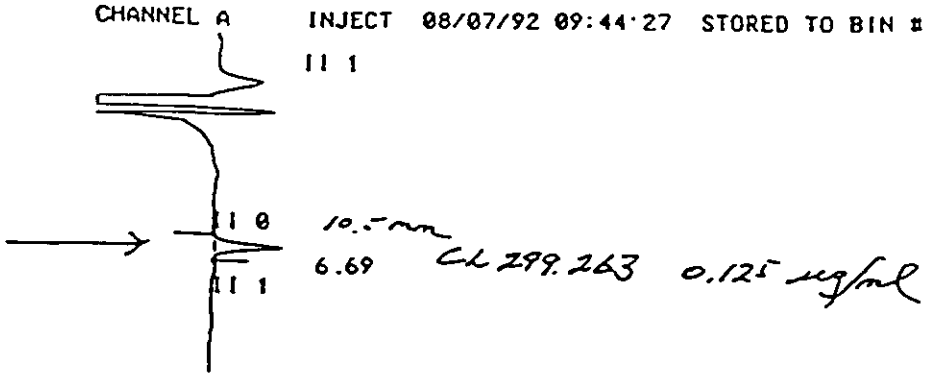
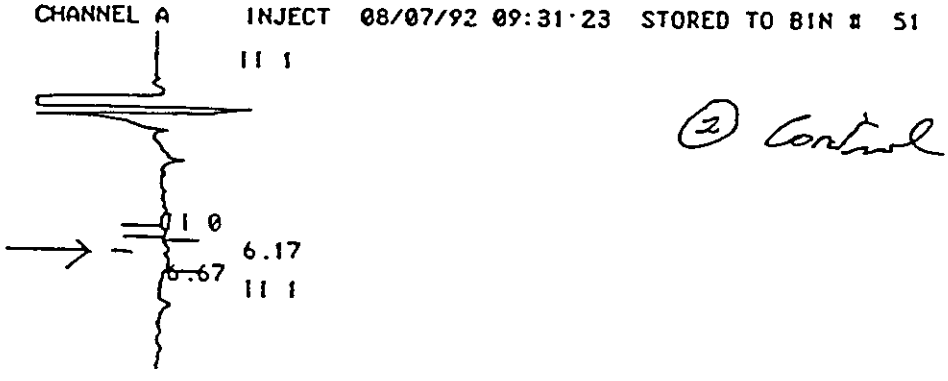
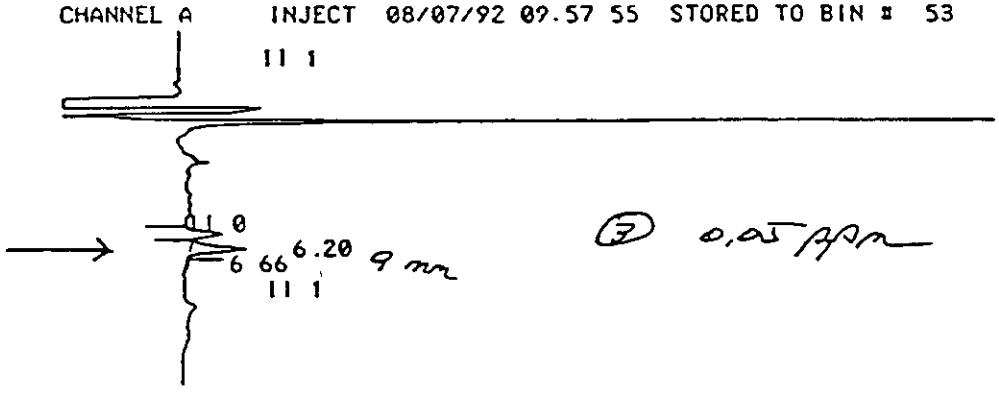
Figure 1: Typical HPLC Chromatograms from the Determination of CL 299,263 Residues in Soybean Seeds Using American Cyanamid Company Method M 2248.01



CL 299,263 Chromatographic Standard Solution F, 25 ng CL 299,263 injected [200 mcL x 0.125 mcg CL 299,263/mL].



Control Soybean Seed (AC 6794.36), 500 mg injected [200 mcL x (5 g/2 mL)], <0.008 ppm apparent CL 299,263 residue found.



Control Soybean Seed (AC 6794.36) fortified at 0.05 ppm CL 299,263, 500 mg injected [200 mcL x (5 g/2 mL)], 0.043 ppm CL 299,263 residue found, 86% recovery.