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 feed. 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 failure.

Consequently, the US FDA developed a GC/MS method for screening and confirmation of melamine and its related analogs. 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

Methods and Materials

Diethylamine (DEA) and 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 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/H$_2$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 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/H$_2$O. 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/H$_2$O. 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 x 0.25 mm x 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**
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Parent ion (m/z)</th>
<th>Excitation storage (m/z)</th>
<th>Excitation amplitude (m/z)</th>
<th>Product ion mass range (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0 - 9.45</td>
<td>345.2</td>
<td>105</td>
<td>60</td>
<td>100 - 380</td>
</tr>
<tr>
<td>9.45 - 10.24</td>
<td>344.2</td>
<td>105</td>
<td>65</td>
<td>100 - 380</td>
</tr>
<tr>
<td>10.24 - 10.90</td>
<td>328.3</td>
<td>105</td>
<td>72</td>
<td>100 - 380</td>
</tr>
<tr>
<td>10.90 - 12.50</td>
<td>327.3</td>
<td>105</td>
<td>80</td>
<td>100 - 380</td>
</tr>
</tbody>
</table>

*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.

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.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Correlation coefficient ($r^2$)</th>
<th>Calibration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanuric acid</td>
<td>0.9997</td>
<td>20-2000</td>
</tr>
<tr>
<td>Ammelide</td>
<td>0.9975</td>
<td>20-2000</td>
</tr>
<tr>
<td>Ammeline</td>
<td>0.9994</td>
<td>20-2000</td>
</tr>
<tr>
<td>Melamine</td>
<td>0.9996</td>
<td>20-2000</td>
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</table>
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 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.

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).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Spiked (µg/g)</th>
<th>Cyanuric acid</th>
<th>Ammelide</th>
<th>Ammeline</th>
<th>Melamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog food</td>
<td>20</td>
<td>132 (5)</td>
<td>104 (6)</td>
<td>102 (10)</td>
<td>119 (7)</td>
</tr>
<tr>
<td>Cat food</td>
<td>20</td>
<td>125 (8)</td>
<td>123 (9)</td>
<td>100 (9)</td>
<td>113 (8)</td>
</tr>
<tr>
<td>Infant milk</td>
<td>5</td>
<td>113 (12)</td>
<td>123 (8)</td>
<td>56 (22)</td>
<td>108 (6)</td>
</tr>
</tbody>
</table>

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.