

CYROMAZINE AND MELAMINE

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I. DETERMINATIVE METHOD

A. INTRODUCTION

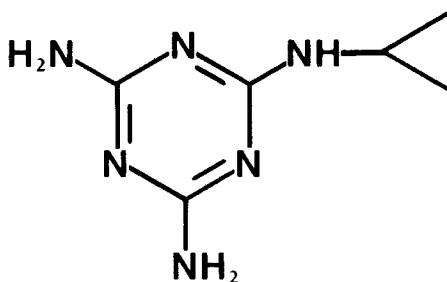
1. Theory and Structure

Cyromazine and melamine are extracted from tissue samples using acetonitrile/water, followed by a cleanup through a C₁₈ Solid Phase Extraction (SPE) device.

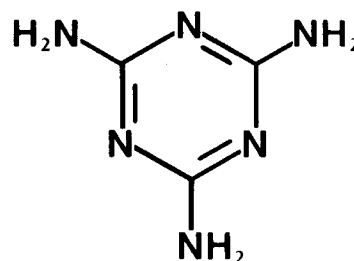
They are then quantitated by High Performance Liquid Chromatography (HPLC) equipped with an amine column and UV detector. This procedure is a modification of the Ciba-Geigy AG-417A method.

Cyromazine: C₆H₁₀N₆
F.W. = 166.18

Melamine: C₃H₆N₆
F.W. = 126.13



CYROMAZINE



MELAMINE

2. Applicability

This method has been evaluated for cyromazine and melamine residues analysis in poultry, red meat tissues, and processed products (ref., *J. Agric. Food Chem.*, 1988, 36, 1009-1012).

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted for any apparatus listed below if necessary.

- a. Bottles, milk dilution, 160 mL (Corning 1368). A 16-oz wide-mouth French square bottle may be optionally used if larger sample is to be used, i.e., 25 g.
- b. Column, Econo-column, polypropylene, 0.7 x 4 cm volume (Bio-Rad #731-1110).
- c. Test tube, Teflon-lined screw-cap (Corning 9825 and 9998).
- d. Vacuum flask, 1 L.
- e. A Vac Elut SPE Processing Station (Analytichem AI 6000).
- f. Graduated cylinders, 50 mL and 100 mL.
- g. Polytron homogenizer (Brinkman Instruments).
- h. N-Evap (Organomation Associates Inc., model 11).
- i. C₁₈ syringe barrel, type SPE (J. T. Baker #7020-3) and 75 mL reservoirs and adaptors (Analytichem #607500 and 636001 respectively).
- j. Syringe glass, 60 mL, with Luer tip.
- k. Vortex mixer.
- l. Optional repipets.
- m. Optional 14-gauge stainless steel cannula for pipetting 50 mL aliquot with glass syringe.

2. Instrumentation

HPLC: Waters, Model WISP 712 equipped with sample injector and automated pump (Model 6000A). A column switching valve (Waters Model 60057) is an advisable optional component.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Acetic acid, 1N, reagent grade.
 - b. Acetonitrile, HPLC grade.
 - c. Water, HPLC grade or at least 10 megohm.
 - d. Methanol, HPLC grade.
 - e. Ammonium hydroxide, reagent grade.
 - f. Sodium hydroxide, 1N aqueous, reagent grade.
 - g. Hydrochloric acid, 0.1N aqueous, reagent grade.
 - h. Acetonitrile/water, 90% v/v: Add 900 mL of acetonitrile to a 1000 mL volumetric flask and dilute to the mark with water.
 - i. Acetonitrile/water, 95% v/v: Add 950 mL of acetonitrile to a 1000 mL volumetric flask and dilute to the mark with water.
 - j. Ammonium hydroxide/methanol, 5% v/v: Add 5 mL of ammonium hydroxide to a 100 mL volumetric flask and dilute to volume with methanol.
 - k. Ammonium hydroxide/methanol, 25% v/v: Add 25 mL of ammonium hydroxide to a 100 mL volumetric flask and dilute to volume with methanol.
 - l. Methanol/water, 50% v/v: Add 50 mL of methanol to a 100 mL volumetric flask and dilute to volume with water.
 - m. Methanol/water, 90% v/v: Add 90 mL of methanol to a 100 mL volumetric flask and dilute to volume with water.
 - n. Resin 1, Dowex 50W-X4 Cation Exchange Resin, 200-400 mesh, Bio-Rad.
 - o. Resin 2, Dowex 1-X8 Anion Exchange Resin, 50-100 mesh, chloride form. (Refer to section E for column preparation.)
 - p. Resin 3, Dowex 50W-X4 Cation Exchange Resin, 50-100 mesh, Bio-Rad.
-

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Cyromazine (96.5%) and melamine (99.0%) standards can be purchased from:

Ciba-Geigy Corp.
410 S. Swing Road
Greensboro, NC 27049
Telephone: (919) 292-7100

2. Preparation of Standards

- a. Stock standard: 1 mg/mL.
 - i. Cyromazine: Place 50 mg of the standard in a 50 mL volumetric flask. Dilute to volume with methanol.
 - ii. Melamine: Place 50 mg of the standard in a 50 mL volumetric flask. Dilute to volume with 50% methanol/water.
- b. Working standard: 10 µg/mL.
 - i. Cyromazine: Add 1 mL of the stock standard (2.a.i.) to a clean 100 mL volumetric flask and dilute to volume with methanol.
 - ii. Melamine: Add 1 mL of the stock standard (2.a.ii.) to a clean 100 mL volumetric flask and dilute to volume with 50% methanol/water.

c. HPLC standards.

Dilute the working standards (2.b.) with the mobile phase as follows:

<i>Cyromazine/Melamine Standard mL of 10 µg/mL standard</i>	<i>Final Volume (mL)</i>	<i>Final Concentration ng/mL</i>
0.5 mL	100	50
1.0 mL	100	100
2.0 mL	100	200

3. Storage Conditions

All standards are to be kept tightly closed in a glass-stoppered bottle and stored at 4° C when not in use.

4. Shelf Life Stability

Stability is based upon the storage conditions listed in section D.3.

- a. Stock standards: One year.
- b. Working standards: Six months.
- c. HPLC standards: Three weeks.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Column Preparation (for resin 2)

- a. In a large beaker, swirl about 100 g of Dowex 1-X8 resin, 50-100 mesh, chloride form, with about 500 mL of 1N acetic acid.
- b. Allow the resin to settle and decant the supernatant liquid together with the suspended fines.

NOTE: Steps b, c, and e may be facilitated with the use of a sintered glass filter.
- c. Wash the resin with 500 mL distilled water as in steps a and b.
- d. Wash the resin twice with 1N sodium hydroxide. The resin will darken in color.
- e. Wash the resin with several portions of distilled water until the supernatant is about neutral or the same pH as the distilled water.
- f. Transfer a slurry of the resin into an Econo-Column sufficient to prepare a 2 mL bed.
- g. Prewash the 1-X8 resin column with 10 mL distilled water before use. This column is ready to use in section E.3.

2. Sample Extraction

- a. Weigh a 10 g sample of tissue into a 160 mL square milk dilution bottle. Optionally, a 25 g sample may be weighed into a 16 oz wide-mouth French square bottle.
- b. Weigh two 10 g portions of tissue blank. Label them as follows:
 - i. Control blank tissue.
 - ii. Control fortified tissue.

Fortify the blank tissue by adding 5 mL of the HPLC standard solution (section D.2.c.). This will yield a recovery of 100 ng/g.
- c. Add 92 mL of 90% acetonitrile/water to the bottle and homogenize for 1 min at a power setting of 6-8 and allow to settle. For the optional 25 g sample, use 230 mL of 90% acetonitrile/water.
- d. Place the sample in a freezer for at least two hours. Warm to room temperature. A clear acetonitrile/water layer will form on the top. Filter the top layer through glass wool. Discard the bottom layer. Extract may be centrifuged, but should not be filtered through filter paper. Some filter papers contain melamine from the manufacturing process.
- e. Load an Econo-Column with a 2 mL bed of resin 1, Dowex 50W-X4. Add about 5 mL of water to the column and place a perforated cap on the column. Insert the Luer tip of a C₁₈ SPE column in the Econo-Column cap; add a few mL of 90% acetonitrile/water to the SPE to prime. Place the SPE adaptor on the SPE and add the syringe barrel reservoir.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- f. Add a 50 mL aliquot of the sample to the reservoir. This is measured with a glass syringe or a graduated cylinder. 50 mL represents a 5 g sample.
 - g. Allow the sample extract to flow through the SPE Econo-Column assembly either by gravity or with vacuum assistance. Flow rate should not exceed 2 drops a second. Discard this liquid.
 - h. Remove the SPE column and reservoir from the assembly and connect a clean reservoir to the Econo-Column cap.
 - i. Wash the ion-exchange column with 50 mL of 0.1N HCl. Discard the wash. Vacuum may be used.
 - j. Wash the ion-exchange column with 50 mL of 90% methanol/water. Discard the wash. Vacuum may be used.
 - k. Wash the ion-exchange column with 10 mL of methanol. Discard the methanol. Vacuum may be used.
 - l. Elute the cyromazine/melamine from the ion exchange resin with 20 mL of 5% ammonium hydroxide/methanol into a 50 mL screw-cap test tube. This step must be done in a fume hood.
 - m. Evaporate the eluate to dryness using air in the N-Evap (40° C bath). This step must be done in a fume hood.
-

3. Sample Cleanup

- a. Fit the Dowex 1-X8 column, resin 2, prepared in section E.1, into the top cap of a second Econo-Column containing a 2 mL bed of Dowex 50W-X4 resin (50-100 mesh), resin 3. The bottom column must be primed with about 5 mL of distilled water prior to connecting these columns together to avoid an air lock.
- b. Dissolve the sample from step 2.m. in 10 mL of distilled water and transfer to the upper anion column (resin 2) either directly or using a reservoir.
- c. Rinse the sample tube with another 10 mL of distilled water and add to the top column or reservoir. Repeat again with 5 mL of distilled water (total of 25 mL of water).
- d. After the aqueous has eluted through the top column, remove this column. Discard this wash.
- e. Rinse the Dowex 50W-X4, 50-100 mesh (bottom column, resin 3), with 10 mL of HPLC grade methanol. Discard this wash.
- f. In a hood, elute the cyromazine and melamine, using 30 mL of 25% ammonium hydroxide/methanol. This is accomplished by using a syringe barrel reservoir or by making three 10 mL additions of the eluent directly to the open column.

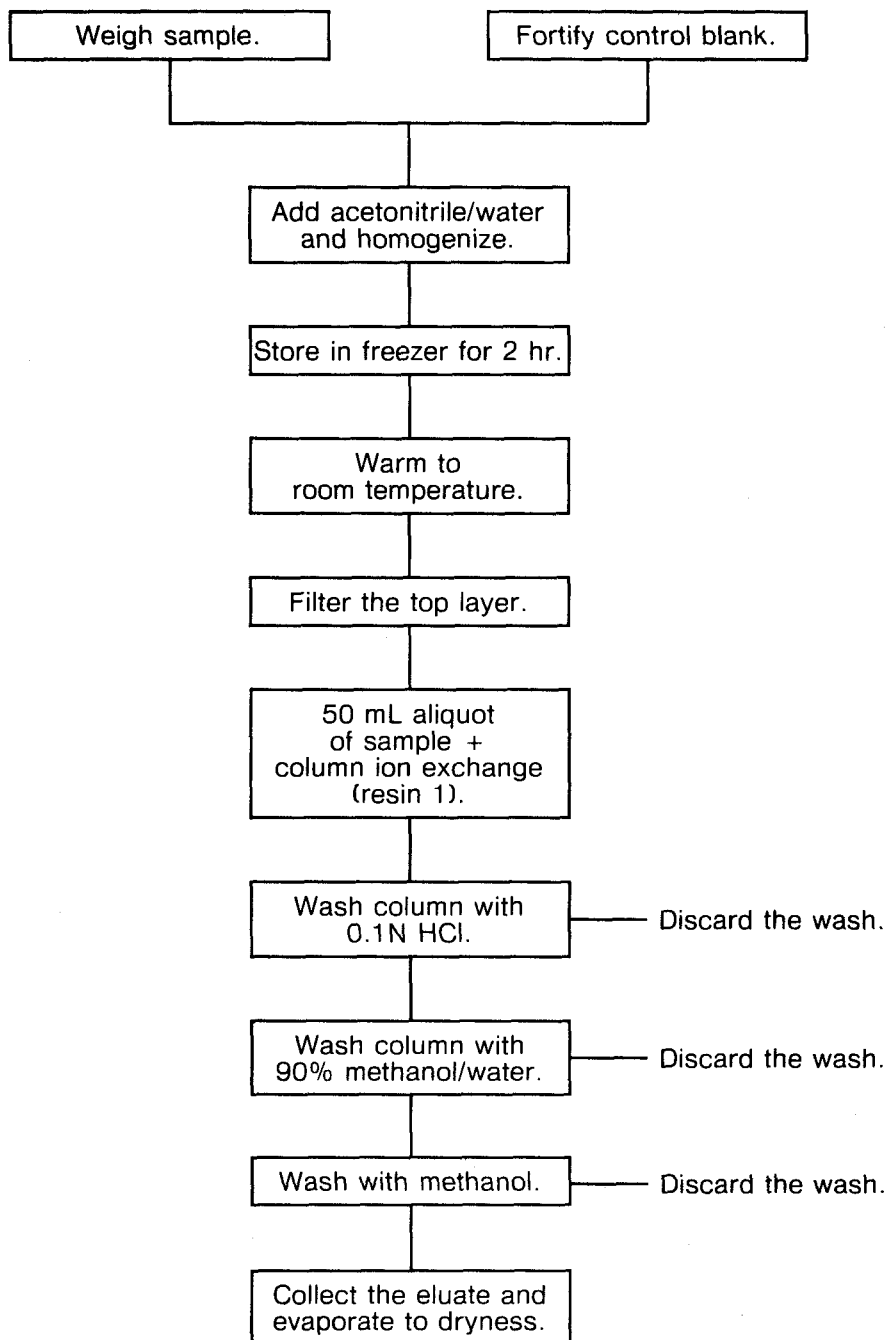
I. DETERMINATIVE METHOD**E. EXTRACTION PROCEDURE (Continued)**

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- g. Collect the eluate in a screw-cap test tube and evaporate as in step 2.m.
 - h. Dissolve the dried sample in 2.5 mL of mobile phase or 90% acetonitrile/water and filter through a Nylon 66 HPLC 0.45 micron filter. Sample is now ready for analysis.
-

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

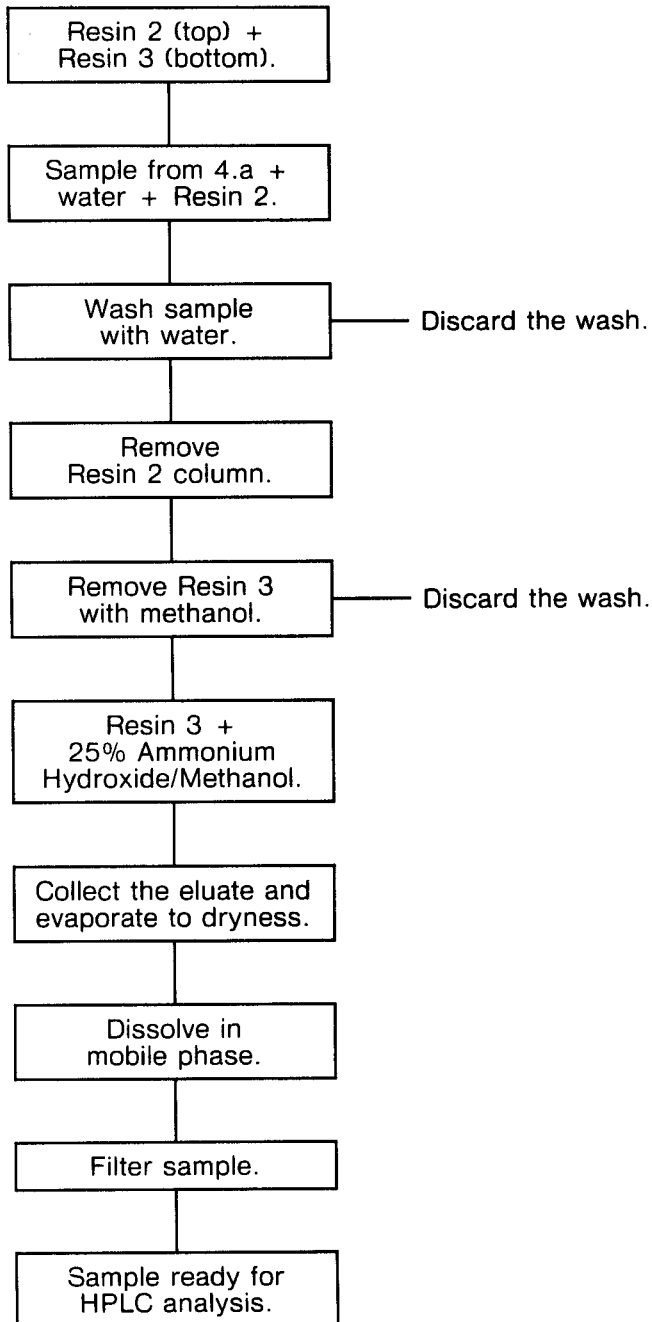
4. Flow Chart Summary a. Sample extraction.



I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Sample cleanup.



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

The following parameters may be modified as necessary to compensate for daily variations in instrument performance characteristics.

- | | |
|---|--|
| a. Mobile phase | 95% acetonitrile/water.
Because dissolved oxygen absorbs UV at 214 nm, the mobile phase is sparged with helium during long automated runs. |
| b. Flow rate | 1.0 mL/min, using 4.6 mm diameter HPLC column. Rate is adjusted down when using microbore column. |
| c. Attenuation | 0.005 AUSF or as needed. |
| d. Detector | UV detector. |
| e. Wavelength | 214 nm. |
| f. Injection volume | 0.025 mL. |
| g. Chart speed | 0.5 to 1.0 cm/min. |
| h. Approximate retention time (for a two-column system) | Cyromazine: 10.2 min.
Melamine: 14.9 min. |
| i. Column | Hibar 250-4 pre-packed with 5 micron NH ₂ . It has been found that frequently a single 250 mm column does not give adequate separation tissue substances. Normally the chromatography is done using two 5 micron NH ₂ columns such as Lichrosorb #50376. |

NOTE: Because of the excessive times needed to elute some of the late-eluting interfering tissue substances, it is helpful to use a column switching valve in the system so that after the compounds of interest have been eluted onto the second column, the first column may have its flow reversed and pumped to waste using a second pump while the chromatography continues through the second column.

NOTE: Waters C₁₈ Sep-Packs may be used in place of the syringe barrels; however, they must be attached to 75 mL reservoirs. The units are then suspended over the reservoirs, which are connected to the caps of the Dowex 50W-X4 200-400 mesh Econo-Column (resin 1).

2. Known Interferences

Processed products can give background peaks that obscure peaks of interest. False positives can occur. GC/MS confirmation of HPLC positive sample is essential.

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

- a. Standardize the HPLC by injecting 25 μL of the standards in section D.2.c under the conditions stated in section F to give a standard curve of 1 to 4 ng on 4.6 mm diameter columns.
- b. Determine the peak heights or areas of the injected standards.
- c. Using the peak data (height or area) and the respective injected amounts of the standard, calculate the least squares standard curves; alternatively, construct the standard curve by plotting peak height vs. standard concentration.
- d. Inject an aliquot of the sample extract from section E.3.h into the HPLC using the same conditions as those for the standards. Determine the cyromazine and melamine in the samples by calculation, using the least squares regression on the peak data from the samples or by reading the values from constructed curves. Recovery corrected values are then obtained by dividing the resulting values by respective fractional recoveries from a spiked sample that has been carried through the analysis.

$$\text{ppb cyromazine} = \frac{\text{ng cyromazine found} / R_c}{\text{g tissue equivalent injected}}$$

$$\text{ppb melamine} = \frac{\text{ng melamine found} / R_m}{\text{g tissue equivalent injected}}$$

$$\text{ng cyromazine/melamine found} = \frac{\mu\text{g}}{\text{mL}} \times \text{final volume (mL)} \times \text{d.f.}$$

where:

R_c and R_m are the fractional recoveries for cyromazine and melamine respectively.

If needed, the factor 1.317 times the melamine value gives the cyromazine equivalent of the melamine.

$\mu\text{g/mL} = \mu\text{g/mL}$ obtained from standard curve.

d.f. = sample volume/aliquot taken.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Cyromazine and Melamine Residues in Animal Tissues	
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.	
3. Procedure Steps	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
C. Reagents		
Acetic acid Hydrochloric acid	Flammable, burn skin upon contact. Ingestion may cause severe corrosion of mouth and G.I. tract. Eye irritation.	Use under well-ventilated hood. Avoid contact with skin, eyes.
Sodium hydroxide Ammonium hydroxide	Very corrosive. Ingestion will result in vomiting, collapse. Will generate extensive amount of heat if solution is made up from bulk.	Same as above.
Acetonitrile	Poisonous, flammable, may cause skin irritation.	Same as above.
Methanol	Flammable.	Same as above.
D. Standards		
Melamine Cyromazine	Toxic by ingestion. Causes skin and eye irritation upon contact.	Same as above.
4. Disposal Procedure		
Acetic acid Hydrochloric acid Ammonium hydroxide Sodium hydroxide	See above.	Flush down sink with lots of water. Use separate sink for acid vs caustic.
Organic solvent mixture/standard	See above.	Segregate chlorinated from nonchlorinated material before disposal by the contract or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Cyromazine/Melamine*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

<i>Compound</i>	<i>Analytical Range (ppb)</i>	<i>Acceptable Recovery %</i>	<i>Repeatability %CV</i>
Cyromazine	25-100	60-115	≤ 20 at 50 ppb
Melamine	25-100	40-115	≤ 30 at 50 ppb

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
a. Sample weight	10 g ± 0.1 g
b. Aliquot taken	50 mL
c. Final volume	2.5 mL
d. Flow rate of SPE column	≤ 2 drops/sec
e. Evaporation temperature	40° C
f. pH of anion exchange resin supernatant	7.5 ± 0.5

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—Triplicate standard curve on each of the cyromazine and melamine standards.
 - (a) Blank.
 - (b) 50 ng/mL.
 - (c) 100 ng/mL.
 - (d) 200 ng/mL.

- ii. Phase II: Fortified samples—Duplicate analyses on fortified samples at 0, 25, 50, and 100 ppb on three consecutive days, one set each of red meat and poultry.

NOTE: Phase I and Phase II may be performed concurrently.

- iii. Phase III: Check samples for analyst accreditation.
 - (a) 14 samples submitted by supervisor, with 7 samples per species (beef and poultry) fortified at least at 50 ppb for poultry and 100 ppb for beef. A blank tissue must also be included.
 - (b) Report data to Chemistry Division.

Notification from Chemistry Division is required to commence official analyses.

- b. Acceptability criteria.
Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

**4. Intralaboratory
Check Samples**

-
- a. System, minimum contents.
 - i. Frequency: At least one check sample biweekly per analyst containing both cyromazine and melamine using different species. At least one out of four of these samples is to be confirmed by mass spectrometry.
 - ii. Random replicates chosen by supervisor or Laboratory QA Officer.
 - iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer for:
 - (a) All replicate findings.
 - (b) Control chart on percent difference between replicates.
 - (c) All % recoveries.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 above if cause was analyst-related.
-

**5. Sample Acceptability
and Stability**

- a. Matrix: Red meat and poultry muscle.
 - b. Sample receipt size, minimum: 30 g.
 - c. Condition upon receipt: Cold (< 10° C).
 - d. Sample storage:
 - i. Time: 6 months.
 - ii. Condition: - 20° C.
-

6. Sample Set

Each set must contain a reagent blank, a blank tissue, a blank tissue fortified at 50 ppb (for poultry) and 100 ppb (for beef) and samples that will be processed on the same day.

7. Sensitivity

- a. Lowest detectable level (LDL): 25 ppb.
 - b. Lowest reliable quantitation (LRQ): 50 ppb (poultry), 100 ppb (beef).
 - c. Minimum proficiency level (MPL): Not determined.
-

II. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory

This method confirms the presence of TMS derivatives of cyromazine and melamine residues extracted from poultry and red meat tissues from the determinative method. The TMS derivatives are formed by heating the residue with BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) in the presence of TMCS (trimethylchlorosilane).

2. Applicability

Refer to Determinative Method, section A.2.

II. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Temp-block module heater, Lab-Line Instruments, Inc., or equivalent.
 - b. Sand for 1.a.
 - c. Conical centrifuge tube with stoppers, 15 mL.
 - d. Volumetric flasks, 10 mL and 200 mL.
 - e. Syringe, 250 μ L.
-

2. Instrumentation

GC/MS: Hewlett Packard 5970B MSD/5890 GC equipped with 30 m DB5 column (J & W Scientific, Inc.), 0.25 mm id, 0.25 micron film thickness.

II. CONFIRMATORY METHOD**C. REAGENTS AND SOLUTIONS****Reagent and
Solution List**

-
- a. Refer to Determinative Method, section C.
 - b. Acetone: Fisher Optima Grade or equivalent.
 - c. Acetone/Methanol, 50% v/v: Mix 250 mL each of acetone and methanol in a clean 500 mL volumetric flask.
 - d. Acetonitrile: Stored in molecular sieve as drying agent.
 - e. Trimethylchlorosilane (TMCS): Pierce Chemical Co., catalog #88530.
 - f. N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% TMCS: Pierce Chemical Co., catalog #38831.
-

II. CONFIRMATORY METHOD

D. STANDARDS

1. Source	Refer to Determinative Method, section D.1.
2. Preparation of Standards	<p>a. Stock standard.</p> <ul style="list-style-type: none">i. Cyromazine stock: 1 mg/mL. Prepare by dissolving 10 mg of the compound in 10 mL of 50% acetone/methanol, v/v.ii. Melamine stock: 0.05 mg/mL. Prepare by dissolving 10 mg of the compound in 200 mL of 50% acetone/methanol, v/v. <p>b. Working standard: 1 μg/mL.</p> <ul style="list-style-type: none">i. Cyromazine standard: Dilute 10 μL of the stock standard (a.i) into 10 mL volumetric flask and bring up to volume with acetone.ii. Melamine standard: Dilute 200 μL of the stock standard (a.ii) into 10 mL volumetric flask and bring up to volume with acetone.
3. Storage Conditions	Refer to Determinative Method, section D.3.
4. Shelf Life Stability	Refer to Determinative Method, section D.4.

II. CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE

1. Derivatization

Refer to determinative method, section E, for column preparation and sample extraction.

- a. Prior to derivatization of the sample, the analyst should verify that the process is functioning. This can be accomplished by derivatizing approximately 100 μL of 1 ppm working standards prepared in section D.
- b. Place the HPLC eluate containing the cyromazine and melamine in a 15 mL conical centrifuge tube. The eluate should represent at least 4 g of 40% of tissue sample.
- c. Evaporate the eluate to dryness under a stream of nitrogen at about 50° C.
- d. Add 100 μL of acetonitrile and mix for 30 sec with a vortex mixer.
- e. Add 100 μL of BSTFA with 1% TMCS.
- f. Mix for 30 sec and heat at 110° C for 1 hour in a sand bath.

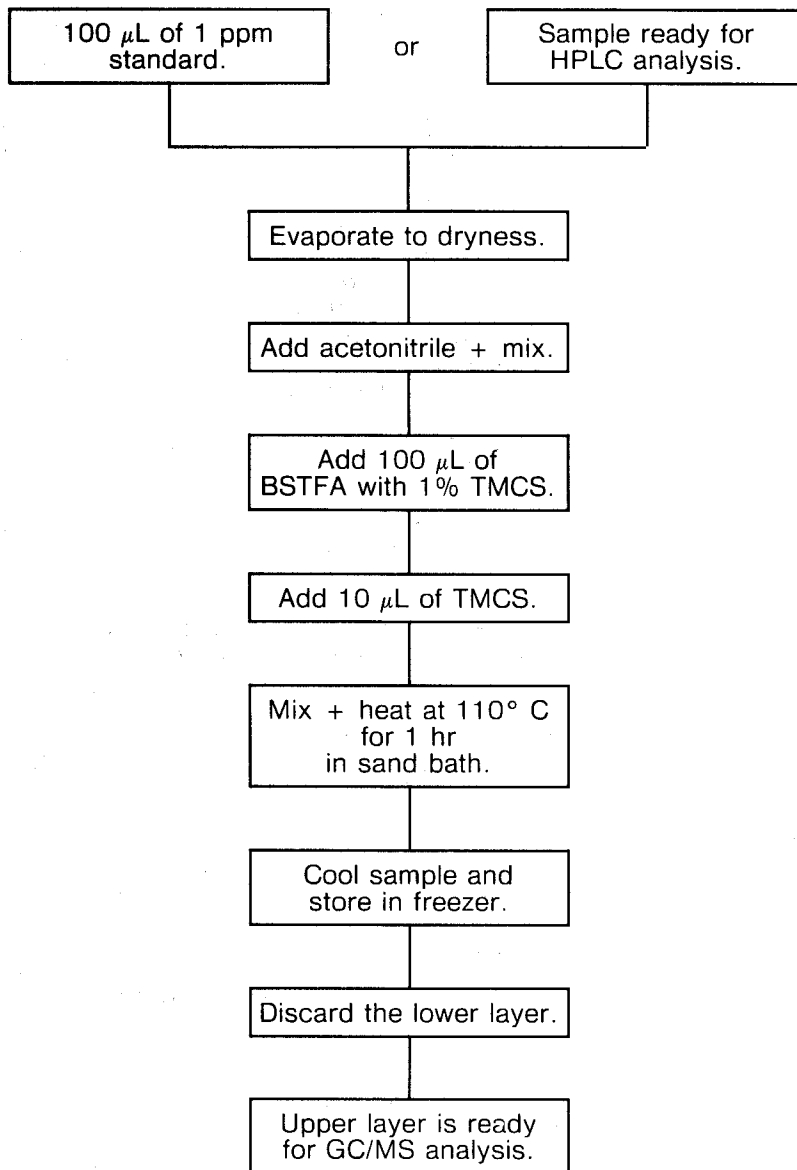
NOTE: This heating will result in the cyromazine derivative with the following ions: 171, 181, 295, and 310. Prolonged heating or heating at a higher temperature will result in the formation of the trisubstituted cyromazine derivative with characteristic ions at 171, 253, 269, 283, 367, and 382. Whether it is more advantageous to drive the reaction to completion and monitor the trisubstituted cyromazine has yet to be determined.

- g. Cool the sample to room temperature and put it in the freezer (approximately -20° C) for 20 min.
 - h. Immediately discard the lower clear liquid organic layer (approximately 100 μL).
 - i. Inject 2.5 μL of the remaining sample, using 0.5 μL acetonitrile plug. Alternatively, directly inject 2.5 μL of the upper organic layer in the bilayer sample in step h above (before discarding the lower organic layer).
 - j. Run approximately 100 ng each of the cyromazine and melamine standards concurrently with the sample by adding 100 μL of 1 ppm working standards into a vial and performing steps b through i above.
 - k. Run a method blank and recovery standard concurrently with the sample by performing steps b through i above. The recovery standard concentration should be within the same range as the concentration expected for the sample.
-

II. CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary Sample derivatization.



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters.

Detector	MS-EI-SIM.
Detector temperature	180-250° C (MS source temperature).
Injection mode	Splitless.
Injection temperature	260° C.
Carrier gas	Helium.
Flow rate	Approximately 30 cm/sec.
Column head pressure	10 psi.
Temperature program	
Initial temperature	100° C.
Hold time	1 min.
Program rate	15°/min.
Final temperature	250° C.
Final hold time	9 min.
Transfer line	270° C.
Splitless injection	1 min vent delay.

b. Mass spectrometer conditions.

Analyzer parameters	Set by autotune program.
Electron energy	70 ev.
Electron multiplier	200-600 v above the autotune voltage.

NOTE: Parameters for Selected Ion Monitoring (SIM)—Set up two separate retention time windows and monitor four ions within each window using 150 msec dwell time per ion.

- Cyromazine—m/z 171, 181, 295, 310.
- Melamine—m/z 171, 197, 327, 342.

2. Required Samples

- Inject 2.5 μ L of standard to check the performance of the instrument.
- Inject 2.5 μ L of acetonitrile to check carryover.
- Inject 2.5 μ L of method blank to check background interferences.
- Inject 2.5 μ L of recovery standard.
- Inject 2.5 μ L of the sample.

NOTE: if the instrument has been performing well, steps 2.a and 2.b can be omitted.

NOTE: The signal to noise ratio of the least abundant ion for either cyromazine or melamine in the recovery standard should at least be equal to 5.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

- a. All four ions for each analyte must be present.

<i>Compound</i>	<i>Ions Monitored</i>
	(+) = Base ion
Cyromazine	171, 181, 295(+), 310
Melamine	171, 197, 327(+), 342

- b. At least two ratios, relative to the most intense ion, must be within 15% of those found in the recovery sample spiked at the level found in the suspect sample. Typical ratios and retention times:

<i>Compound</i>	<i>Relative Retention Time (min)</i>	<i>Ions</i>	<i>Relative Ratio</i>
Cyromazine	11.38	171/295	0.416
		181/295	0.482
		310/295	0.816
Melamine	10.54	171/327	0.629
		197/327	0.135
		342/327	0.608

- c. Retention time must agree within 5 seconds of that found for the fortified sample.

<i>Compound</i>	<i>Retention Time (min)</i>
Cyromazine	11.38
Melamine	10.54

- d. Blank should show no interferences.
 e. Postulated structure of confirming ions.

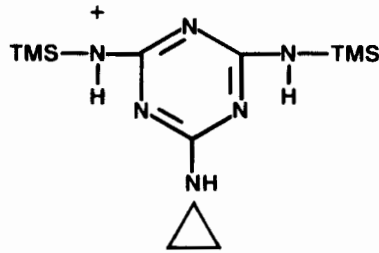
Refer to facing page.

II. CONFIRMATORY METHOD

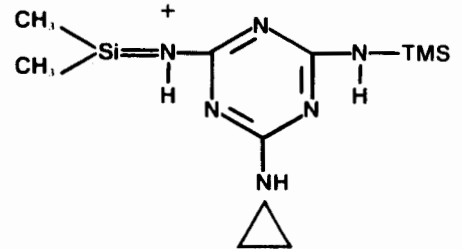
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Postulated ion fragments.

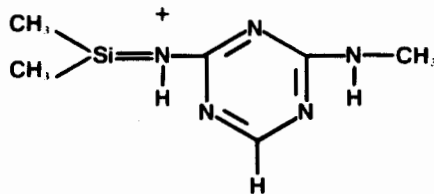
CYROMAZINE



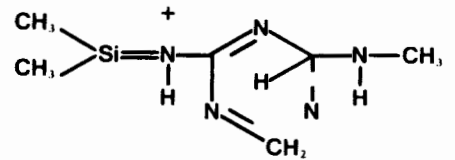
m/z = 310



m/z = 295

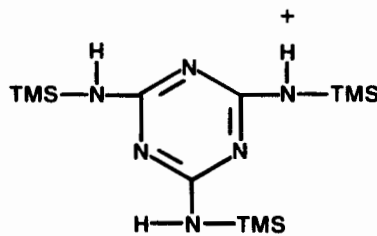


m/z = 181

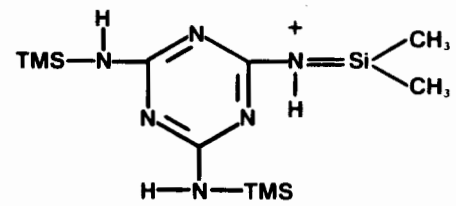


m/z = 171

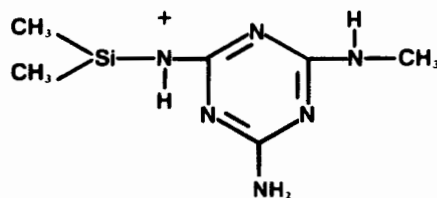
MELAMINE



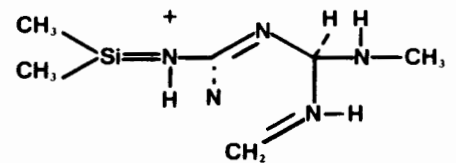
m/z = 342



m/z = 327



m/z = 197



m/z = 171

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Procedure Steps

Refer to Determinative Method, section H, in addition to the following:

	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
C. Reagents		
Acetone Methanol	Vapors are irritating to the skin, eyes, and respiratory system.	Perform all work in an efficient hood. Avoid contact with skin, eyes.
E. Derivatization		
BSTFA + 1% TCMS TCMS	Combustible liquid. It is also a suspected mutagen/carcinogen.	Same as above.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards	a. No false positives at 0 ppb for both compounds. b. No false negatives at 50 ppb or higher in poultry or 100 ppb or higher in beef for both compounds.	
2. Critical Control Points and Specifications	Refer to Determinative Method, section J.2, in addition to the following:	
	<i>Record</i>	<i>Acceptable Control</i>
	a. Derivatization	110° C ± 2° C for 1 hr ± 0.1 hr
	b. Sample cooling	- 15° C to - 30° C. The derivatives are stable for at least 20 hrs within the temperature range.
	c. GC unit	Verify to ensure that the injector of the system is clean and free of contaminants by injecting 2 µL of BSTFA + 1% TMCS.
3. Readiness To Perform	a. Familiarization. <ul style="list-style-type: none"> <li data-bbox="476 1035 1358 1087">i. Phase I: Standards—Analyze three 50 ppb (mixture of cyromazine and melamine) per day for a three-day period. <li data-bbox="476 1115 1358 1245">ii. Phase II: Fortified samples—Analyze three chicken samples and three beef samples at 100 ppb. Demonstrate that the coefficient of variation for the relative ion ratios is within 10% for each species. Also make sure the method blank is free of interferences. Repeat experiment for two more days. NOTE: Phase I and Phase II may be performed concurrently. <ul style="list-style-type: none"> <li data-bbox="465 1329 1358 1486">iii. Check samples for analyst accreditation. <ul style="list-style-type: none"> <li data-bbox="511 1381 1358 1486">(a) Three samples for each species, including one blank. If fortified, the sample should be fortified at 50 ppb for poultry and 100 ppb for beef. The same check sample can be used in both the determinative and confirmatory methods. <li data-bbox="511 1514 1058 1541">(b) Report data to the Chemistry Division. b. Acceptability criteria. Refer to section F.3.	

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples	Refer to Determinative Method, section J.4.
5. Sample Acceptability and Stability	Refer to Determinative Method, section J.5.
6. Sample Set	Each set must contain a method blank, a blank tissue fortified at the level found in the presumptive suspect sample, and all positive samples found above the concentration of interest from the determinative method.
7. Sensitivity	<ul style="list-style-type: none">a. Lowest detectable level (LDL): Not applicable.b. Lowest reliable confirmation (LRC): 50 ppb (poultry), 100 ppb (beef).
