



Determination of Melamine, Ammeline, Ammelide and Cyanuric Acid in Infant Milk-Based Formula and Other Food and Feed Products Using the Varian 220-MS Ion Trap GC/MS/MS and V:Results™ GC/MS Software

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Introduction

In 2007, several pet food manufacturers recalled their products after finding melamine contamination, which caused serious illnesses in animals that consumed the food. In a follow-up investigation, the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) found melamine and its analogs, cyanuric acid, ammeline and ammelide (Figure 1), in various food and feed ingredients, including bakery meal, pet food, swine, poultry and fish feed¹. In September 2008, it was reported that milk products, especially infant formula, were contaminated with melamine in China. The melamine sickened at least 15,000 infants across the country and killed at least four. Allegedly, melamine was added to the milk formula and other vegetable protein products, such as wheat gluten and rice protein, to artificially increase the apparent protein levels due to its high nitrogen content. Although melamine itself may have low or no toxicity, it is believed that melamine and its analogs form insoluble crystals in urine, causing kidney stones and eventual acute renal failure².

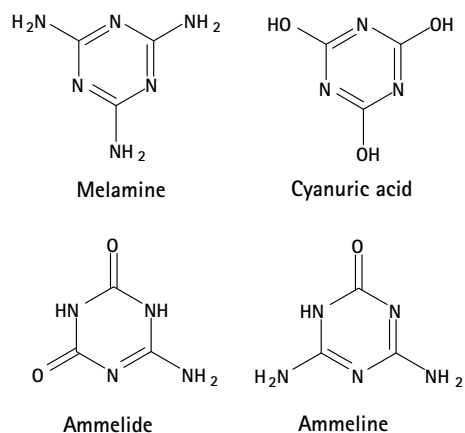


Figure 1. Melamine and related analogs.

Consequently, the US FDA developed a GC/MS method for screening and confirmation of melamine and its related analogs¹. However, the method was not evaluated for

quantitative analysis. In this note, we evaluated and developed a method using the Varian 220-MS ion trap mass spectrometer (Figure 2) to determine melamine and its analogs qualitatively and quantitatively based on the framework of the original FDA method.

Instrumentation

- Varian 220-MS Ion Trap Mass Spectrometer
- 431-GC gas chromatograph
- 8400 Trap AutoSampler
- Pierce Reacti-Therm/Reacti-Vap sample preparation system
- V:Results GC/MS software

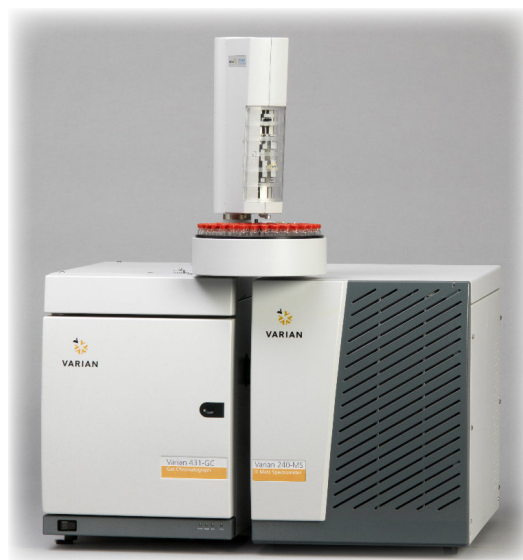


Figure 2. Varian 220-MS ion trap mass spectrometer with 431-GC gas chromatograph and 8400 AutoSampler.

Methods and Materials

Diethylamine (DEA) and pyridine (Sigma-Aldrich Co.)
Acetonitrile (Acros)
Extraction solvent: 10:40:50 DEA/water/acetonitrile

Sylating reagent: BSTFA with 1% TMCS:bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Supelco)
 Melamine and cyanuric acid (Sigma-Aldrich Co.)
 Ammelide and ammeline (TCA America)
 Dry dog food, cat food, and infant milk- based formula from a local supermarket.

Extraction Procedure

Approximately 0.5 g of a representative sample was weighed into a scintillation vial and extracted with 10 mL of extraction solvent (10:40:50 DEA/H₂O/ acetonitrile). The sample was mixed thoroughly, sonicated for 30 min, and centrifuged for 10-30 min at 15,000 rpm. The supernatant fluid was filtered using a 0.45 µm membrane.

Trimethylsiloxane (TMS) Derivatives

Transfer 200 µL filtrate from previous step to a 2 mL vial; evaporate to dryness at 70 °C with a low flow stream of dry nitrogen using the sample preparation system. Add 200 µL of pyridine and 200 µL BSTFA with 1% TMCS to the GC vial. Vortex to mix and incubate at 70 °C for 45 min.

Standard Curve

Prepare a stock solution containing melamine and its analogs (cyanuric acid, ammelide and ammeline) at 250 µg/mL 20:80 (v/v) in a mixture of DEA/H₂O. Dilute the stock solution to prepare calibration standards at 0.04, 0.1, 0.4, 1, 4, and 10 ppm in 20:80 (v/v) DEA/H₂O. Transfer 200 µL of each individual standard into in a 2.0 mL GC vial. Follow the same TMS-derivatization procedure as used for sample preparation in pyridine. Vortex to mix, and incubate at 70 °C for 45 min. The final concentration of the derivatized standards is 20, 50, 200, 500, 2000, and 5000 ppb.

GC Conditions

Column: FactorFour™ VF-Xms, 30 m × 0.25 mm × 0.25 µm, with 5 m EZ-Guard™ (Part no. CP9018)

Inlet Temperature: 280 °C

Injection Volume: 1 µL

Carrier Gas Flow: Helium at 1 mL/min

Injection Mode: Splitless

Oven Program: 75 °C for 1 min to 300 °C at 15 °C/min, and hold 4 min for a total run time of 20 min

MS Conditions

Filament Delay: 6 min

Manifold Temp: 50 °C

Transfer Line Temp: 280 °C

Trap Temp: 220 °C

Emission Current: 15 µamp

Full Scan Mass Range: 100-400

MS/MS Parameters*

Time (min)	Parent ion (m/z)	Excitation storage (m/z)	Excitation amplitude (m/z)	Product ion mass range (m/z)
7.0 - 9.45	345.2	105	60	100 - 380
9.45 - 10.24	344.2	105	65	100 - 380
10.24 - 10.90	328.3	105	72	100 - 380
10.90 - 12.50	327.3	105	80	100 - 380

*Using non-resonant waveforms

Results and Discussion

Melamine and its analogs, cyanuric acid, ammelide and ammeline, were analyzed in both full scan and MS/MS operation modes. The multiple reaction monitoring (MRM) trace of the TMS derivatives of these four compounds is shown in Figure 3.

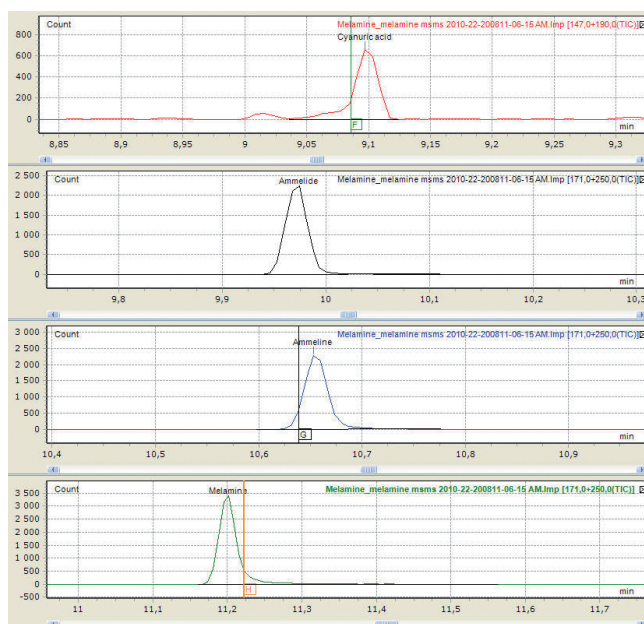


Figure 3. MRM of melamine and analogs in MS/MS operation mode at 10 ppb concentration.

This method provided excellent separation and identification of all melamine analogs. The quantitative determination of melamine was conducted from 20 to 2000 ppb in MS/MS mode. The calibration results are included in Table 1 and Figure 4. All compounds showed excellent linearity in MS/MS mode. The product ion spectra of melamine and its analogs are illustrated in Figure 5.

Table 1. Calibration of melamine and analogs.

Compound	Correlation coefficient (r ²)	Calibration range
Cyanuric acid	0.9997	20-2000
Ammelide	0.9975	20-2000
Ammeline	0.9994	20-2000
Melamine	0.9996	20-2000

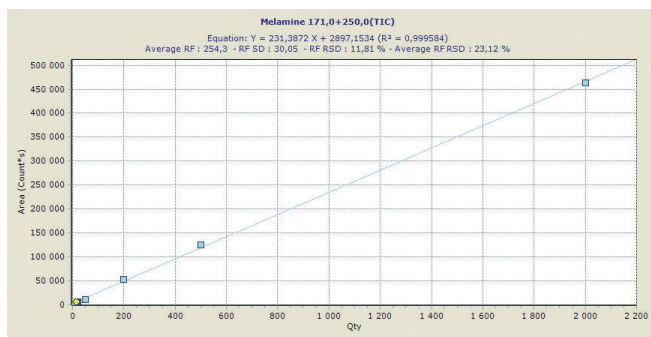


Figure 4. Calibration of melamine in MS/MS mode.

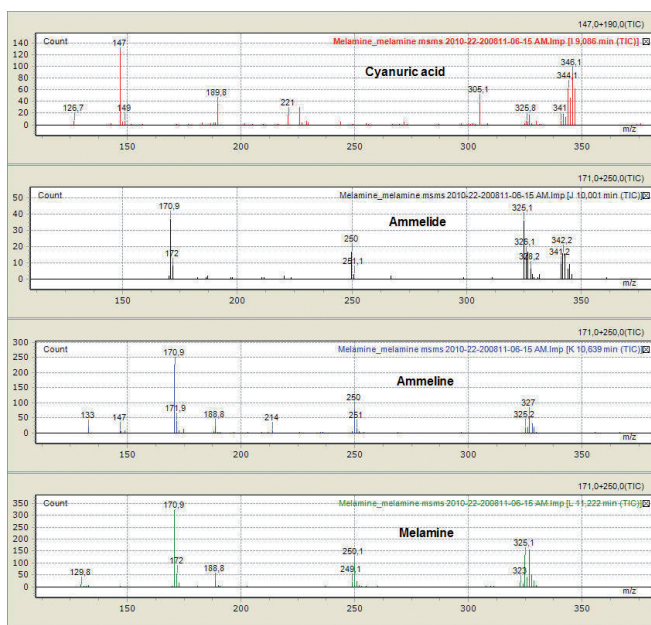


Figure 5. MS/MS product ion spectra of melamine and its analogs.

Three matrices were used to evaluate the robustness of this method. Melamine and its analogs (20 µg/g) were spiked in dry dog and cat foods and 5 µg/g was spiked in infant milk-based formula. The total ion chromatogram (TIC) of the spiked infant formula extract in full scan is shown in Figure 6. As indicated in the chromatogram, significant matrix

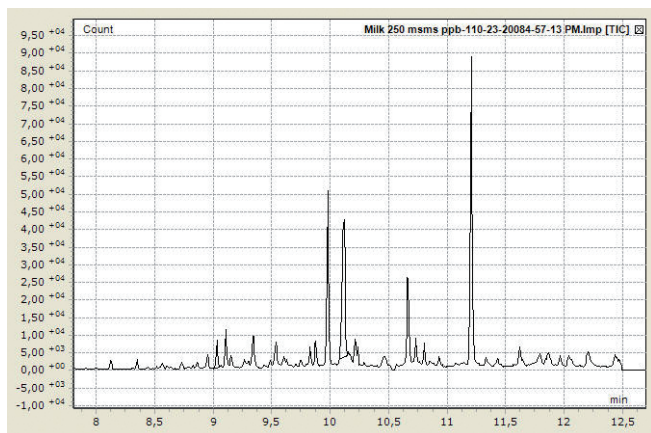


Figure 6. EIC of melamine and analogs spiked at 250 ppb in infant milk extract in full scan mode.

interference was observed in full scan acquisition even when displaying the extracted ion chromatogram (EIC). In MS/MS mode, the interference from the matrix was eliminated, as shown in Figure 7 with the infant milk-based formula extract.

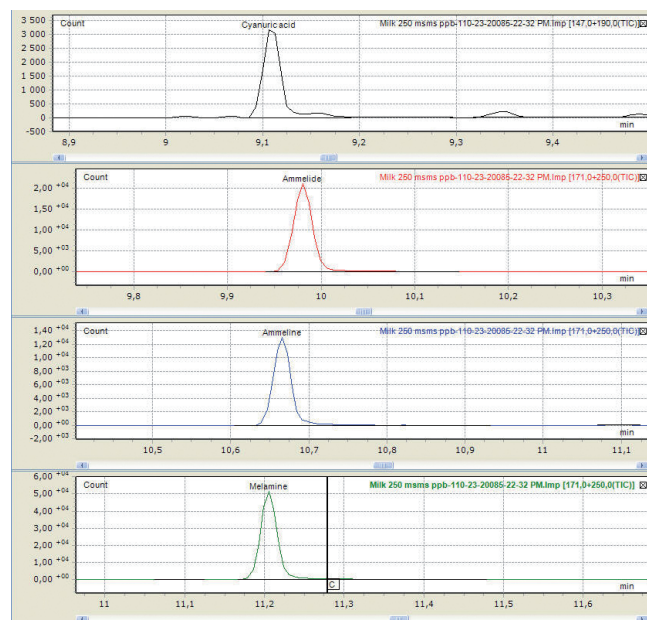


Figure 7. MRM of melamine and analogs spiked at 250 ppb in the infant formula extract in MS/MS mode.

Recovery studies of melamine, cyanuric acid, ammelide and ammeline were conducted in all three matrices at different concentrations (Table 2). Most compounds showed good recovery with excellent %RSD. Recovery of ammeline in infant milk was poorer overall with higher %RSD. This may have been due to variable extraction efficiency of ammeline from the infant milk matrix. Use of an internal standard, such as 2,6-diamino-4-chloropyrimidine, may alleviate the variation.

Table 2. Recovery and %RSD of melamine, cyanuric acid, ammelide and ammeline in MS/MS mode in dry dog food (n=3), dry cat food (n=3) and infant formula (n=6).

Average percentage recovery (%RSD)					
Matrix	Spiked (µg/g)	Cyanuric acid	Ammelide	Ammeline	Melamine
Dog food	20	132 (5)	104 (6)	102 (10)	119 (7)
Cat food	20	125 (8)	123 (9)	100 (9)	113 (8)
Infant milk	5	113 (12)	123 (8)	56 (22)	108 (6)

Conclusions

The method reported here, using the Varian 220-MS ion trap GC/MS/MS, screened and quantitated melamine and its analogs, cyanuric acid, ammelide and ammeline, in pet foods and infant formula at a concentration as low as 0.4

µg/g, which was 25 times lower than the FDA recommended level. MS/MS significantly eliminated matrix interference, providing an extra layer of confidence to positively identify and quantify target analytes. The linearity ranges studied were from 20 to 2000 ppb, and the detection limit was below 10 ppb for all four compounds.

References

1. FDA (2009) GC-MS Screen for the presence of Melamine, Ammeline, Ammelide and Cyanuric acid. US Food and Drug Administration, Silver Springs, MD, USA.
2. FDA (2009) Interim melamine and analogues safety/risk assessment. US Food and Drug Administration. US Food and Drug Administration, Silver Springs, MD, USA.

These data represent typical results.

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