Application Note # CA-270126

Determination of Melamine, Ammeline, Ammelide and Cyanuric Acid in Infant Milk-Based Formula and Other Food and Feed Products Using the 300-MS Triple Quadrupole GC/MS/MS

Introduction

In September 2008, it was reported that milk products, especially infant formula, were contaminated with melamine in China. The melamine sickened at least 300,000 infants across the country and killed at least 6. 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 [1]. FDA set thresholds:

- At or below 1 part per million (ppm) for melamine and cyanuric acid in infant formula (Nov. 2008)
- 2.5 parts per million (ppm) for melamine in foods other than infant formula (Oct. 2008)

Melamine and related analogues

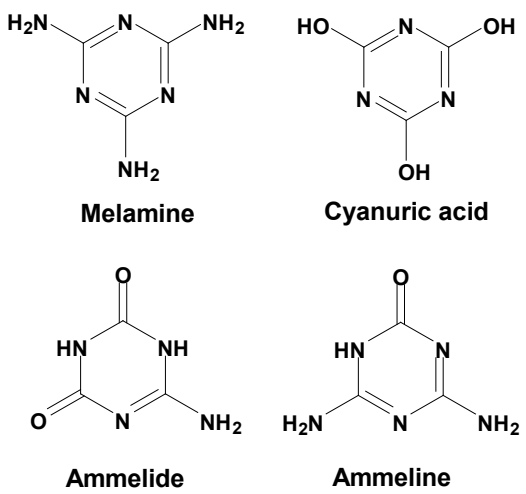

Figure 1: Melamine and related compounds.

Consequently, the US FDA developed a GC/MS method for the analysis of melamine and related analogues [2]. The minimum reporting level of this method was lowered from 10 to 2.5 µg/g to accommodate this new regulation. In this note, we evaluated and developed a method using the Bruker 300-MS triple quadrupole mass spectrometer (Figure 2) to determine melamine and related compounds quantitatively at ultra-trace levels based on the framework of the original FDA method.

Figure 2: Bruker 300-MS triple quadrupole mass spectrometer with 450-GC gas chromatograph and CTC Combi PAL autosampler.

Instrumentation

- 300-MS triple quadrupole mass spectrometer
- 450-GC gas chromatograph
- CTC Analytics Combi PAL™ autosampler
- Pierce Reacti-Therm/Reacti-Vap sample preparation system

Reagents and Standards

- Diethylamine (DEA) (Sigma-Aldrich Co.)
- 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 (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/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 a Pierce Reacti-Therm/Reacti-Vap sample preparation system. Add 200 µL of pyridine, 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 related compounds (cyanuric acid, ammelide and ammeline) at 250 µg/mL 20:80 (v/v) in a mixture of DEA/H_2O. Dilute the stock solution to prepare calibration standards at 0.2, 1, 2, 4, 10, 40, 100, 400, 1000 and 2000 ppb in 20:80 (v/v) DEA/H_2O. Transfer 200 µL of each individual standard into 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 concentrations of the derivatized standards are 0.1, 0.5, 1, 2, 5, 20, 50, 200, 500 and 1000 ppb, which correspond to 0.002, 0.01, 0.02, 0.04, 0.1, 0.4, 1, 4, 10 and 20 µg/g of sample concentration, respectively.

GC Conditions

Column: FactorFour™ VF-5ms™ capillary column, 30 m × 0.25 mm x 0.25 µm
Inlet Temperature: 220 °C
Injection Volume: 1 µL
Carrier Gas Flow: Helium at 1 mL/min
Injection Mode: Splitless
Oven Program: 75 °C for 4 min to 300 °C at 15 °C/min, hold 4 min, and to 320 °C at 20 °C/min, for a total run time of 24 min

MS Conditions

Filament delay: 9 min
Manifold Temp: 50 °C
Transfer Line Temp: 280 °C
Source Temp: 200 °C

Results and Discussion

Melamine and related compounds, cyanuric acid, ammelide and ammeline, were analyzed in MS/MS operation modes. The total ion chromatogram (TIC) of multiple reaction monitoring (MRM) trace of the TMS derivatives of these four compounds at 2 ppb is shown in Figure 3.
This method provided excellent separation and identification of all compounds. The quantitative determination of melamine was conducted from 0.1 to 1000 ppb in MS/MS mode. The calibration results are included in Table 1 and Figure 4.

MS/MS Parameters

Time (min)	Q1 (m/z)	Q3 (m/z)	Collision Energy (V)	Dwell Time (S)
9.0-12.5	345.2	147.1	10	0.075
	345.2	215.1	10	0.075
12.5-15.0	344.2	171.1	10	0.05
	344.2	189.1	10	0.05
	328.2	171.1	10	0.05
	328.2	189.1	10	0.05
	327.3	171.1	10	0.05
	327.3	189.1	10	0.05

Excellent separation and identification of compounds

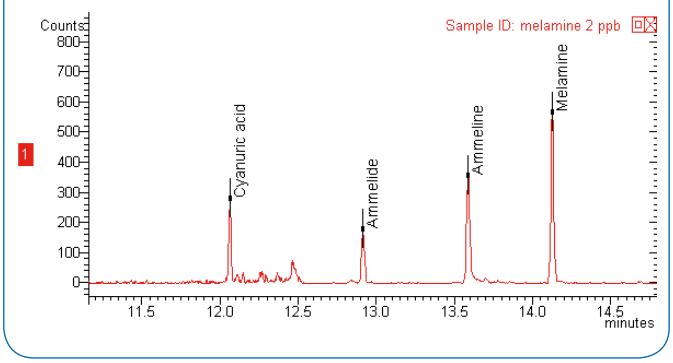

Figure 3: TIC of melamine and related compounds in MS/MS operation mode at 2 ppb concentration.

Table 1: Calibration of melamine and related compounds.

Compound	Correlation Coefficient	Calibration Range (ppb)
Cyanuric acid	0.9916	2-2000
Ammelide	0.9970	1-1000
Ammeline	0.9992	1-1000
Melamine	0.9995	0.1-1000

Excellent linearity in MS/MS mode

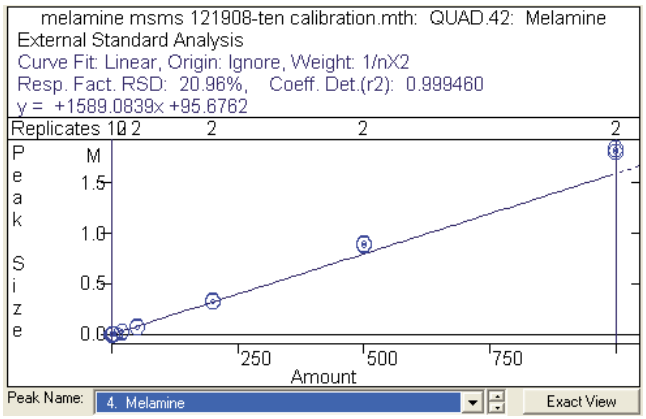

Figure 4: Calibration curve for melamine, 0.1 to 100 ppb, in MS/MS mode.

All compounds showed excellent linearity in MS/MS mode. Under these analytical conditions, melamine can be determined quantitatively as low as 0.1 ppb, which is equivalent to 0.002 µg/g in the sample (Figure 5).

Melamine determination at 0.1 ppb

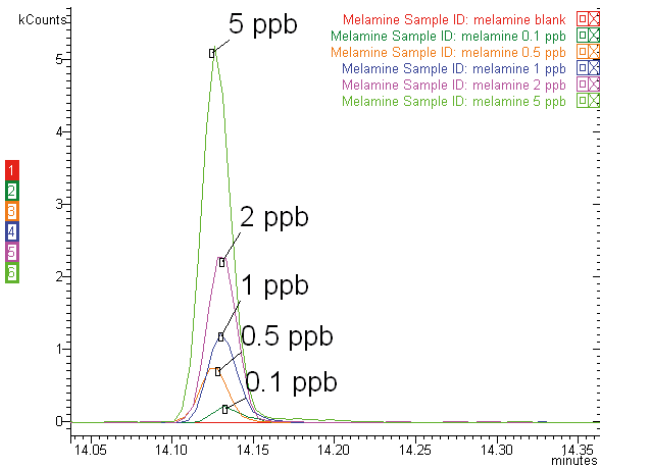

Figure 5: Chromatograms of melamine at 0.1, 0.5, 1, 2, and 5 ppb, which are equivalent to 0.002, 0.01, 0.02, 0.04 and 0.1 µg/g in the sample, respectively.

Three matrices were used to evaluate the robustness of this method. Melamine and related compounds (5 µg/g) were spiked in dry dog and cat foods; 2.5 µg/g were spiked in infant milk-based formula (Figure 6). Recoveries were found to be between 90-120% for melamine, ammeline, ammelide, and cyuranic acid in the three matrices studied [3]. Ion ratios of melamine, ammeline and ammelide were determined by using the two product ions at m/z 171 and m/z 189; the ion ratio of cyanuric acid was determined by ions at m/z 147 and 215 (Table 2). There are no qualification mandate criteria in the FDA method. However, the Chinese National Standard requires a maximum allowed %RSD based on the relative ion abundance. If the ion ratio is less than 10%, the maximum RSD is < 50%; if ion ratio is 10%-20%, the maximum RSD is <30%; if the ion ratio is 20-50%, the maximum RSD is <25%; if ion ratio is >50%, the maximum RSD is <20% [3].
The results in Table 2 meet the requirement of the Chinese National Standard.

Robustness of the method

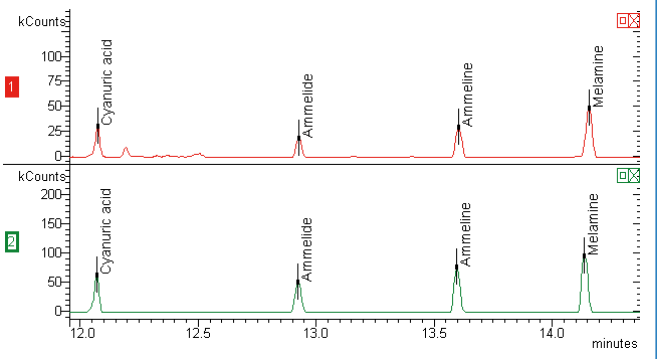

Figure 6: Chromatograms of melamine related compounds spiked in infant formula and dog food at 2.5 µg/g and 5 µg/g, respectively.

Table 2: Ion ratio of melamine and related compounds.

Com-pound	Melamine	Ammeline	Ammelide	Cyanuric Acid
Ion Ratio	189 to 171	189 to 171	189 to 171	215 to 147
Ratio	19.6%	19.1%	20.8%	32.2%
RSD	18.8%	15.7%	21.5%	22.8%

Conclusion

The reported method using the 300-MS triple quadrupole GC/MS/MS can quantitate melamine at ultratrace concentration levels: as low as 0.002 µg/g, which is:

- More than 1000 times lower than the FDA current minimum reporting level of 2.5 µg/g AND
- 500 times more sensitive than the FDA mandated threshold of 1 ppm in infant formula

This method can also quantify ammeline, ammelide and

cyanuric acid at the concentrations of 0.02, 0.02 and 0.04 µg/g, respectively. MS/MS significantly eliminated matrix interference, and provides an extra layer of confidence to positively identify and quantify target analytes.

References

[1] Interim melamine and analogues safety/risk assessment, Department of Health and Human Services, US Food and Drug Administration.

[2] Jonathan J. Litzau, Gregory E. Mercer, Kevin J. Mulligan. GC-MS Screen for the presence of Melamine, Ammeline, Ammelide and Cyanuric acid. US Food and Drug Administration Laboratory Information Bulletin No. 4423, Oct 2008.

[3] Determination of melamine in raw milk and daily products. China National Standard http://www.aqsiq.gov.cn/ztlm/nf/rdgz/200810/P020081008418357021863.pdf

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Instrumentation & Software
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