M-1586
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09/25/85

AMERICAN CYANAMID COMPANY
AGRICULTURAL RESEARCH DIVISION
CHEMICAL DEVELOPMENT
P. O. Box 400
Princeton, New Jersey 08540

Recommended Method of Analysis

CL 263,499 Herbicide: GC Method for the Determination of CL 263,499 Residues in Soybean Plant, Seed and Straw

A. Principle

CL 263,499 residues are extracted from soybean plant, seed and straw using acidic aqueous methanol. After filtration, the extract is pH-adjusted and partitioned with methylene chloride. The methylene chloride is partitioned with pH 9 buffer. After adjustment to acidic pH, the aqueous buffer is partitioned with methylene chloride. The methylene chloride is evaporated and the residue dissolved in methanol-water. The methanol-water solution is passed through a disposable micro ion exchange column and CL 263,499 eluted with dilute hydrochloric acid solution. The acidic aqueous solution is then partitioned with methylene chloride, the methylene chloride evaporated and the residue dissolved in a measured amount of methanol. The CL 263,499 residue is quantified using trimethylanilinium hydroxide, an on-column methylating agent, and gas chromatography. A 3% OV-17 column and a thermionic nitrogen-selective detector are used to measure residues versus an external standard. The validated sensitivity of the method is 0.1 ppm for each soybean commodity.

B. Reagents

1. Analytical Standard: CL 263,499 [2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-5-ethyl-3-pyridine carboxylic acid] obtained from American Cyanamid Company, Agricultural Research Division, P. O. Box 400, Princeton, New Jersey 08540.

2. Solvents, Specially Purified: "Distilled in Glass", Burdick and Jackson Laboratories, Incorporated, or equivalent.

   a. Methylene Chloride
   b. Methanol
   c. Acetone

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

   a. Potassium Phosphate, monobasic, crystal
   b. Sodium Phosphate, dibasic, anhydrous powder
   c. Hydrochloric acid, concentrated
   d. Sodium Hydroxide, 50% w/w solution

4. TMAH Methylation Reagent: Trimethylanilinium hydroxide, 0.2M in methanol, Cat. No. 680400, Regis Chemical Company.

5. Solutions:

   a. 1M Hydrochloric Acid: Add 84 ml of concentrated HCl to a 1-liter volumetric flask containing 500 ml of distilled water and dilute to the mark with distilled water.

   b. Extraction Solvent, 1% 1M Hydrochloric Acid, 39% Water in Methanol: Mix 40 ml of 1% 1M HCl with 1560 ml of distilled water and add to 2400 ml of methanol.

   c. 0.05M Hydrochloric Acid: Add 4.2 ml of concentrated HCl to a 1-liter flask containing 500 ml of distilled water and dilute to the mark with distilled water.

   d. 25% w/w Sodium Hydroxide: Mix 50 ml of a 50% w/w sodium hydroxide solution with 50 ml of distilled water.

   e. pH 9.0 Buffer: Dissolve 13.6 g of potassium phosphate, monobasic and 14.1 g of sodium phosphate, dibasic in 4 liters of distilled water. Using a pH meter and 25% sodium hydroxide solution, adjust the buffer solution to pH 9.0.

6. GC Column Material: 3% OV-17 on 100-120 mesh Supelcoport, Cat. No. 1-1754, Supelco, Incorporated.

C. Apparatus

1. Balance, Analytical: Mettler, weighing to nearest 10 mcg.

2. Balance, Toploading Pan: Sartorius, weighing to nearest 0.01 g.

3. Assorted Glassware: General laboratory.

4. Sorvall Omni-Mixer and Mason Jars: One-quart capacity, DuPont Instruments, Newton, Connecticut.

5. Rotary Flash Evaporator: Buchler Instruments, Model PF-10DN or equivalent equipped with a heated water bath maintained at 35°C in which the evaporation flask can be partially submerged.

6. Filtering Flasks: 500-ml capacity, Corning Glass Works.

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

8. Filter Paper: Glass fiber, 11-cm diameter, Whatman, Incorporated.

9. pH Meter: Orion Research, Model 701A or equivalent.

10. Separatory Funnels: Squibb type with Teflon stopcocks, 1000-ml, 500-ml, 250-ml capacity, Kontes Glass Company.

11. Plastic Syringe: Becton-Dickinson, 30-cc, Disposable, Luer-lok tip.

12. GC Syringe: Hamilton, Model 701-N, 10-mcl capacity.

13. Gas Chromatograph: Tracor, Model 550 equipped with a Model 702 thermionic nitrogen-phosphorus detector.

14. GC Column: 182-cm x 2-mm I.D., glass, packed with 3% OV-17 on 100-120 mesh Supelcoport. The column was packed using a slight vacuum. Silylated glass-wool plugs were placed at each end. The column was conditioned overnight at 275°C with a carrier gas flow of 30 ml/minute.

15. Solid Phase Extraction Column: J. T. Baker Chemical Company, 3-ml quaternary amine ion-exchange, Cat. No. 7091-3.

D. Preparation of Standard Solutions

1. Stock Solution

Weigh accurately 10 ± 0.01 mg of CL 263,499 analytical standard into a 100-ml volumetric flask. Dilute to the mark with acetone and mix well. This is Standard Solution A and contains 100 mcg/ml.

2. Standard Fortification Solutions

a. Pipet a 20-ml aliquot of Standard Solution A into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution B and contains 20 mcg/ml.

b. Pipet a 10-ml aliquot of Standard Solution A into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution C and contains 10 mcg/ml.

c. Pipet a 10-ml aliquot of Standard Solution C into a 100-ml volumetric flask. Dilute to the mark with acetone and mix. This is Standard Solution D and contains 1 mcg/ml.

NOTE: All standard solutions for GC must contain 50 mcl of TMAH per milliliter.

E. Gas Chromatographic Conditions

1. Instrument: Tracor Model 550 gas chromatograph.

2. Detector: Model 702 thermionic nitrogen-phosphorus detector. Bead setting should be adjusted to give a peak height of approximately 30% full scale deflection (FSD) for a 5.0 ng injection of CL 263,499.

3. Column: 182 cm x 2 mm I.D., glass, packed with 3% OV-17 on 100-120 mesh Supelcoport.

4. Instrument Conditions:

a.	Column Temperature	220°C
b.	Injection Port Temperature	300°C
c.	Detector Temperature	270°C
d.	Outlet Temperature	250°C
e.	Hydrogen Flow Rate	2.5 ml/min
f.	Helium Carrier Flow Rate	35 ml/min
g.	Air Flow Rate	120 ml/min
h.	Retention Time	approximately 4 min.
i.	Electrometer	set to give a peak height of approx. 30% FSD for a 5.0 ng injection

5. Recording Integrator: Hewlett-Packard Model 3380A

a.	Chart Speed	0.5 cm/min
b.	Attenuation	set to give approx. 30% FSD for a 5.0 ng injection of CL 263,499
c.	Area Reject	1000
d.	Slope Sensitivity	1 mV/min

F. Linearity Check

1. Pipet 5-, 10-, 15- and 20-ml aliquots of Standard Solution C into separate 100-ml volumetric flasks. Dilute to volume with acetone. These solutions contain 0.5, 1.0, 1.5 and 2.0 mcg/ml, respectively, of CL 263,499.

2. Pipet 2.0-ml aliquots of each concentration into separate vials, add 100 mcl of TMAH and inject 5-mcl aliquots of each solution into the GC.

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

G. Sample Preparation

1. Pulverize sufficient dry ice in a Hobart food chopper to thoroughly chill the bowl and the blade.

2. Add the pre-frozen sample in portions of sufficiently small size 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. Allow the sample to stand in a freezer overnight for the dry ice to dissipate completely.

4. 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 set of samples analyzed.

1. Weigh a 20-g subsample of soybean control material into a quart-blendor container.

2. Add by pipet a volume of standard fortification solution appropriate to the fortification level to be tested.

3. Add the solution dropwise and mix the sample well before adding the extraction solvent.

4. Continue with the extraction and cleanup steps as described in the following sections.

I. Extraction

1. Accurately weigh 20 g of soybean material into a 1-quart Mason jar.

2. Extract sample with 200 ml of 1% 1M HCl, 39% water in methanol at medium speed for 15 minutes.

3. Vacuum filter the extract through a Buchner funnel fitted with a 11-cm glass fiber filter paper. Add 100 ml extractant to the blendor jar, replace lid and shake to thoroughly rinse the jar. Filter the rinsate.

4. Transfer the filtrate to a 500-ml beaker.

J. Methylene Chloride Partition

1. Adjust the filtrate to pH 2.0 (± 0.1) using a pH meter and 1M HCl.

2. Transfer the pH 2 solution to a 500-ml separatory funnel. Gently partition the extract with 100 ml of methylene chloride for 30 seconds. Collect the lower methylene chloride layer in a 500-ml beaker*.

3. Repeat the extraction twice more using 100 ml of methylene chloride each time, combining the methylene chloride fractions in the beaker. Discard the upper aqueous methanol phase.

K. Buffer Partition Cleanup

1. Transfer the 300 ml of methylene chloride to a 1-L separatory funnel. Add 200 ml of pH 9 buffer to the separatory funnel and gently partition the methylene chloride, shaking for 1 minute. Allow a few minutes for the phases to separate*.

2. Discard the lower methylene chloride phase. Collect the upper aqueous buffer layer in a 500-ml beaker. Adjust the buffer to pH 2.0 (± 0.1) with 1M HCl using a pH meter.

3. Transfer the pH 2 solution to a 500-ml separatory funnel and partition with 100 ml of methylene chloride for 30 seconds. Collect the lower methylene chloride layer in a 1-L round bottom flask. Repeat the partition three more times using 100 ml of methylene chloride each time, combining the methylene chloride fractions in a 1-L round bottom flask.

* The lower methylene chloride layer may emulsify at this point. The emulsion can be broken by passing the methylene chloride phase through a powder funnel filled with glass wool. Repeat the process if necessary to thoroughly deemulsify the methylene chloride. Use the separatory funnel to remove water separated from the methylene chloride.

4. Evaporate the methylene chloride just to dryness on a rotary evaporator at 35°C. Dissolve the residue in 20 ml of methanol. Quantitatively transfer the methanol to a 100-ml round bottom flask. Rinse the 1-L flask twice more with 20 ml of methanol each time, using ultrasonication to dissolve and transfer the residue.

5. Evaporate the 60 ml of methanol on a rotary evaporator just to dryness.

L. Solid Phase Extraction Column Cleanup

1. Add 1 ml of methanol to the flask, carefully dissolving the residue. Add 24 ml of distilled water to the methanol and swirl.

2. Prepare a 3-ml Baker solid phase extraction column by passing 10 ml of methanol through the column followed by 10 ml of distilled water using a 30-cc disposable plastic syringe fitted to the top of the column. Do not allow the column to go dry.

3. Pass the 25-ml aqueous methanol sample solution from step L.1 through the extraction column using the 30-cc syringe. The eluant should not exceed a rate of about 2 drops per second. Discard the eluant. Add 15 ml of 0.05M HCl to the 30-cc syringe, passing the solution through the extraction column at a rate not to exceed about 2 drops per second. Collect the eluant in a 50-ml beaker.

M. Methylene Chloride Partition

1. Add 20 ml of distilled water to the eluant and ensure that the pH is 2.0 (± 0.1) using a pH meter.

2. Transfer the pH 2 solution to a 250-ml separatory funnel. Partition the solution three times with 100 ml of methylene chloride, collecting the methylene chloride extracts in a 500-ml round bottom flask.

3. Evaporate the methylene chloride just to dryness on a rotary evaporator.

4. Dissolve the residue in 4.0 ml of methanol for analysis by GC.

N. Gas Chromatographic Analysis

1. Transfer a 2.0-ml aliquot of a 1.0 mcg/ml standard of CL 263,499 to a small vial. Evaporate the acetone with the aid of a stream of nitrogen and pipet 2.0 ml of methanol into the vial. Add 100 mcl of TMAH methylation reagent to the standard vial (all GC solutions should contain 50 mcl TMAH/ml solution).

2. Transfer a 1.0-ml aliquot of the sample solution to a small vial.

3. After obtaining a stable GC response to the standard, add 50 mcl of TMAH to the sample solution vial and inject a 5.0-mcl aliquot of the sample into the GC. After addition of TMAH, the sample should be injected within 10 minutes.

4. Compare the peak height with that obtained from a 5.0-mcl injection of a GC solution containing 1.0 mcg/ml of CL 263,499 (external standard).

5. If the sample peak goes off scale, dilute to an appropriate volume with methanol and reinject. NOTE: All final (diluted) extracts prepared for GC should contain 50 mcl of TMAH per milliliter of solution.

6. Alternate injections of standard and sample and use the average peak height of the standards bracketing a sample for calculation.

O. Calculations

For each sample calculation, use the sample peak height and the average peak height value resulting from measurement of the external standard peak height obtained before and after the sample injection 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 working standard

W = Weight of sample taken for analysis in grams

V1 = Volume of extracting solvent added to sample in ml

V2 = Aliquot of extract taken for analysis in ml

V3 = Volume of methanol added to dissolve the residue for chromatographic analysis in ml

V4 = Volume of sample solution injected in mcl

V5 = Volume of working standard solution injected in mcl

C(STD) = Concentration of working standard solution in mcg per ml

D.F. = Dilution factor

Figures M-1586.A to M-1586.C show typical chromatograms for determining CL 263,499 residues in soybean plants, straw and seed, respectively.

Figure M-1586.A: Typical Chromatograms of Fortified and Control Soybean Plant

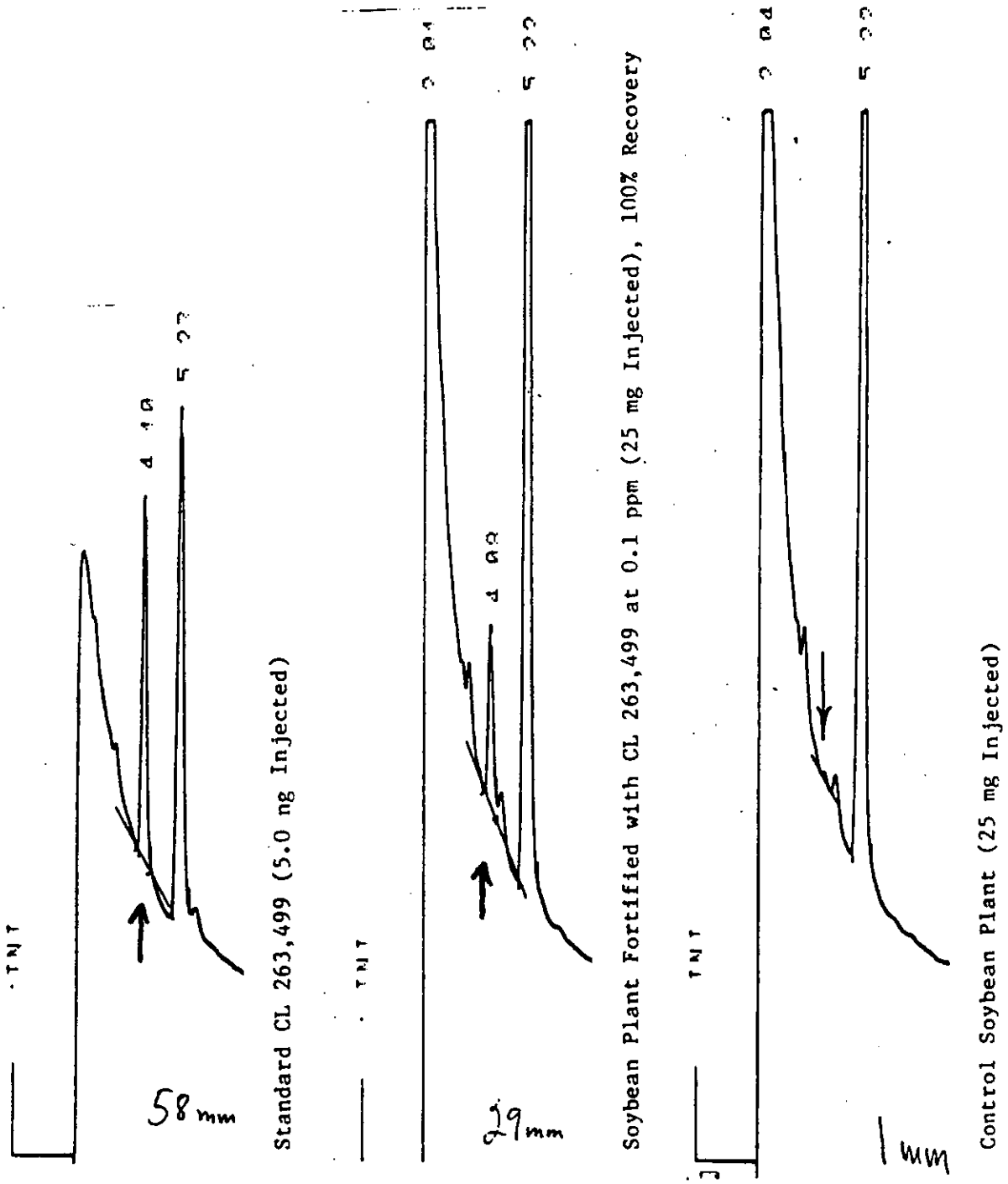

Figure M-1586.B: Typical Chromatograms of Fortified and Control Soybean Straw

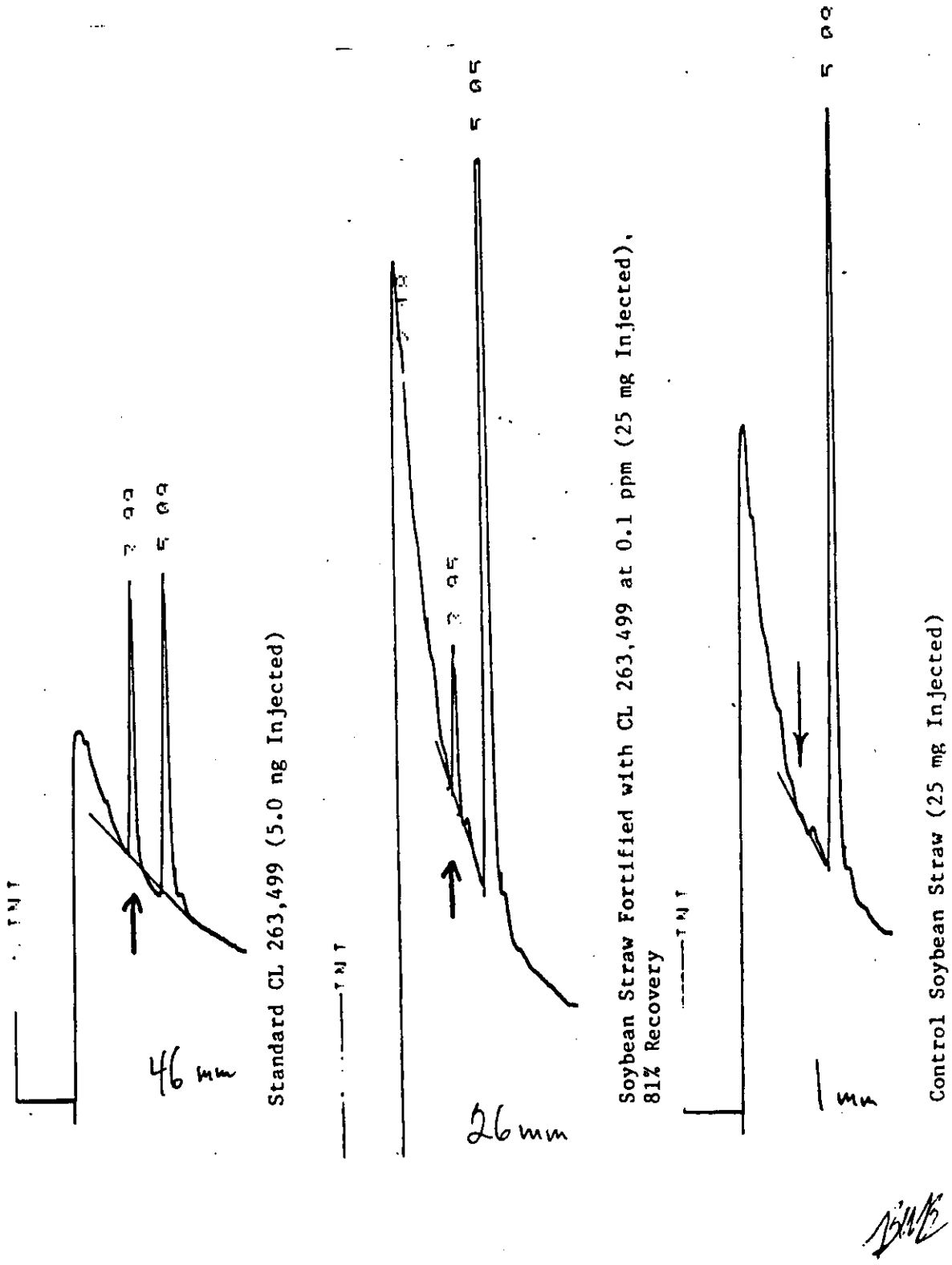

Figure M-1586.C: Typical Chromatograms of Fortified and Control Soybean Seed

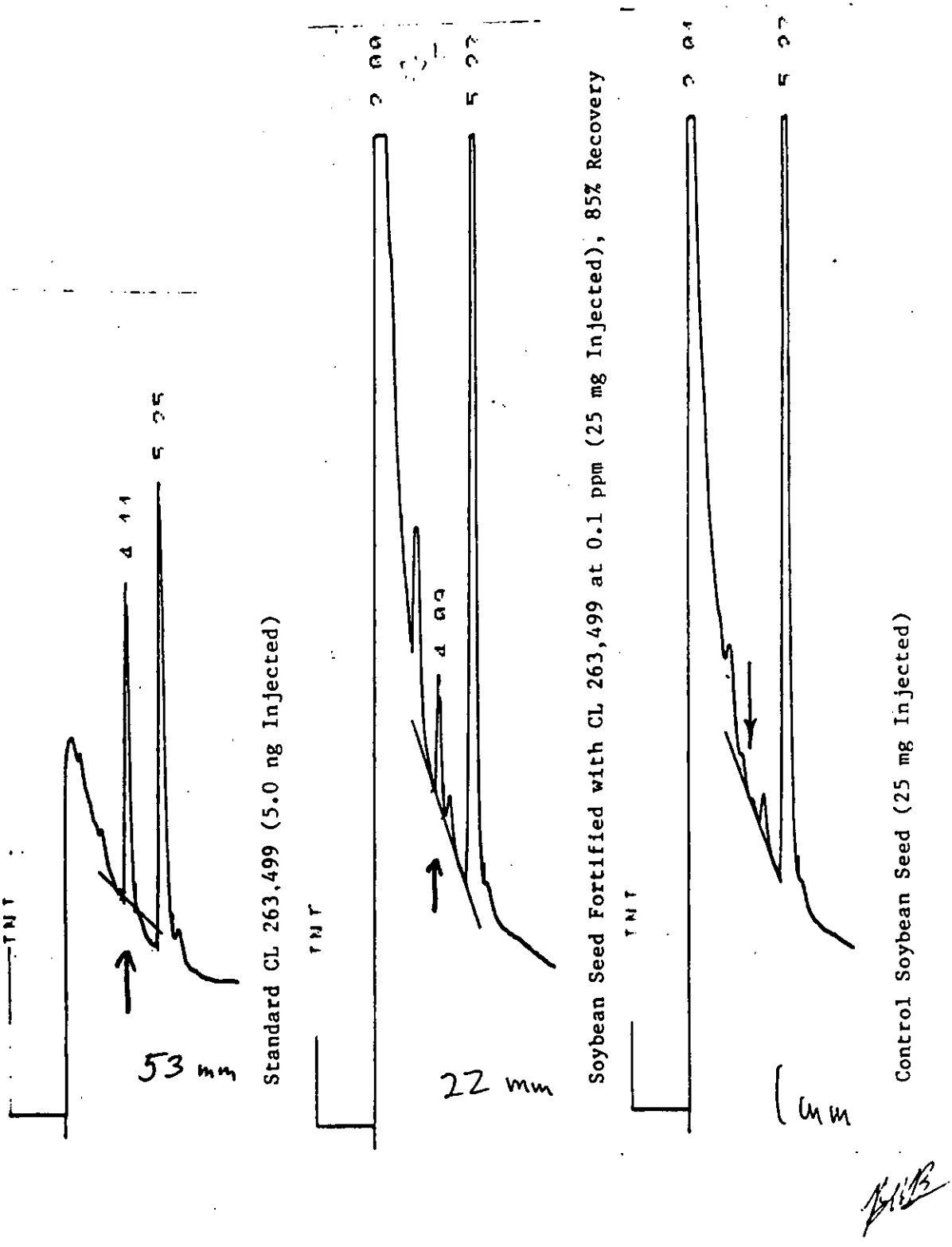

TABLE I

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: SOYBEAN PLANT
ANALYZED FOR: CL 263,499
METHOD USED: M-1586
VALIDATED SENSITIVITY: 0.10 PPM
RESPONSE MEASURED AS: PEAK HEIGHT IN MILLIMETERS
V4 = V5 = : 5.0

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RECOVERY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION					STAND. SOLN.	RESPONSES		APPARENT	
SAMPLE NUMBER	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	C(Std) mcg/ml	R(SAMP) units	R(STD) units	PPM FOUND	PERCENT RECOVERY
4920.48	0	0	0	20	300	300	4.0	1	1.00	-N.M.	58	<0.007	
4920.51	0	0	0	20	300	300	4.0	1	1.00	-N.M.	48	<0.008	
										AVERAGES		0.007	
4920.48	2.00	1.00	0.100	20	300	300	4.0	1	1.00	29	58	0.100	100.0
4920.49	2.00	1.00	0.100	20	300	300	4.0	1	1.00	28	58	0.097	96.5
										AVERAGES		0.098	98.3
4920.50	4.00	1.00	0.200	20	300	300	4.0	1	1.00	49	45	0.218	108.9
4920.50	4.00	1.00	0.200	20	300	300	4.0	1	1.00	36	43	0.167	83.7
										AVERAGES		0.192	96.3
4920.51	0.27	73.55	0.993	20	300	300	20.0	1	1.00	42	51	0.824	82.9
4920.51	0.27	73.55	0.993	20	300	300	20.0	1	1.00	34	45	0.756	76.1
										AVERAGES		0.790	79.5
4920.49	2.72	73.55	10	20	300	300	200.	1	1.00	47	57	8.246	82.4
4920.49	2.72	73.55	10	20	300	300	200.	1	1.00	45	51	8.824	88.2
										AVERAGES		8.535	85.3

N.M.= not measurable (less than 2 units), corresponding, for control samples, to the limits given above in the APPARENT-residue column. These limits are calculated by insertion of the number 2 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

Control samples are indicated by a minus sign before the R(SAMP) value.

TABLE II

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: SOYBEAN SEED
ANALYZED FOR: CL 263,499
METHOD USED: M- 1586
VALIDATED SENSITIVITY: 0.10 PPM
RESPONSE MEASURED AS: PEAK HEIGHT IN MILLIMETERS
V4 = V5 = : 5.0

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RECOVERY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION					STAND. SOLN.	RESPONSES		APPARENT	
SAMPLE NUMBER	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	C(Std) mcg/ml	R(SAMP) units	R(STD) units	PPM FOUND	PERCENT RECOVERY
4920.45	0	0	0	20	300	300	4.0	1	1.00	-N.M.	46	<0.009	
4920.44	0	0	0	20	300	300	4.0	1	1.00	-N.M.	53	<0.008	
4920.22	0	0	0	20	300	300	4.0	1	1.00	-N.M.	52	<0.008	
										AVERAGES		0.008	
4920.44	2.00	1.00	0.100	20	300	300	4.0	1	1.00	22	51	0.086	86.3
4920.44	2.00	1.00	0.100	20	300	300	4.0	1	1.00	19	48	0.079	79.2
										AVERAGES		0.082	82.8
4920.45	4.00	1.00	0.200	20	300	300	4.0	1	1.00	45	51	0.176	88.2
4920.45	4.00	1.00	0.200	20	300	300	4.0	1	1.00	43	48	0.179	89.6
										AVERAGES		0.177	88.9
4920.46	10.0	1.00	0.500	20	300	300	10.0	1	1.00	47	57	0.412	82.5
4920.46	10.0	1.00	0.500	20	300	300	10.0	1	1.00	43	56	0.384	76.8
										AVERAGES		0.398	79.7
4920.22	0.27	73.55	0.993	20	300	300	20.0	1	1.00	57	62	0.919	92.6
4920.22	0.27	73.55	0.993	20	300	300	20.0	1	1.00	46	61	0.754	75.9
										AVERAGES		0.836	84.3

N.M.= not measurable (less than 2 units), corresponding, for control samples, to the limits given above in the APPARENT-residue column. These limits are calculated by insertion of the number 2 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

Control samples are indicated by a minus sign before the R(SAMP) value.

TABLE III

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: SOYBEAN STRAW
ANALYZED FOR: CL 263,499
METHOD USED: M- 1586
VALIDATED SENSITIVITY: 0.10 PPM
RESPONSE MEASURED AS: PEAK HEIGHT IN MILLIMETERS
V4 = V5 = : 5.0

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RECOVERY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION					STAND. SOLN.	RESPONSES		APPARENT	
SAMPLE NUMBER	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	C(Std) mcg/ml	R(SAMP) units	R(STD) units	PPM FOUND	PERCENT RECOVERY
4920.41	0	0	0	20	300	300	4.0	1	1.00	-N.M.	66	<0.006	
4920.42	0	0	0	20	300	300	4.0	1	1.00	-N.M.	43	<0.009	
										AVERAGES		0.007	
4920.41	2.00	1.00	0.100	20	300	300	4.0	1	1.00	31	62	0.100	100.0
4920.42	2.00	1.00	0.100	20	300	300	4.0	1	1.00	26	63	0.083	82.5
4920.40	2.00	1.00	0.100	20	300	300	4.0	1	1.00	18	40	0.090	90.0
										AVERAGES		0.091	90.8
4920.47	4.00	1.00	0.200	20	300	300	4.0	1	1.00	48	47	0.204	102.1
4920.43	4.00	1.00	0.200	20	300	300	4.0	1	1.00	47	55	0.171	85.5
										AVERAGES		0.188	93.8
4920.47	10.0	1.00	0.500	20	300	300	10.0	1	1.00	43	47	0.457	91.5
4920.43	10.0	1.00	0.500	20	300	300	10.0	1	1.00	40	55	0.364	72.7
										AVERAGES		0.410	82.1
4920.41	0.27	73.55	0.993	20	300	300	20.0	1	1.00	53	56	0.946	95.3
4920.42	0.27	73.55	0.993	20	300	300	20.0	1	1.00	45	56	0.804	80.9
4920.40	0.27	73.55	0.993	20	300	300	20.0	1	1.00	34	41	0.829	83.5
										AVERAGES		0.860	86.6

N.M.= not measurable (less than 2 units), corresponding, for control samples, to the limits given above in the APPARENT-residue column. These limits are calculated by insertion of the number 2 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

Control samples are indicated by a minus sign before the R(SAMP) value.