



## Application Note 01864

# 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

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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 related compounds, cyanuric acid, ammeline and ammelide (Figure 1), in various food and feed ingredients, including bakery meal, pet food, swine, poultry and fish feed (1). In September 2008, it was reported that milk products, especially infant formula, were contaminated with melamine in China. The melamine sickened at least 60,000 infants across the country and killed at least 4. 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 related compounds will form insoluble crystals in urine, causing kidney stones and eventual acute renal failure (2).

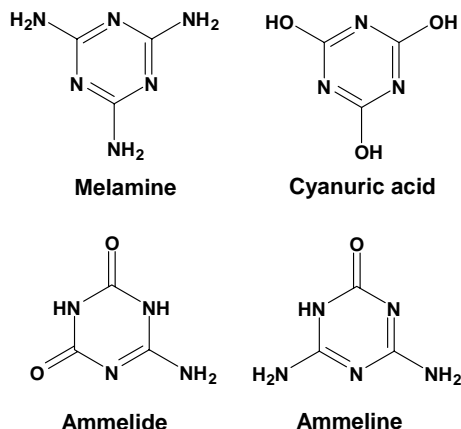


Figure 1. Melamine and related compounds.

Consequently, the US FDA developed a GC/MS method for the screening and confirmation of melamine and related compounds (1). 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 related compounds qualitatively and quantitatively based on the framework of the original FDA method.

### Instrumentation

- Varian 220-MS ion trap mass spectrometer
- Varian 431-GC gas chromatograph
- Varian CP-8400 AutoSampler
- Pierce Reacti-Therm/Reacti-Vap sample preparation system



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

### Reagents and Standards

- Diethylamine (DEA) (Sigma-Aldrich Co.)
- Pyridine (Sigma-Aldrich Co.)
- Acetonitrile (Acros)
- Extraction Solvent 10:40:50 DEA/water/acetonitrile
- Silylating Reagent: BSTFA with 1% TMCS : bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Supelco)
- Melamine (Sigma-Aldrich Co.)
- Ammelide (TCA America)
- Ammeline (TCA America)
- Cyanuric Acid (Sigma-Aldrich Co.)
- Dry dog food, cat food, and infant milk-based formula purchased from a local supermarket

### Method

**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/H<sub>2</sub>O/ 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- $\mu$ m membrane.

**Trimethylsiloxane (TMS) Derivatives.** Transfer 200- $\mu$ L filtrate from previous step to a 2-mL vial; Evaporate to dryness at 70 °C with a low flow stream of dry nitrogen using a Pierce Reacti-therm/ Reacti-Vap sample preparation system. Add 200  $\mu$ L of pyridine, 200- $\mu$ L 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 related compounds (cyanuric acid, ammelide and ammeline) at 250  $\mu$ g/mL 20:80 (v/v) in a mixture of DEA/H<sub>2</sub>O. Dilute the stock solution to prepare calibration standards at 0.04, 0.1, 0.4, 1 and 4 ppm in 20:80 (v/v) DEA/H<sub>2</sub>O. Transfer 200  $\mu$ L 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 extract concentration of the derivatized standards are 20, 50, 200, 500 and 2000 ppb.

### GC Conditions

Column: Varian FactorFour™ VF-Xms™ capillary column, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m, with 5 M EZ-guard™ (Varian Part Number CP9018)

Inlet Temperature: 280 °C  
 Injection Volume: 1  $\mu$ L  
 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  $\mu$ amps  
 Full Scan Mass Range: 100-400

### MS/MS Parameters\*

| Time (min) | Parent Ion (m/z) | Excitation Storage (m/z) | Excitation Amplitude (V) | 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                      |
| 10.24-10.9 | 328.3            | 105                      | 72                       | 100-380                      |
| 10.9-12.50 | 327.3            | 105                      | 80                       | 100-380                      |

\*Using non-resonant waveforms.

## Results and Discussion

Melamine and related compounds, 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.

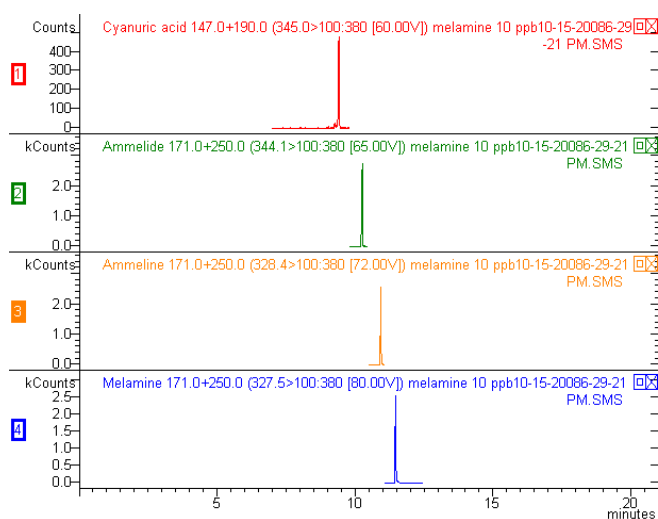


Figure 3. MRM of melamine and related compounds in MS/MS operation mode at 10 ppb concentration.

Table 1. Calibration of melamine and related compounds.

| Compound      | Correlation Coefficient | Calibration Range |
|---------------|-------------------------|-------------------|
| Cyanuric acid | 0.9998                  | 20-2000           |
| Ammelide      | 0.9977                  | 20-2000           |
| Ammeline      | 0.9994                  | 20-2000           |
| Melamine      | 0.9996                  | 20-2000           |

This method provided excellent separation and identification of all melamine related compounds. The quantitative determination of melamine was conducted from 20 to 2000 ppb (0.4 to 40  $\mu$ g/g) 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 related compounds are illustrated in Figure 5.

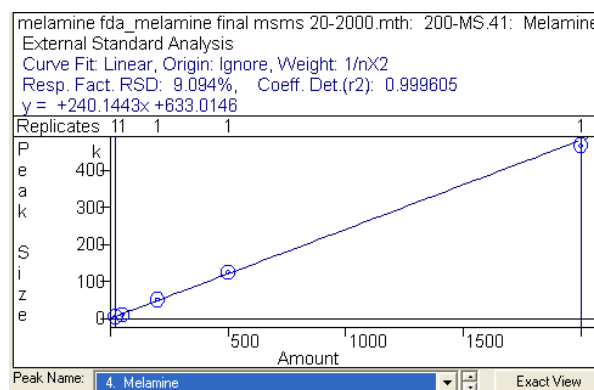


Figure 4. Calibration of melamine in MS/MS mode.

Spectra Plots - 10/24/2008 10:17 AM

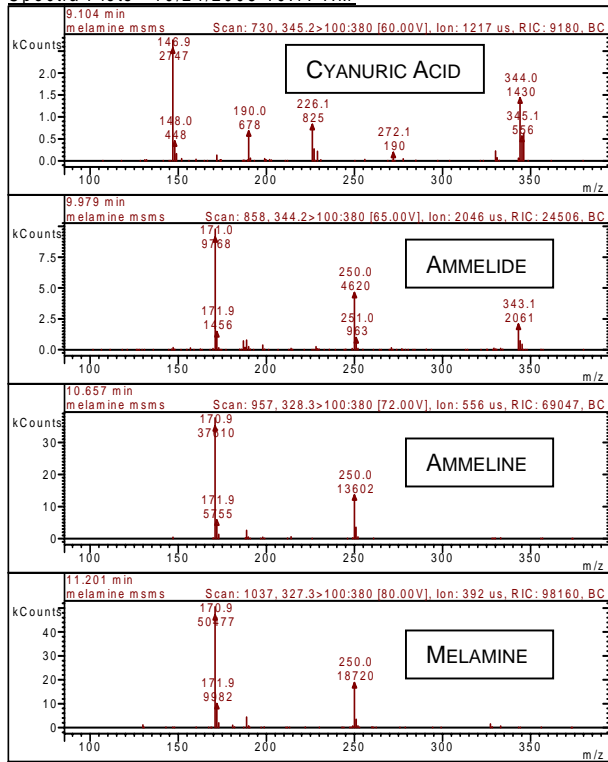


Figure 5. MS/MS product ion spectra of melamine and related compounds.

Three matrices were used to evaluate the robustness of this method. Melamine and related compounds (20 µg/g) were spiked in dry dog and cat foods; 5 µg/g were spiked in infant milk-based formula. The total ion chromatogram (TIC) of a spiked infant formula extract in full scan is displayed in Figure 6. As indicated in the chromatogram, significant matrix interference was observed in full scan acquisition even when displaying the extracted ion chromatogram (EIC). In MS/MS mode, the interference from the matrix is eliminated as shown in Figure 7 in the infant milk based formula extract.

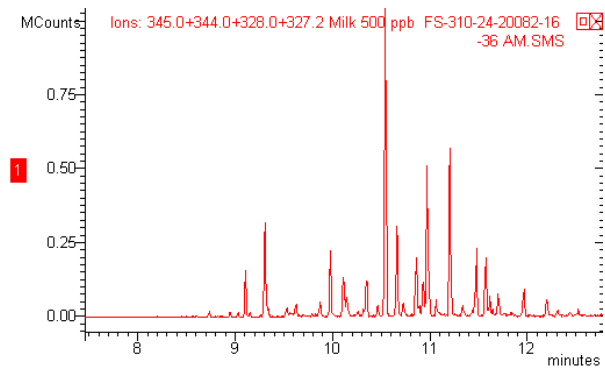


Figure 6. EIC of melamine and related compounds spiked at 500 ppb in infant milk extract in full scan mode.

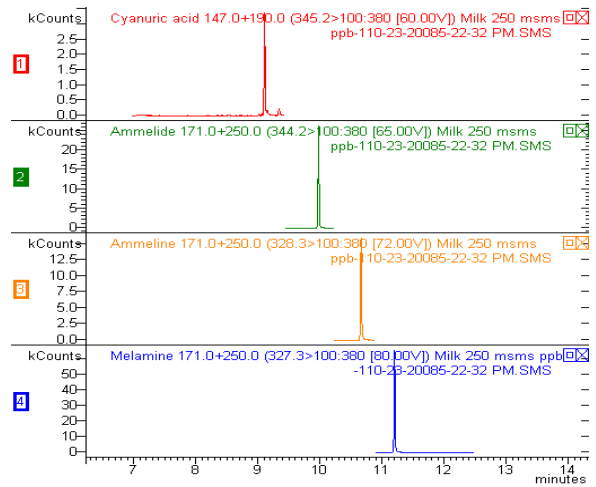


Figure 7. MRM of melamine and related compounds spiked at 500 ppb in the infant formula extract in MS/MS mode.

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. Ammeline showed slightly high %RSD. This may be 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            | 105 (3)       | 110 (1)  | 97 (2)   | 110 (2)  |
| Cat Food                           | 20            | 116 (4)       | 94 (1)   | 99 (3)   | 116 (1)  |
| Infant Milk                        | 5             | 116 (7)       | 101 (6)  | 86 (22)  | 110 (6)  |

Conclusion

The reported method using the Varian 220-MS ion trap GC/MS/MS can screen and quantitate melamine and related compounds, cyanuric acid, ammelide and ammeline, in pet foods and infant formula at a concentration as low as 0.4 µg/g, which is 25 times lower than the FDA minimum reporting level of 10 µg/g. MS/MS significantly eliminated matrix interference, providing an extra layer of confidence to positively identify and quantify target analytes.

## References

1. GC-MS Screen for the presence of Melamine, Ammelide, and Cyanuric acid. US Food and Drug Administration.
2. Interim melamine and analogues safety/risk assessment, Department of Health and Human Services, US Food and Drug Administration.  
<http://www.cfsan.fda.gov/~dms/melamra.html>

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