BIOCHEMISTRY DEPARTMENT

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EDITION 7/15/83		
SUBMITTED BY: J. Smith, T. Boone		APPROVED BY

1.0 SCOPE

This method is used for extraction, clean-up, and final determination of cyromazine and its metabolite, melamine, in crop samples. The limit of detection for cyromazine is 0.05 ppm and for melamine is 0.04 ppm, equivalent to 0.05 ppm of cyromazine (see chemical structures in Figure 1). This method involves modifications of AG-402, used for the determination of cyromazine and melamine in crops.

2.0 PRINCIPLE

Residues of cyromazine and melamine are extracted by refluxing chopped crop samples in 10% water:methanol for two hours. An aliquot of the extract is evaporated to the aqueous, diluted with 0.1M hydrochloric acid and cleaned up by partition with dichloromethane and hexane, and by cation exchange chromatography. An additional cleanup by anion exchange chromatography is proposed for those samples where cleanup is insufficient. Cyromazine and melamine are determined by High Performance Liquid Chromatography (HPLC) on a LiChrosorb-NH_2 column using 90% acetonitrile:water as the mobile phase. The method is outlined in Figure 2.

3.0 APPARATUS

3.1 Bottles, Boston round, narrow mouth, 16-oz.

3.2 Column, Econo-Column®, polypropylene, 0.7 X 4 cm bed volume (BioRad Cat. No. 731-1110) and Econo-Column® reservoir, glass, 500-ml capacity (BioRad Cat. No. 737-9010).

3.3 Concentration tube, conical, 50-ml capacity.

3.4 Condenser, Allihn, bulb type, 30-cm jacket.

3.5 Flask, round bottom, 500-ml and 250-ml.

3.6 Flask, erlenmeyer, 250-ml.

3.7 Flask, vacuum, 1000-ml.

3.8 Food chopper, Hobart or equivalent.

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3.9 Graduated cylinder, 100-ml and 250-ml.

3.10 Heating mantle, 500-ml, Glas-Col.

3.11 Rotary evaporator, Büchi or equivalent.

3.12 Separatory Funnel, 250-ml, with Teflon stopcock.

3.13 Variable transformer.

4.0 REAGENTS

4.1 Acetic Acid 1N (Reagent Grade).

4.2 Acetonitrile (HPLC grade).

4.3 Ammonium Hydroxide (conc.)

4.4 Bio-Rex 9 Anion Exchange Resin, 50-100 mesh, BioRad. (See preparation page 5).

4.5 Dichloromethane (HPLC-Grade).

4.6 5% (v/v) Ammonium Hydroxide (conc.)/Methanol.

4.7 25% (v/v) Ammonium Hydroxide (conc.)/Methanol.

4.8 Dowex 50W-X4 Cation Exchange Resin, 50-100 mesh, BioRad. (Pre-washed and stored in distilled-deionized water).

4.9 Dowex 50W-X4 Cation Exchange Resin, 200-400 mesh, BioRad. (Pre-washed and stored in distilled-deionized water).

4.10 Methanol (HPLC grade).

4.11 Sodium Hydroxide 1N solution.

4.12 10% (v/v) Water/Acetonitrile.

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4.13 10% (v/v) Water/Methanol.

4.14 Water, ~~distilled, deionized~~. HPLC

5.0 PROCEDURE

5.1 Sample Preparation

Crop samples are chopped into small pieces in a Hobart food chopper prior to extraction.

5.2 Extraction

5.2.1 Weigh a 25-gram sample of chopped crop sample into a 500-ml round bottom flask.

5.2.2 Add 250 ml of 10% water:methanol to the flask, attach to an Allihn condenser, and reflux for two hours at a variable transformer setting of 70.

5.2.3 Cool the refluxed sample to room temperature and allow particulate matter to settle. Decant supernatant into a 16-oz. Boston round bottle.

NOTE: Extracts should not be filtered through filter paper. Some filter papers have been found to be contaminated by traces of melamine in the manufacturing process.

5.3 Partition Cleanup

5.3.1 Decant a 5-gram aliquot of the 10% water:methanol extract into a 500-ml round bottom flask and evaporate on a rotary evaporator (40°C bath) until only aqueous remains.

5.3.2 Add 100 ml of 0.1M hydrochloric acid and 50 ml of dichloromethane to the round bottom flask, stopper and shake vigorously for 1 minute.

5.3.3 Transfer the contents of the round bottom flask to a 250-ml separatory funnel and allow phase separation. Discard the lower dichloromethane phase.

5.3.4 Add 50 ml of fresh dichloromethane to the separatory funnel, shake for 1 minute, allow phase separation, and then discard the lower dichloromethane phase.

5.3.5 Add 50 ml of hexane to the separatory funnel and shake vigorously for 1 minute then allow phase separation. The lower aqueous acid solution will be loaded onto the cation exchange column in Section 5.4.

5.4 Ion Exchange Chromatography

5.4.1 Fit the Econo-Column reservoir onto the Econo-Column containing a 2-ml bed of Dowex 50W-X4 (200-400M), resin and connect to a 1-liter vacuum flask using a 1-hole rubber stopper.

5.4.2 Using slight vacuum, wash the ion exchange resin with 10 ml of distilled-deionized water.

5.4.3 Load the entire aqueous-acid solution from Section 5.3.5 containing cyromazine and melamine onto the ion exchange column. Discard the eluate.

5.4.4 Wash the ion exchange resin with 50 ml of 10% water:acetonitrile, 50 ml of 10% water:methanol, and 10 ml of methanol. Discard the eluates.

5.4.5 Remove the Econo-Column and Econo-Column reservoir from the vacuum flask and place in a well-ventilated hood.

5.4.6 Elute cyromazine and melamine from the ion-exchange resin using 20 ml of 5% NH_4OH (conc):methanol into a 50-ml graduated concentration tube (gravity flow).

5.4.7 Evaporate the eluate to dryness using a rotary evaporator.

5.4.8 Dissolve sample in methanol for HPLC injection (Section 6.0) or in 10 ml of distilled-deionized water if additional cleanup is required (anion exchange cleanup Section 5.5.2).

5.5 Anion Exchange Chromatography (Only if cleanup insufficient for final determination)

5.5.1 BioRex 9 Resin Preparation

5.5.1.1 In a large beaker, swirl about 100 grams of BioRex 9 resin, 50-100 mesh, chloride form, with about 500 ml of 1N acetic acid.

5.5.1.2 Allow the resin to settle and decant the supernatant liquid together with suspended fines.

5.5.1.3 Wash the resin with distilled water as in steps 5.5.1.1 and 5.5.1.2.

5.5.1.4 Wash the resin two times with 1N sodium hydroxide. The resin will now be a dark brown color.

5.5.1.5 Wash the resin with several portions of distilled water until the supernatant water is about neutral (pH 7-8).

5.5.1.6 Transfer a slurry of the resin into an Econo-Column sufficient to prepare a 2-ml bed.

5.5.1.7 Prewash the BioRex 9 resin with 10-ml of distilled water before use.

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5.5.2 Sample Clean-up by Anion Exchange Chromatography

5.5.2.1 Fit the BioRex 9 column, prepared in Section 5.5.1.7, into the top cap of a second econo-column containing a 2-ml bed of Dowex 50W-X4 resin (50-100 mesh).

5.5.2.2 Transfer the aqueous sample from Section 5.4.8 (10 ml) to the upper BioRex 9 cleanup column, allowing the aqueous to simultaneously drain through the lower Dowex 50-X4 column (discard aqueous).

5.5.2.3 Rinse the sample tube with 10-ml of distilled water and repeat Section 5.5.2.2.

5.5.2.4 Rinse the tube with an additional 5-ml of distilled water and repeat Section 5.5.2.2.

5.5.2.5 After the aqueous phase has eluted through the lower Dowex 50-X4 resin remove the top BioRex 9 column and rinse the Dowex column with 10-ml of HPLC grade methanol (discard methanol.

5.5.2.6 Attach an econo-column reservoir to the Dowex 50-X4 column and place in a well-ventilated hood.

5.5.2.7 Elute Cyromazine and Melamine from the column using 30-ml of 25% NH_4OH (conc.)/methanol. Collect eluate in a 250-ml round bottom flask. Evaporate eluate to dryness using a rotary evaporator (40°C bath).

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5.5.2.8 Dissolve the residue in Section 5.5.2.7 in an appropriate volume of methanol for HPLC analysis and proceed with Section 6.0.

6.0 HPLC DETERMINATION OF CYROMAZINE AND MELAMINE

Cyromazine and melamine residues are determined simultaneously by High Performance Liquid Chromatography (HPLC) on a LiChrosorb-NH_2 column using 90% acetonitrile:water as the mobile phase. HPLC conditions are given in Table I.

6.1 Standardization

6.1.1 Prepare stock solutions containing 50 mg of cyromazine in 50 ml of methanol and 50 mg of melamine in 50 ml of 50% water/methanol. Make serial dilutions of mixtures of these stock solutions with methanol to obtain solutions in a working range of 0.03 to 1.0 ng each per µl.

6.1.2 Standardize the HPLC under conditions stated in Table I by making 10-µl injections to give standard chromatograms in the range of 0.3-10 ng for each compound.

6.1.3 Determine the peak heights or areas of injected standards. Typical chromatograms for standards are presented in Figure 3 .

6.1.4 Enter the standardization data into an appropriate electronic calculator (e.g., Texas Instruments TI-55) to calculate least squares standard curves. Alternatively, construct standard curves, plotting peak heights vs nanograms injected.

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6.2 Detection of Sample Residues

6.2.1 Dissolve the residue from Section 5.4.8 or 5.5.2 in an appropriate volume of methanol.

6.2.2 Inject an aliquot of sample extract into the HPLC using same conditions as for standards. Compare the peak height for the unknown sample with the standard curves to determine the nanograms of cyromazine and melamine in the aliquot injected. Typical chromatograms for carrots and celery are shown in Figures 4 and 5.

6.2.3 Calculate the residue results in ppm by the equations below:

Cyromazine:

$$\text{ppm} = \frac{\text{ng cyromazine found}}{\text{mg sample injected}} \div R$$

Melamine:

$$\text{ppm} = \frac{\text{ng melamine found} \times 1.317}{\text{mg sample injected}} \div R$$

where R is the recovery factor determined using fortified control samples carried through the procedure (100% = 1.0, etc.). The factor 1.317 converts melamine residues to cyromazine equivalents.

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7.0 DISCUSSION

To date, this method has been used for the analysis of celery, carrot, and pepper samples. Recovery data of cyromazine and melamine from fortified control samples from these crops are listed in the table below:

	Recovery % From Fortified Samples			
Fortification Level (PPM)	0.05	0.40	1.0	2.0
Cyromazine	100	85	75	94
	85	83		88
	76			
	77			
Melamine	100	74	80	104
	82	92		88
	77			
	68			

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TABLE I: HPLC OPERATING CONDITIONS FOR DETERMINATION OF CYROMAZINE AND MELAMINE

Instrument:	Waters, Model 6000A solvent pump and variable volume sample injector
Column:	LiChrosorb-NH_2, HIBAR-II 10 µM particle size, (E. M. Merck) 4.0 x 250 mm
Mobile Phase:	90% Acetonitrile:Water
Flow Rate:	0.5 ml/min.
Temperature:	Ambient
Detection:	Variable wavelength UV detector set at 214 nm (Waters)
Minimum Detection Limit:	0.3 ng
Injection Volume:	10-30 microliters
Chart Speed:	0.5 cm/min.
Retention Time:	≃ 9.6 min. Cyromazine ≃ 13.4 min. Melamine

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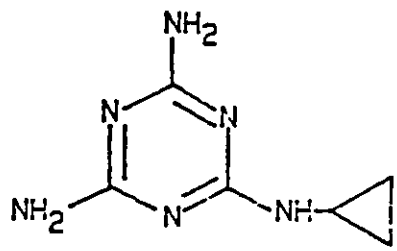
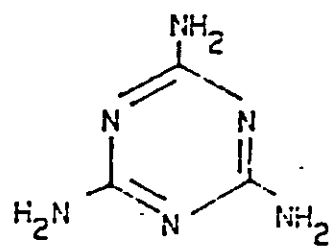
FIGURE 1: CHEMICAL NAMES AND STRUCTURES



Cyromazine

N-Cyclopropyl-1,3,5-triazine-2,4,6-triamine

$C_6H_{10}N_6$



Melamine

1,3,5-Triazine-2,4,6-triamine

$C_3H_6N_6$

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FIGURE 2: FLOW DIAGRAM FOR THE ANALYTICAL PROCEDURE FOR DETERMINATION OF CYROMAZINE AND MELAMINE RESIDUES IN CROPS

25 grams Crop + 250 ml 10% water:methanol
Reflux 2 hours
↓
Cool, decant supernatant (16-oz. bottle)
↓
Aliquot 5-g sample, evaporate to aqueous and add 100 ml 0.1 M HCl
↓

Partition Cleanup:
Add 50 ml DCM, shake (discard DCM)
Add 50 ml DCM, shake (discard DCM)
Add 50 ml Hexane, shake (discard hexane)
Load aqueous phase on Dowex column
↓

Dowex Column Cleanup 200-400 mesh slight vacuum:
Wash resin with 10-ml distilled water
Load aqueous from partition step
Wash with 50 ml of 10% water:acetonitrile → Discard
Wash with 50 ml of 10% water:methanol
Wash with 10 ml of methanol
↓

Elute cyromazine and melamine
Using 20 ml 5% NH_4OH:methanol
↓
Evaporate to Dryness
↓

Additional Cleanup:
Dissolve in 10 ml water
↓
Anion Exchange (BioRex 9) cleanup
↓
Collect aqueous through 50-100 mesh Dowex ion exchange resin (discard aqueous). Rinse Dowex with 10-ml HPLC methanol (discard methanol)
↓
Elute Dowex (30 ml 25% NH_4OH:methanol; collect)
↓
Evaporate to dryness then dissolve in methanol
↓

Dissolve in methanol
↓
HPLC Analysis cyromazine and melamine

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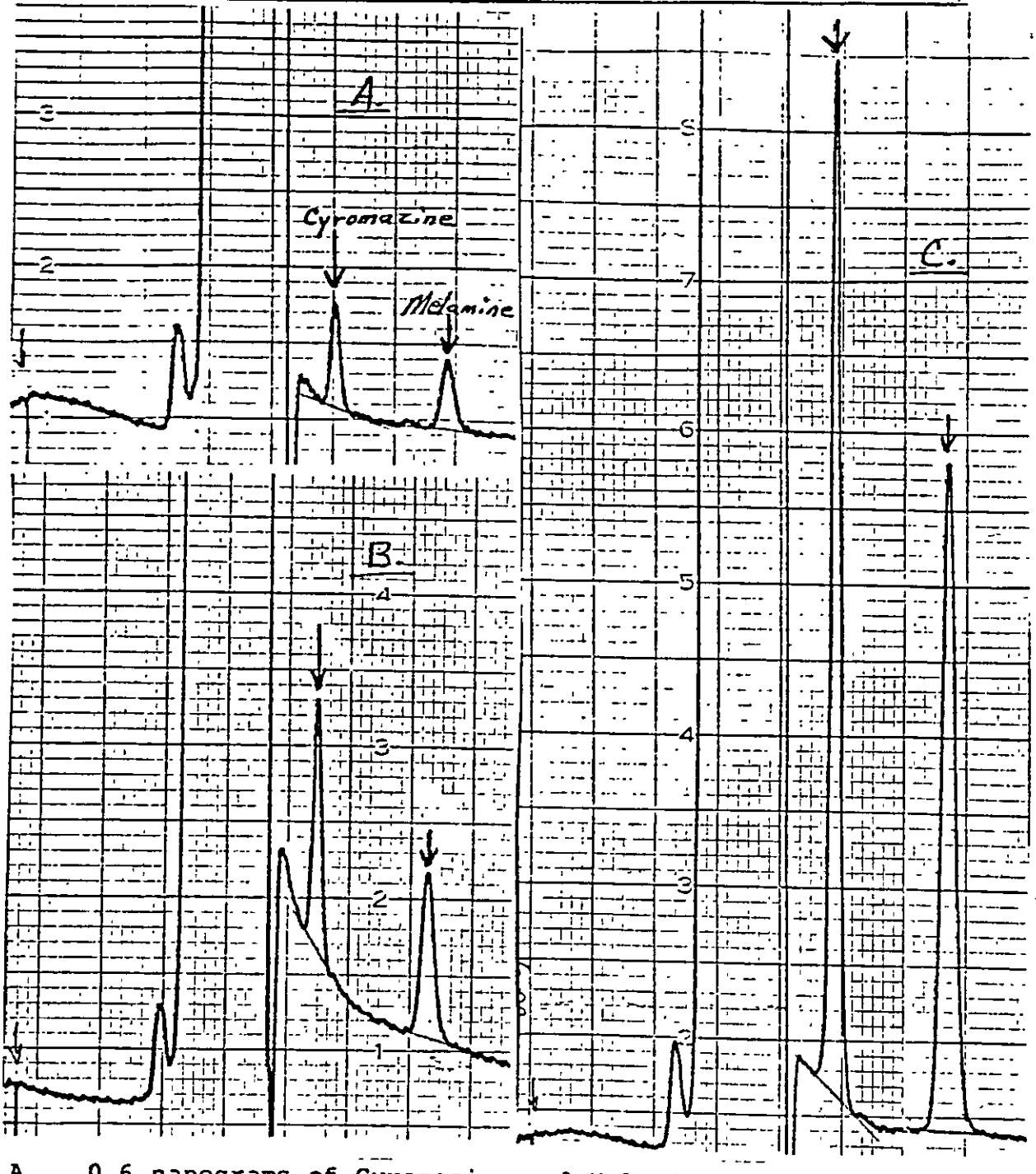
FIGURE 3: TYPICAL CHROMATOGRAMS FOR CYROMAZINE AND MELAMINE STANDARDS USING HPLC-UV DETECTION (214 NANOMETERS)



A. 0.6 nanograms of Cyromazine and Melamine
B. 1.5 nanograms of Cyromazine and Melamine
C. 6.0 nanograms of Cyromazine and Melamine

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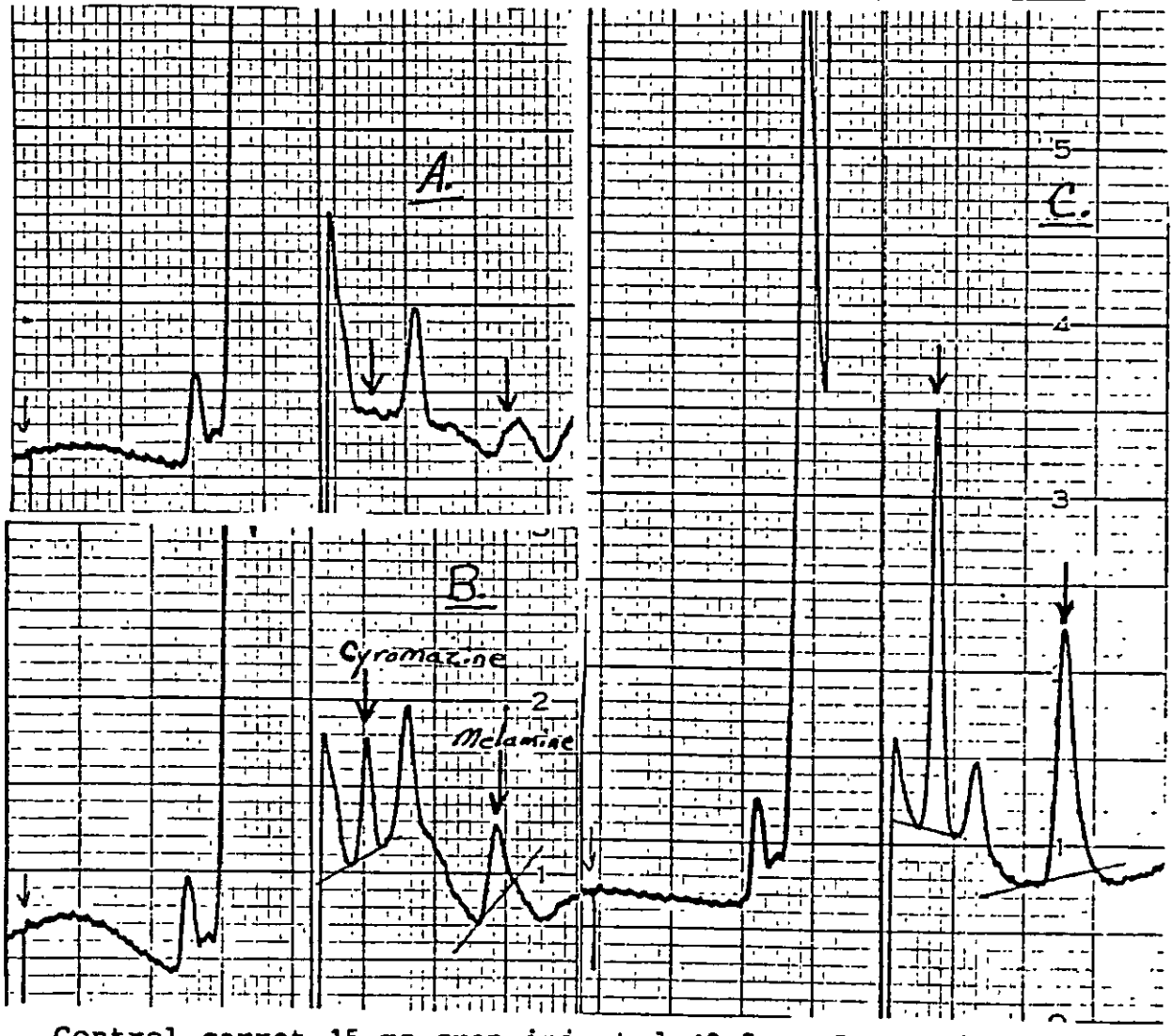
FIGURE 4: TYPICAL CHROMATOGRAMS FOR CYROMAZINE AND MELAMINE IN CARROTS USING HPLC-UV DETECTION (214 NANOMETERS)



A. Control carrot 15 mg crop injected <0.3 ng Cyromazine found (<0.05 ppm) <0.3 ng Melamine found (<0.05 ppm).

B. Control carrot + 0.05 ppm of Cyromazine and Melamine 15 mg crop injected 0.64 ng of Cyromazine found (85% recovery), 0.61 ng of Melamine found (82% recovery).

C. Field-treated carrot (10 foliar applications at 0.125 lbs. a.i./A) 0-day PHI 15 mg crop injected 2.2 ng of Cyromazine found 0.18 ppm, 2.0 ng of Melamine found 0.21 ppm.

Reference: AG-A 7387 (BioRex 9 anion exchange resin cleanup used).