United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science

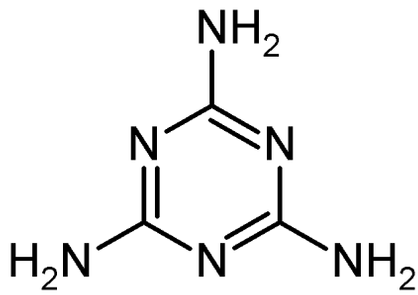
CLG-MEL1.01		Page 1 of 24
Title: Determination and Confirmation of Melamine by LC/MS/MS		
Revision: 01	Replaces: CLG-MEL1.00	Effective: 12/02/2008

Contents

A. INTRODUCTION

1. Theory and Structure

Melamine is extracted from ground beef tissue with an acetonitrile/water (50:50, v/v) solution followed by a methylene chloride defat washing. The extract is cleaned through a Waters Oasis MCX column. Quantitative analysis of melamine is performed by LC/MS-MS with hydrophilic interaction chromatography (HILIC) and electrospray ionization in positive ion mode.



2. Applicability

This method is applicable for the determination and confirmation of Melamine in ground beef at ≥ 50 ppb using triple quad and linear ion trap (See section B.2.a and B.2.b), and ready to eat (RTE) products at ≥1 ppm using linear ion trap (see section B.2.b).

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

SPE vacuum manifold - Supelco.

SPE Columns - Oasis, MCX 150 mg / 6 mL, Waters.

Centrifuge - Eppendorf 5810.

Centrifuge - Galaxy 14D, VWR.

50 mL glass disposable centrifuge tubes - Kimble 73785-50.

15 mL glass disposable centrifuge tubes - Kimble 73785-15.

Glass disposable culture tubes 13 mm x 100 mm - VWR 47729-572.

Microfilterfuge tubes 0.2 µm Nylon-66 - Rainin 7016-021.

N-EVAP - Organomation 112t.

2. Instrumentation

   a. Triple Quad (TQD)

      Liquid Chromatograph - Waters Acquity UPLC, Waters Co.

      Analytical Column - Waters Acquity UPLC, BEH HILIC Silica 1.7 µm, 2.1 x 100 mm, Waters.

      Mass Spectrometer - Waters TQD, Waters Co.

   b. Linear Ion Trap (LTQ)

      Liquid Chromatograph – Agilent 1200 HPLC, Agilent Technologies.

      Analytical Column - Waters Acquity UPLC, BEH HILIC Silica 1.7 µm,

      2.1x100 mm, Waters.

      Mass Spectrometer – Thermo Finnigan LTQ, Thermo Finnigan.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents

   a. Methanol – Optima HPLC Grade, CAT No A454-4, Fisher.

   b. Acetonitrile – Optima, 0.2µm filtered, CAT No A996-4, Fisher.

   c. Water – Optima, 0.5µm filtered, CAT No W7-4, Fisher.

   d. Methylene Chloride – 99.8%, GC, CAT No 66741, Fluka.

   e. Ammonium Formate – 96% min, CAT No JTM530-8, J T Baker.

   f. Ammonium Hydroxide – ACS, CAT No AX1303-6, EMD.

   g. Hydrochloric Acid – 1.0 ± 0.002 N, CAT. No VW3202-1, VWR.

2. Solutions

   a. Extraction Solvent: Acetonitrile: water 1:1 (v/v):

      Mix well 500 mL acetonitrile and 500 mL Optima water.

   b. 20 mM ammonium formate in water:

      Prepare 1 liter by diluting approximately 1.25 grams of Ammonium Formate in 1 liter of Optima water.

   c. 0.1N Hydrochloric Acid:

Dilute 100 mL 1N Hydrochloric Acid to 1 liter with Optima water.

d. 5% (v/v) ammonium hydroxide in acetonitrile:

Dilute 5 mL ammonium hydroxide into 95 mL Optima water. Mix well.

e. 50% (v/v) methanol in Optima water:

Dilute 50 mL methanol into 50 mL of Optima water. Mix well.

D. STANDARDS

1. Reference Standard

Common Name: Melamine.

Chemical Name: 2,4,6-Triamino-1,2,5-Triazine.

Chemical Formula: $C_3H_6N_6$.

Molecular Weight: 126.12.

Source: TCI CAT No T0337.

Storage: Store in a closed container in a cool dry place.

2. Preparation of Melamine Standard Solutions

a. Stock Standard (1 mg/mL):

Weigh 10 mg melamine in a 10 mL volumetric flask and bring to volume with 50% (v/v) methanol in Optima water.

b. Working standards

i. 10 µg/mL:

Pipet 100 µL stock standard into a 10 mL volumetric flask and bring to volume with acetonitrile.

ii. 100 µg/mL:

Pipet 1000 µL stock standard into a 100 mL volumetric flask and bring to volume with acetonitrile.

c. LC/MS-MS standard curve solutions:

Dilute 0, 10, 25, 50, and 75 µL of the 10 µg/mL working standard into 5 tubes to 10 mL with acetonitrile, as shown in the following table:

Calibration Level	Volume working standard solution (μL)	Volume acetonitrile (μL)	Calibration standard concentration (ng/mL) (ppb)
0	0	10000	0
1	10	9990	10
2	25	9975	25
3	50	9950	50
4	75	9925	75

Storage and Stability:

Store stock standard solution (1 mg/mL) tightly closed at room temperature. This solution is stable for 1 year.

Store working standard solution (10 µL/mL) tightly closed at room temperature. This solution is stable for 1 month.

Standard curve solutions are stable for 3 weeks.

E. SAMPLE PREPARATION

Sample size: 25 grams.

Blend sample until homogeneous.

Sample type: ground beef.

RTE products : e.g. cheese dogs, sausage, chicken fingers, meatballs, baby foods (meats), etc.

Store samples in glass containers at -20°C.

F. ANALYTICAL PROCEDURE

1. Extraction

   a. Weigh 5 ± 0.05 g of thawed homogenized sample in duplicate into a 50 mL centrifuge tube.

      Ground beef :, weigh two 5.0 ± 05 g of blank tissue into 50 mL centrifuge tubes. Use the first tube as a negative blank and fortify the second tube as a recovery by adding 25 µL of working standard (10 µg/mL) for a 50 ppb recovery.

      RTE products: weigh two 5.0 ± 05 g of blank tissue into 50 mL centrifuge tubes. Use the first tube as a negative blank and fortify the second tube as a recovery by adding 50 µL of working standard (100 µg/mL) for a 1 ppm recovery.

b. Add 25 mL extraction solvent, acetonitrile/water 1:1 solution, into each tube and vortex or shake vigorously for approximately 1 min or until sample is thoroughly mixed.

c. Centrifuge at 2500 rpm for 10 min. Transfer 5 mL of the supernatant into a 15 mL centrifuge tube.

2. Methylene Chloride Wash

a. Add 250 µL of 1N HCl to the tube and add enough methylene chloride to fill the tube to 2 cm from the top. Cap and shake for approximately 1 min. Check the pH of the solution with a pH strip, the pH should be below 6.0. Adjust pH if necessary with 1N HCl.

b. Centrifuge at 2500 rpm for 10 min. Transfer the upper layer to a 15 mL glass culture tube.

c. Extract the sample in the centrifuge tube a second time by adding approximately 4 mL Optima water. Cap and shake for approximately 1 min.

d. Centrifuge at 2500 rpm for 10 min. Transfer the upper layer to the glass culture tube containing the first transfer.

3. SPE Cleanup

Note: For elutions in steps a, b, c, and e below use gravity flow, do not use vacuum.

a. Condition the Oasis MCX with 5 mL methanol followed by 5 mL Optima water.

b. Load sample onto the column. Discard the eluate.

c. Wash column with 5 mL 0.1 N HCl followed by 2 mL methanol. Discard eluates.

d. Aspirate the column for approximately 30 sec using vacuum. Discard eluate.

e. Elute the sample by gravity with 5 mL 5% (v/v) ammonium hydroxide in acetonitrile into a glass culture tube.

f. Gently evaporate sample to about 1.5 mL on N-EVAP at approximately 60 °C.

Ground Beef: transfer quantitatively into 2 mL volumetric flask by washing culture tube with acetonitrile and bring to volume. Mix well.

RTE Products: transfer quantitatively into 25 mL volumetric flask by washing culture tube with acetonitrile and bring to volume. Mix well.

g. Transfer a portion of the sample to a microfilterfuge tube and centrifuge at

8000 rpm for 5 minutes. Transfer about 300 µL of the filtered solution to an LC injection vial containing a glass insert.

4. Instrumental Settings

Note: The following instrument parameters may be optimized.

a. Triple Quad (TQD)

i. LC Parameters:

Flow: 0. 4 mL/min.

Injection volume: 2 µL

Autosampler Temp: 20 °C

Run Time: 6 min.

Column Temp.: 45 °C

Solvent A: 20 mM ammonium formate

Solvent B: Acetonitrile

HPLC Mobile Phase Gradient Table:

Run time (min)	Flow rate (mL/min)	Solvent A %	Solvent B (%)
0	0.4	10	90
2.0	0.4	10	90
2.1	0.4	50	50
3.1	0.4	50	50
3.2	0.4	10	90
8.0	0.4	10	90

ii. MS Parameters:

The LC-MS-MS electrospray (ESI) conditions are optimized by tuning with a 10 ng/µL solution of Melamine dissolved in 90:10 (v/v) acetonitrile:ammonium formate buffer (20 mM) at 0.4 mL/min.

Optimize for m/z 127 for parent and m/z 85 for product

Spray Voltage: 3.5 kV

Desolvation flow: 900 L/hr

Cone flow: 20 L/hr

Capillary Temperature: 350 °C

MRM: Parent 127; Daughters 85 and 68

ESI Positive ion mode

b. Linear Ion Trap (LTQ)

i. LC Parameters:

Flow: 0. 350 mL/min

Injection volume: 1.0 µL

Autosampler Temp: off

Run Time: 10 min.

Column Temp.: 45 °C

Solvent A: 20 mM ammonium formate

Solvent B: Acetonitrile

HPLC Mobile Phase Gradient Table:

Run time (min)	Flow rate (mL/min)	Solvent A %	Solvent B (%)
0	0.350	5	95
4.0	0.350	50	50
4.1	0.350	5	95
10.0	0.350	5	95

ii. MS Parameters

The LC-MS-MS electrospray (ESI) conditions are optimized by tuning with a 10 ng/µL solution of Melamine dissolved in 90:10 (v/v) acetonitrile:ammonium formate buffer (20 mM) at 0.350 mL/min.

Optimize for m/z 127 for parent and m/z 85 for product

Source Voltage: 4.5 kV

Capillary Voltage: 4.0 V

Sheath flow: 78

Aux flow: 30.0

Sweep flow: 19.0

Capillary Temperature: 300 °C

ESI Positive ion mode

G. DETERMINATION AND CONFIRMATION

Note: Because final dilution of the sample equals to approximately 0.5 grams per mL, each fortified sample is equivalent to half of its value in tissue. (Example; a found 50 ng/mL is equivalent to ~100 ng/g in tissue)

1. Determination Triple Quad (TQD)

   a. Determine retention times and peak areas for all standards, controls, and samples.

   b. The Chromatographic peak (127 → 85 transition) of the standard and fortified sample should have a signal to noise ratio greater than 3.

   c. Using a linear regression analysis, construct a standard curve. Plot peak area (area counts m/z 127 → 85) (y-coordinate) against standard concentration (ng/mL) (x-coordinate). Calculate slope, intercept, and correlation coefficient (r) of the regression line. Samples may be quantified only if the correlation coefficient calculated equals or exceeds 0.995 and sample response is within the standard calibration curve.

   d. Identification of melamine in a sample requires that its chromatogram shows a peak eluting with a retention time within ± 5% of that determined for the melamine peak in the fortified sample.

   e. Calculate melamine concentration in samples using the equation:

      Melamine (ppb) = ((Area for m/z 85 – Y intercept)/Slope) * (Dilution Factor/Weight)

      Note: Dilution factor ≈ 2

2. Confirmation Triple Quad (TQD)

   a. Product ion abundance ratios must match that of the external standard within 10% absolute difference. The following dissociated ions are monitored;

m/z 127 → 85

m/z 127 → 68

b. The solvent blank analyzed after the recovery must be negative for melamine.

c. The tissue blank must be negative for melamine.

d. The fortified tissue must be positive for melamine.

3. Determination Linear Ion Trap (LTQ)

a. Determine retentions times and peak areas for the daughter ion at 85 m/z for all standards, controls, and samples.

b. The Chromatographic peak for daughter ion 85 m/z of the standard and fortified sample should have a signal to noise ratio greater than 20.

c. Using a linear regression analysis, construct a standard curve. Plot peak area (area counts m/z 85) (y-coordinate) against standard concentration (ng/mL) (x-coordinate). Calculate slope, intercept, and correlation coefficient (r) of the regression line. Samples may be quantified only if the correlation coefficient calculated equals or exceeds 0.995 and sample response is within the standard calibration curve.

d. Identification of melamine in a sample requires that its chromatogram shows a peak eluting with a retention time within ± 5% of that determined for the melamine peak in the fortified sample.

e. Calculate melamine concentration in samples using the equation:

Melamine (ppb) = ((Area for m/z 85 – Y intercept)/Slope) * (Dilution Factor/Weight)

Note: Dilution factor ≈ 2

4 Confirmation Linear Ion Trap (LTQ)

a. The full MS2 spectrum obtained from the molecular ion at m/z 127 in the samples should visually match the spectrum obtained form the standards.

b. At least three structurally-significant ions are present (m/z 68, 110, 85, and 127).

c. There should be general correspondence between the relative abundances of the samples and those of the standards.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment: Protective clothing, eyewear, and gloves.

2. Hazards

Reagent	Hazard	Recommended Safe Procedures
Methanol	Poison, flammable.	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Keep away from heat, sparks, and open flames.
Acetonitrile	Flammable, Irritant, harmful if swallowed, inhaled or absorbed through the skin.	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Keep away from heat, sparks, and open flames.
Methylene Chloride	Harmful if swallowed or inhaled. Causes eye, skin, and respiratory track irritation. Potential cancer hazard.	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Keep away from heat, sparks, and open flames. Keep from contact with oxidizers.
Ammonium Formate	Irritant. Hygroscopic.	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation.
Ammonium Hydroxide	Corrosive. Causes burns. Harmful if swallowed	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Store in caustic corrosives area. Keep from contact with oxidizers.
Hydrochloric Acid	Corrosive. Causes burns. Fatal if inhaled or swallowed.	Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Store in acid corrosives area. Store away from oxidizers and alkalis.

3. Disposal Procedures

Reagent	Hazard	Recommended Safe Procedures
Methanol	See section 2 above	Collect waste in a sealed container and store in a cool, well ventilated. Flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acetonitrile	See section 2 above	Collect waste in a ground tightly closed container. Keep from contact with oxidizing

Reagent	Hazard	Recommended Safe Procedures
		materials. Store in a cool, well ventilated flammables area.
Methylene Chloride	See Section 2 above	Collect waste in tightly closed container. Keep from contact with oxidizing materials. Store in a cool, dry, well ventilated area.
Hydrochloric Acid	See section 2 above	Collect waste in a sealed container and store in a cool, well ventilated away from incompatibles. Acid liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Ammonium Formate	See section 2 above	Collect waste in a sealed container and store in a cool, well ventilated, away from oxidizers. Dispose in accordance with local, state, and Federal regulations.
Ammonium Hydroxide	See section 2 above	Collect waste in sealed container and store in a cool, well ventilated room, away from oxidizing agents, acids, halogen, silver nitrite, dimethyl sulfate, silver oxide. Dispose in accordance with local, state and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	Sample Analytical Range	Acceptable Recovery	Acceptable Repeatability (CV)
Melamine	25 - 100 ng/g (ground beef) 0.5 - 2 µg/g (RTE products)	70 -110%	20%

2. Critical Control Points and Specifications

	Record	**Acceptable Control**
a.	Sample weight	5.0 ± 0.05 g
b.	Evaporation	Avoid evaporation to dryness
c.	Sample pH (Section F.2.a)	pH < 6.0

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards- Generate a standard curve in duplicate on

three working days at the following levels:

0 ng/mL

10 ng/mL

25 ng/mL

50 ng/mL

75 ng/mL

ii. Phase II: Fortified samples. Analyze in duplicate on three different days five samples fortified at the following levels:

(a) Ground beef: 0 ppb and 50 ppb

(b) RTE Products: 0 ppm and 1ppm

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

iii. Phase III: Check samples for analyst accreditation.

(a) Six unknown samples fortified at levels from 0 to 100 ppb (ground beef) and 0.5 to 2.0 ppm (RTE products), one of which should at the zero level. Fortification scheme is provided and dispensed by supervisor/designee or Quality Assurance Manager (QAM).

(b) Report analytical findings to supervisor.

(c) A letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1-3 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one blind check sample per analyst, per week, when samples are analyzed.

ii. Records to be maintained by analyst and reviewed by supervisor

b. Acceptability criteria.

If unacceptable values are obtained,

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: fresh ground beef and RTE products (e.g. cheese dogs, sausage, chicken fingers, meatballs, baby food (meat)).

b. Sample receipt size: minimum: 30 g.

c. Condition upon receipt: Cold (< 10° C).

d. Shelf life: 6 months.

e. Storage Condition: Store samples in glass containers at -20° C.

6. Sample Set

All of the standards, samples, and quality control samples are to be analyzed in a single automated run.

Each set must contain:

a. Solvent blank

b. Standard solutions

c. A negative blank tissue

d. A fortified blank tissue at 50 ppb for ground beef, 1 ppm for RTE products

e. Sample extracts

7. Sensitivity

Minimum proficiency level (MPL): 50 ppb (ground beef); 1 ppm (RTE products)

J. WORKSHEET

Following is an example of a worksheet.

MELAMINE BY HPLC/MS-MS

Method: CLG-MEL1._____

Date Started:_____ **Analyst:**_____

Date Completed:_____ **Reviewer:**_____

Number	Sample ID	Sample Amount (5.0 ± 0.05) g	pH (< 6) Sample at Section F.2.a.	ppb $_{melamine}$	RT (min)	Fortification (ppb)	% Recovery
1	Blank						
2	Recovery						
3	Internal Check						
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							

REAGENTS	IDENTIFICATION N°
Stock Std Soluion	
Working Std Sol.	
Acetonitrile/Water 1:1 (v/v)	
20 nM Ammonium Formate	
0.1 N Hydrochloric Acid	
5% Ammonium Hydroxide/ACN	
50% methanol in water	
1 N Hydrochloric acid	
0.1 N Hydrochloric Acid	

EQUIPMENTS	IDENTIFICATION N°
Balance	
Pipets	
LC/MS-MS	
Strata X-C	
Pipets	
pH strips Lot No.	

K. APPENDIX

1. Chromatograms

   a. Melamine External Standard at 50 ppb

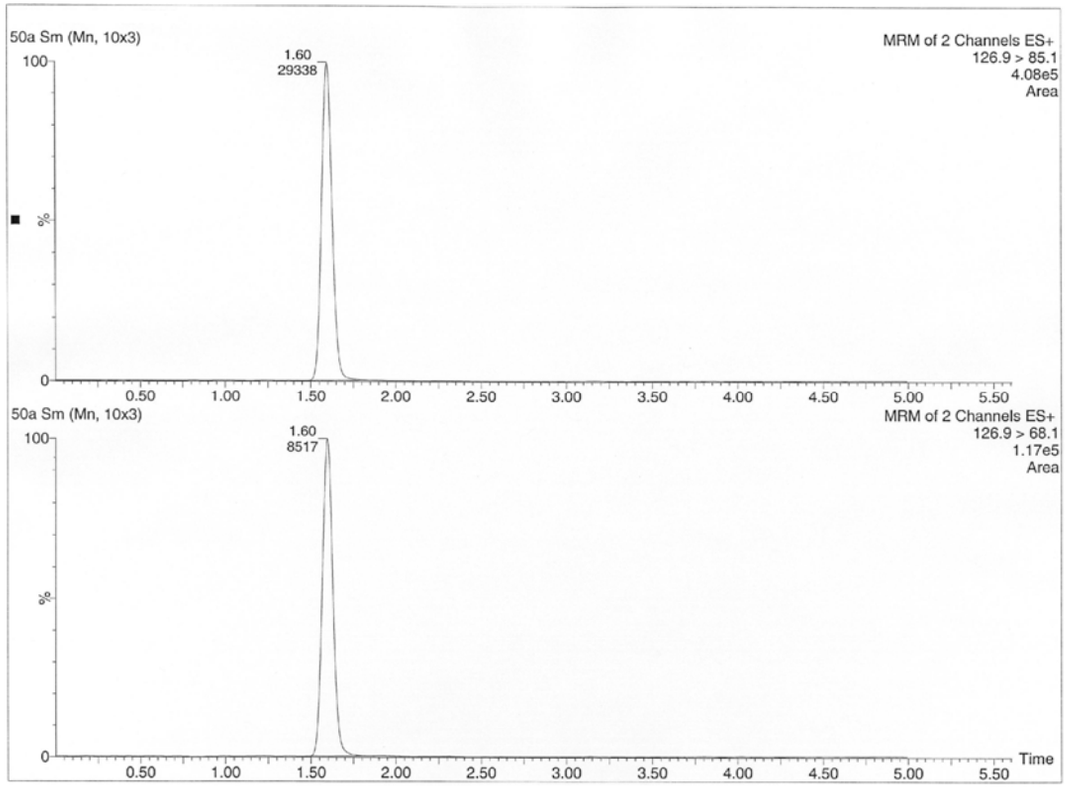


Figure 1. TQD Spectra of 50 ppb Melamine External Standard

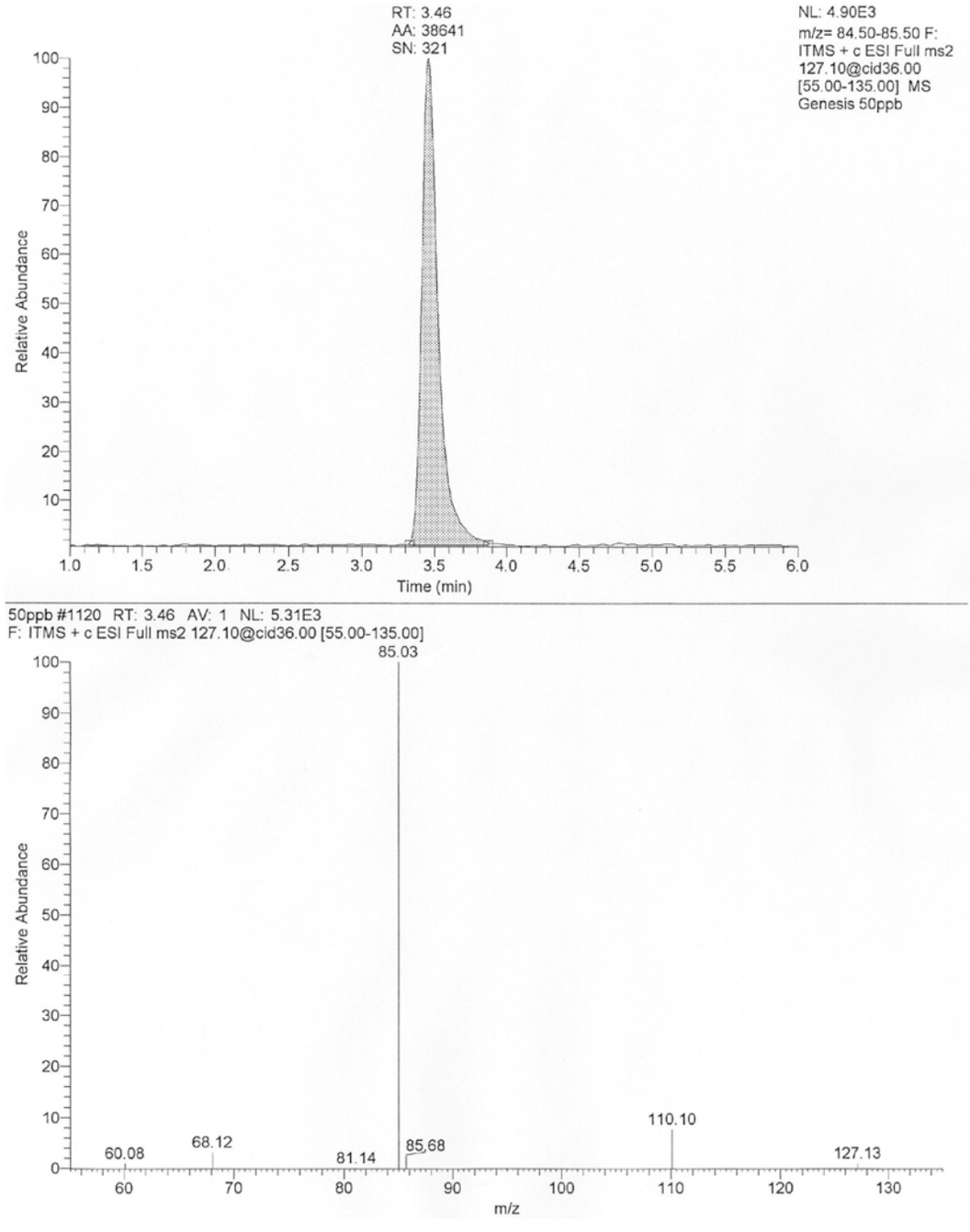


Figure 2. LTQ Spectra of 50 ppb Melamine External Standard

b. Negative Control (Blank) and Recovery Samples (Melamine 50 ppb), Ground Beef

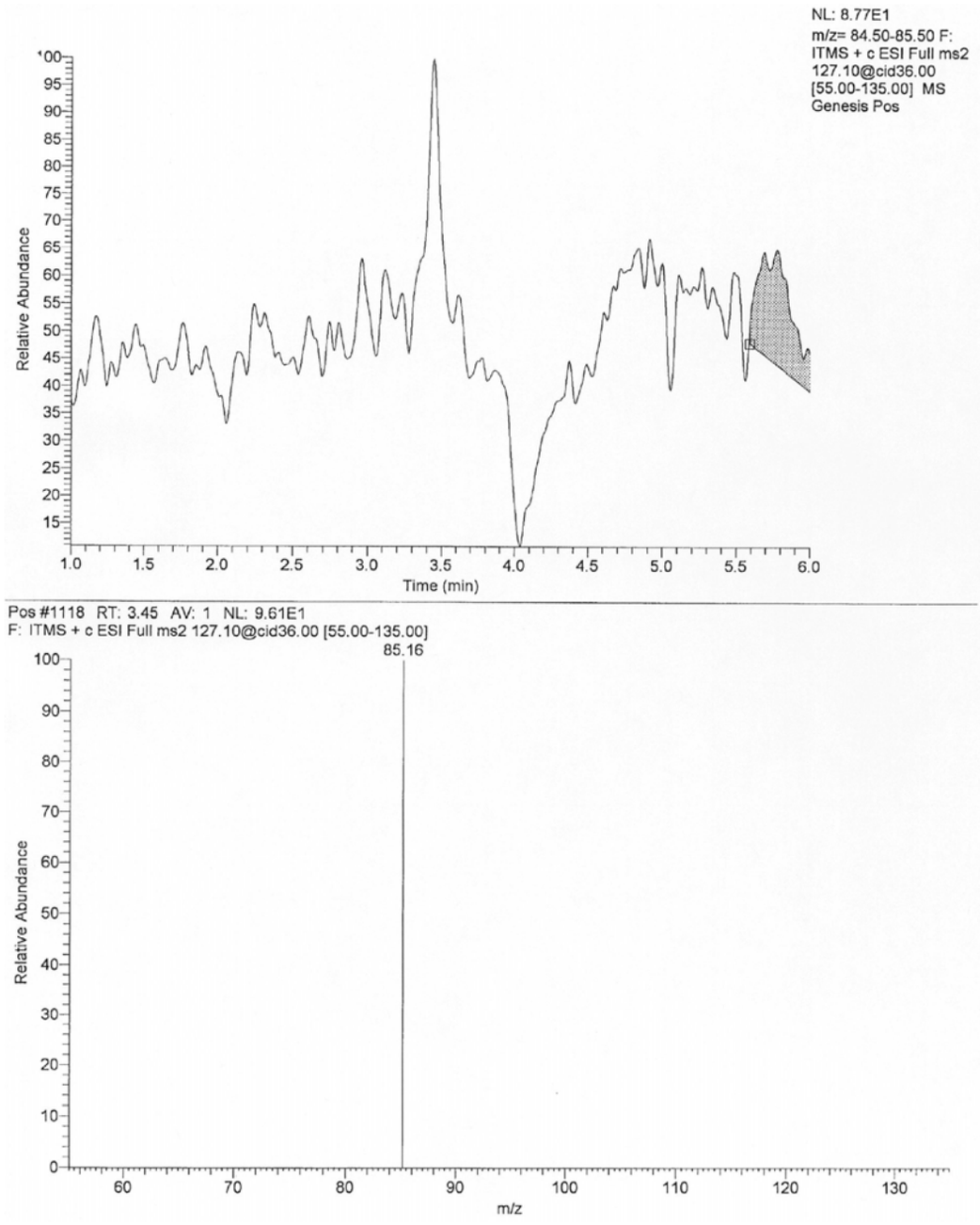


Figure 3. LTQ Spectra of Negative Control (Blank), Ground Beef

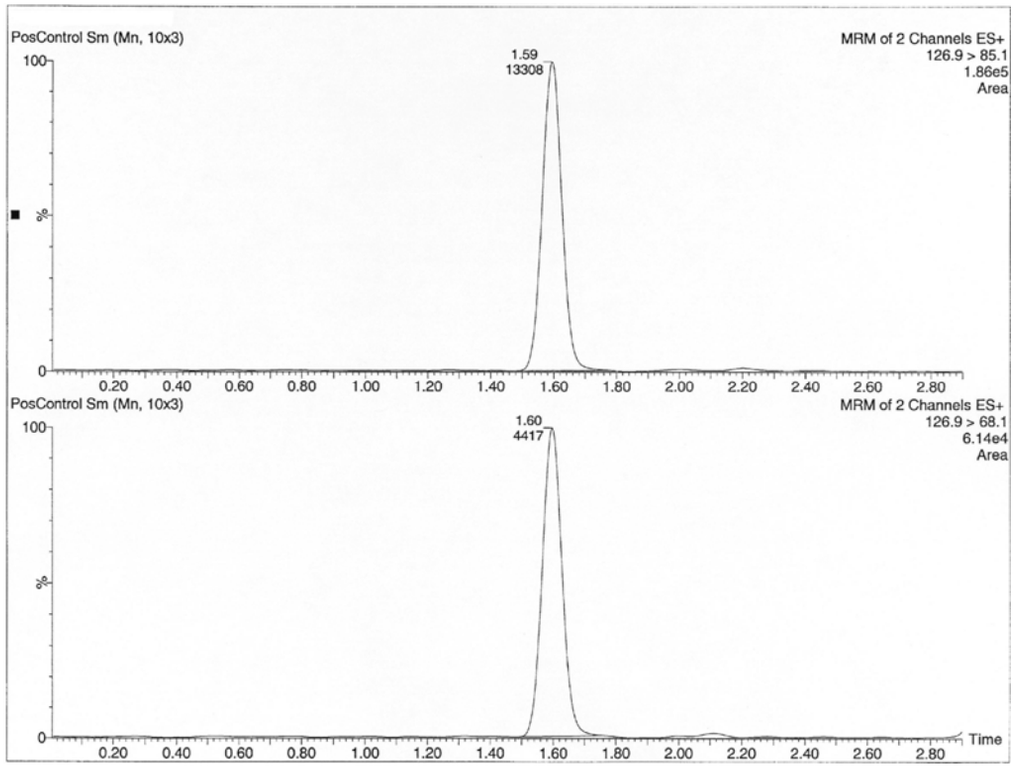


Figure 4. TQD Spectra of 50 ppb Melamine Recovery, Ground Beef

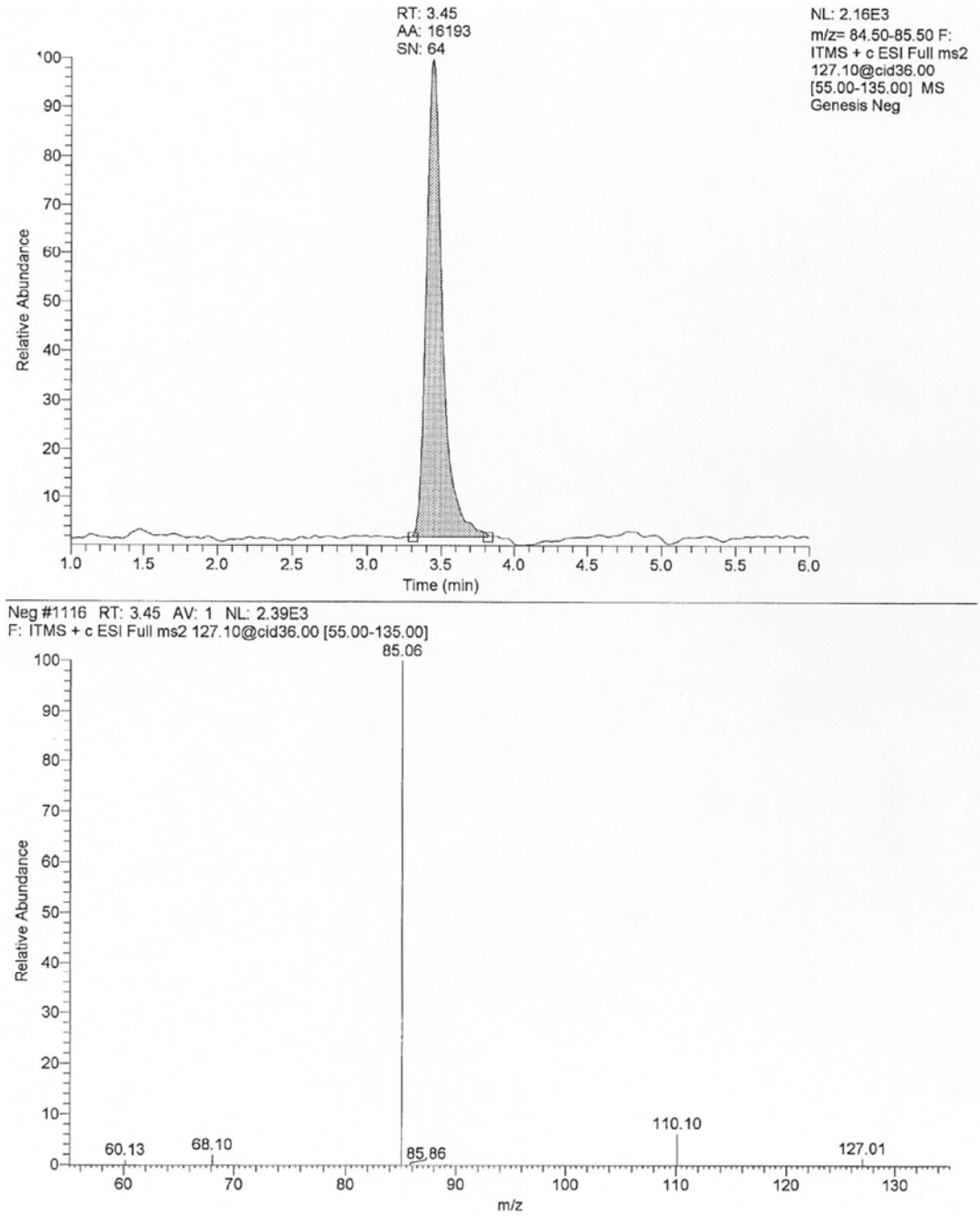


Figure 5. LTQ Spectra of 50 ppb Melamine Recovery, Ground Beef

c. Negative Control (Blank) and Recovery Samples (Melamine 1 ppm), in Veal Baby Food

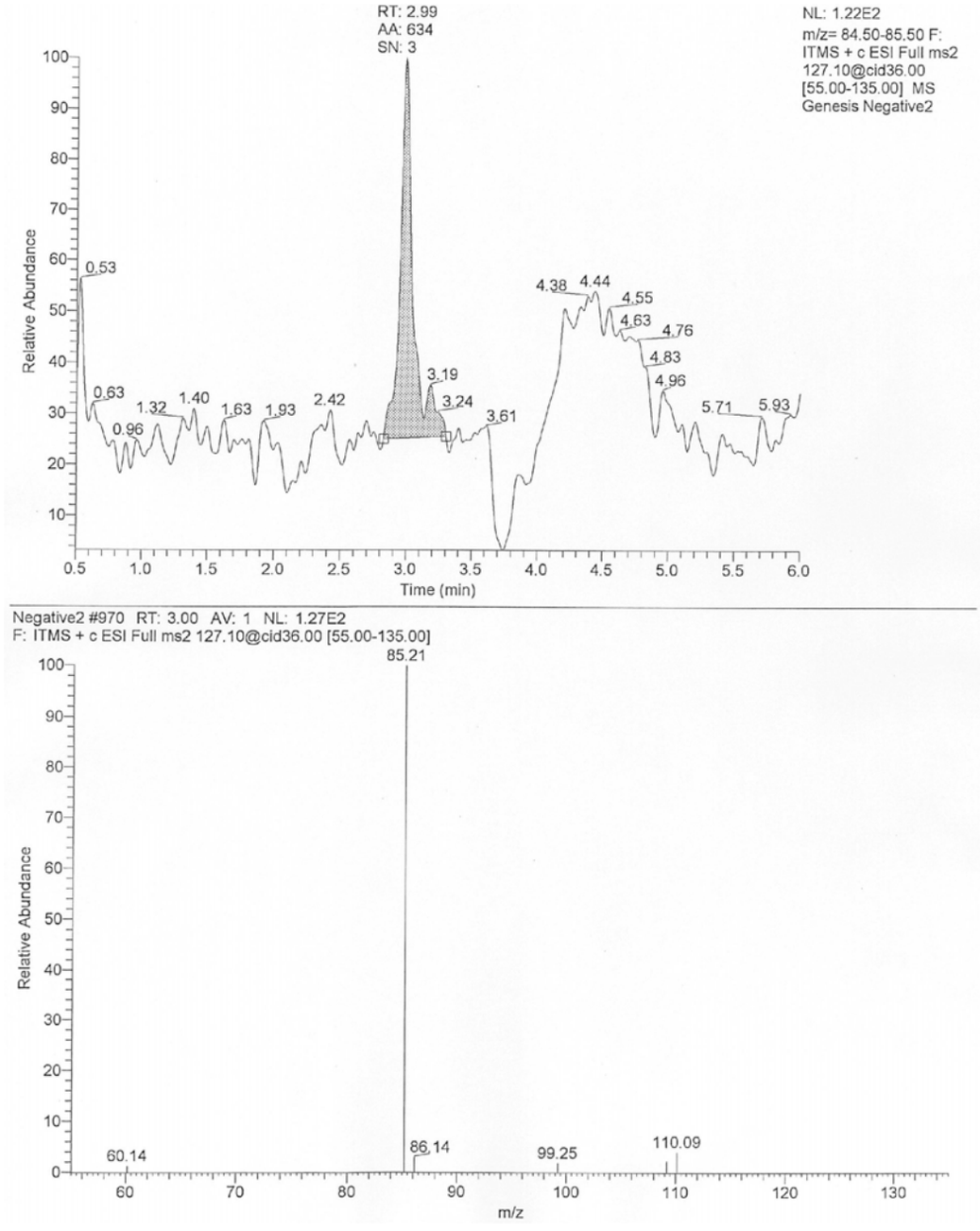


Figure 6. LTQ Spectra of Negative Control (Blank), Veal Baby Food

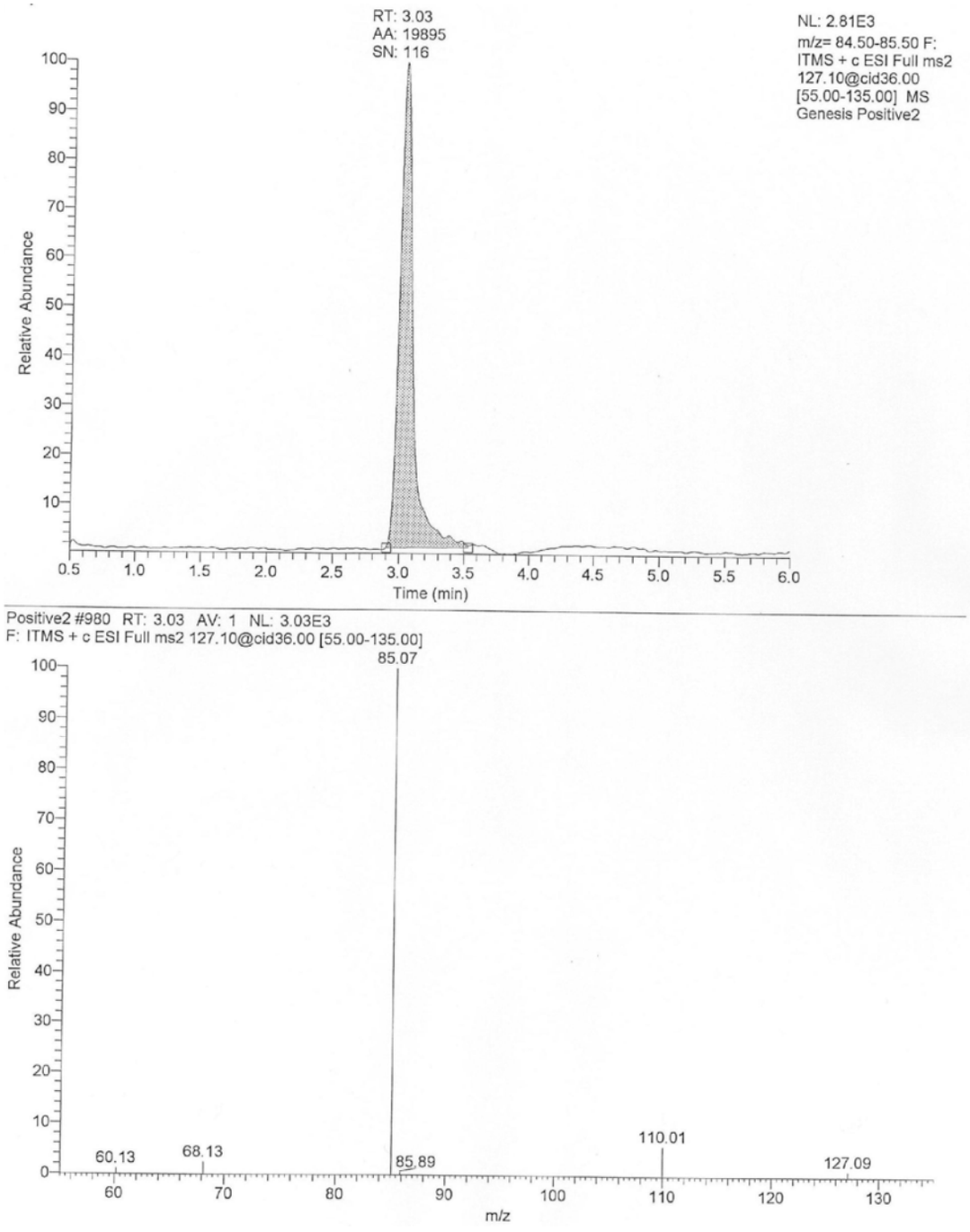
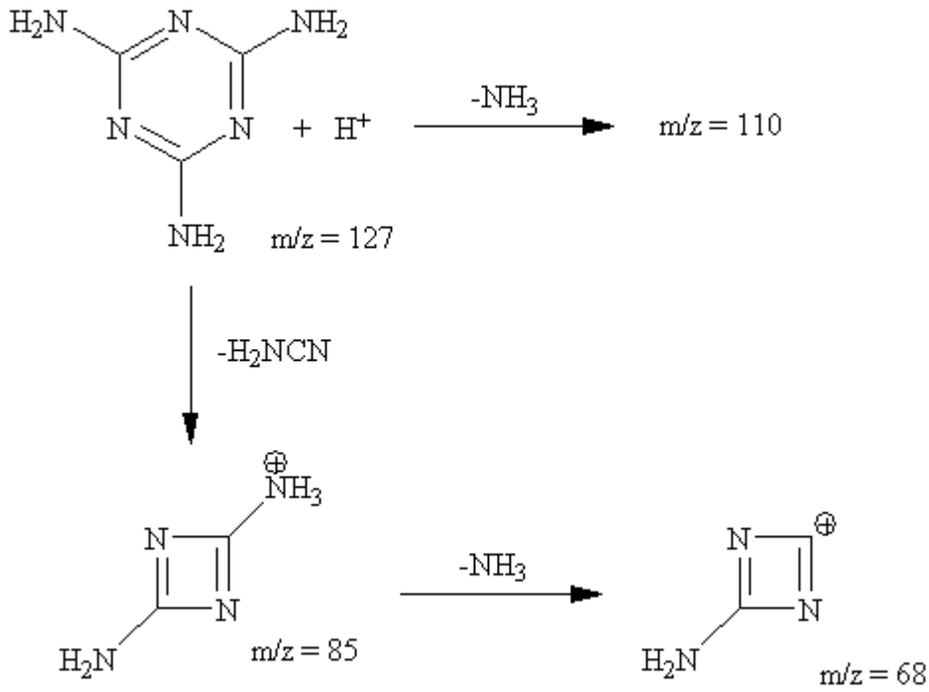


Figure 7. LTQ Spectra of 1 ppm Melamine Recovery, Veal Baby Food

2. Proposed Fragmentation Pattern of Melamine Product Ions



3. References

a. Anderson, W.C. Turnipseed, S.B., Karbiwnyk, C.M and Mandson,M.R. *"Determination of Melamine Residues in Catfish Tissue by TripleQuadrupole LC/MS-MS with HILIC Chromatography."* FDA, Denver, CO. Food and Drug Administration Laboratory Information Bulletin LIB No. 4396. Volume 23, May 2007

b. *"LC-MS/MS Method for the Analysis of Melamine in Porcine Meat Tissue"* California Health and Food Safety Laboratory, University of California at Davis, posted 4/30/2007, http://www.cahfs.ucdavis.edu

L. APPROVALS AND AUTHORITIES

Approvals on file.

Issuing Authority: Director, Laboratory Quality Assurance Division.