Application Note GCMS-03

Reliable high-throughput quantification of melamine and related analogs below regulatory limits in food using the EVOQ GC-TQ

Introduction

Melamine is a molecule that presents a high content of nitrogen, 67% by mass. It has many industrial applications in the form of melamine or melamine resins. For example, it can be found in kitchenware, whiteboards, laminate flooring, colorant or fire retardant additives. In recent years, melamine has been fraudulently added to food and feed in order to increase their nitrogen ratios. The main reason for these fraudulent activities is to provide the food item with apparent higher protein content since many protein tests are only based on nitrogen content detection.

In 2007, after the death of a large number of animals, melamine was found in wheat gluten destined to pet-food manufacturing. Later in 2008, a food scandal occurred in China involving milk and infant formula which was adulterated with melamine, leading to kidney stones and other renal failure among young children.

According to a WHO report from 2009 and a report from the Chinese Ministry of Health, 294,000 infants had been affected by melamine-contaminated infant formulae by the end of November 2008. More than 50,000 infants were hospitalized and six deaths have been confirmed.

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Melamine Analogs	
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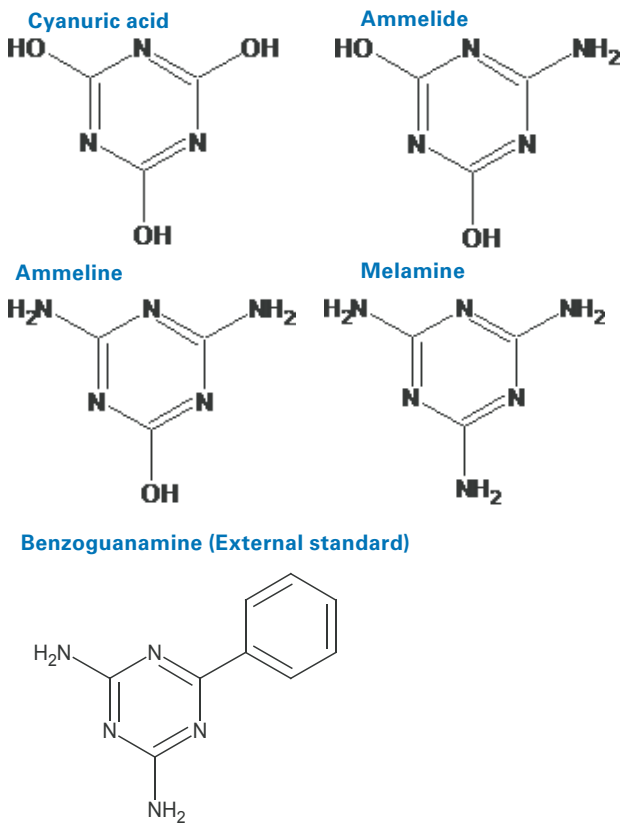
Chemical structure of melamine and its analogs



Taking into account the available occurrence data, the level of 2.5 mg/kg in foodstuff was chosen as an appropriate level to distinguish between the unavoidable background presence of melamine and unacceptable adulteration. For infant formula, this level was lowered to 1 mg/kg, as described in Commission Regulation (EU) No 594/2012, amending Regulation 1881/2006/EC. These levels take into account the need to ensure a large margin of safety.

The development of a specific and sensitive method based on Selected Reaction Monitoring (SRM) with a GC-MS/MS system was conducted to ensure unambiguous identification of this compound in foodstuff. With the goal of having a fast and high-throughput method, a rapid sample preparation was used without any SPE cleanup.

Since it was proven that the toxicity of melamine increased in the presence of cyanuric acid, it was decided to investigate broader contamination profiles, i.e., including not only melamine but also its analogs using the EVOQ GC-TQ. Structures of these compounds are illustrated above.

The calibration standards ranged from 0.5 to 10 mg/kg of melamine and analogs. All analyzed food samples (unknown, blank and supplemented) were submitted to solid / liquid extraction, by adding 4 mL of a H_2O / Acetonitrile mixture (50/50, v/v), and agitated for 30 sec., followed by 30 min. sonication and centrifugation for about 10 min. at 2000 g. The obtained liquid phase was filtered through a 0.45 µm nylon filter, and 50 µL of filtrate was then spiked with benzoguanamine (external standard). This solution was evaporated, dissolved in 50 µL of MSTFA and then submitted to derivatization by heating 60°C for 45 min.

GC-MS/MS Conditions

The samples and calibration solutions were injected with the following GC and MS/MS parameters. For ionization, filament current was set to 80 µA. Argon was used as the collision gas at a pressure of 2.0 mTorr.

Chromatographic conditions

Column	ZB5 MS 30 m x 0.25 mm x 0.25 µm
Gas	Helium, 1.0 mL/min constant flow
Injector	280°C, Splitless
Injection volume	2 µL

Oven temperature program

Rate (°C/min)	Temperature(°C)	Hold (min)
-	120	1
10	320	2

Instrument

Gas chromatography (436 GC, Bruker) coupled to a triple quadrupole mass spectrometer (EVOQ GC-TQ, Bruker).

Material and reagents

Melamine, ammeline, ammelide, cyanuric acid and benzoguanamine were purchased from BCP Instruments (Irigny, France), $^{13}C_3$-$^{15}N_3$ labeled melamine and cyanuric acid from Cluzeau (Sainte-Foy-La-Grande, France). Mixed solutions containing melamine and its three analogs were made in H_2O / diethylamine (80/20, v/v). Benzoguanamine solution was prepared in pyridine, and $^{13}C_3$-$^{15}N_3$ labeled melamine and cyanuric acid solution were prepared in ultrapure water. Diethylamine, acetonitrile, MSTFA and pyridine were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Sample preparation

100 mg of sample (ground into a fine powder) was used for the analysis. The internal standard mixture was added to the sample at a concentration of 2 mg/kg. A blank food sample (containing no traces of melamine), and a range of various supplemented food samples used for calibration were prepared using the same protocol.

MS Conditions

Ionisation mode	Electron Impact, 70eV
Source temperature	250°C
Transfer Line Temperature	310°C
Acquisition mode	SRM
Quadrupoles Resolutions	Standard on Q1 (i.e. 2.0 amu width) Unit on Q3 (i.e. 0.7 amu width)

The various transitions and collision energies are summarized in the table below.

SRM Transitions monitored for each target analyte

Compound	Transition 1 (CE, V)	Transition 2 (CE, V)
$^{13}C_{3}$-$^{15}N_3$ Cyanuric Acid	351.1>147.0 10 V	-
Cyanuric Acid	345.1>147.0 10 V	345.1>188.0 5 V
Ammelide	344.1>171.0 15 V	344.1>189.0 10 V
Ammeline	343.1>171.0 15 V	343.1>189.0 15 V
$^{13}C_{3}$-$^{15}N_3$ Melamine	348.1>173.0 20 V	-
Melamine	342.1>171.0 20 V	342.1>327.1 10 V
Benzo-guanamine	331.1>171.0 20 V	331.1>316.1 10 V

For acquisition, windows of 3 minutes width were centered at the retention time for each compound, and scan times were set by the software, between 60 and 300 ms, adjusted for peak width (in sec), desired number of data points per peak, and number of transitions simultaneously monitored.

Chromatogram: blank (top) and spiked (bottom) sample of powdered milk

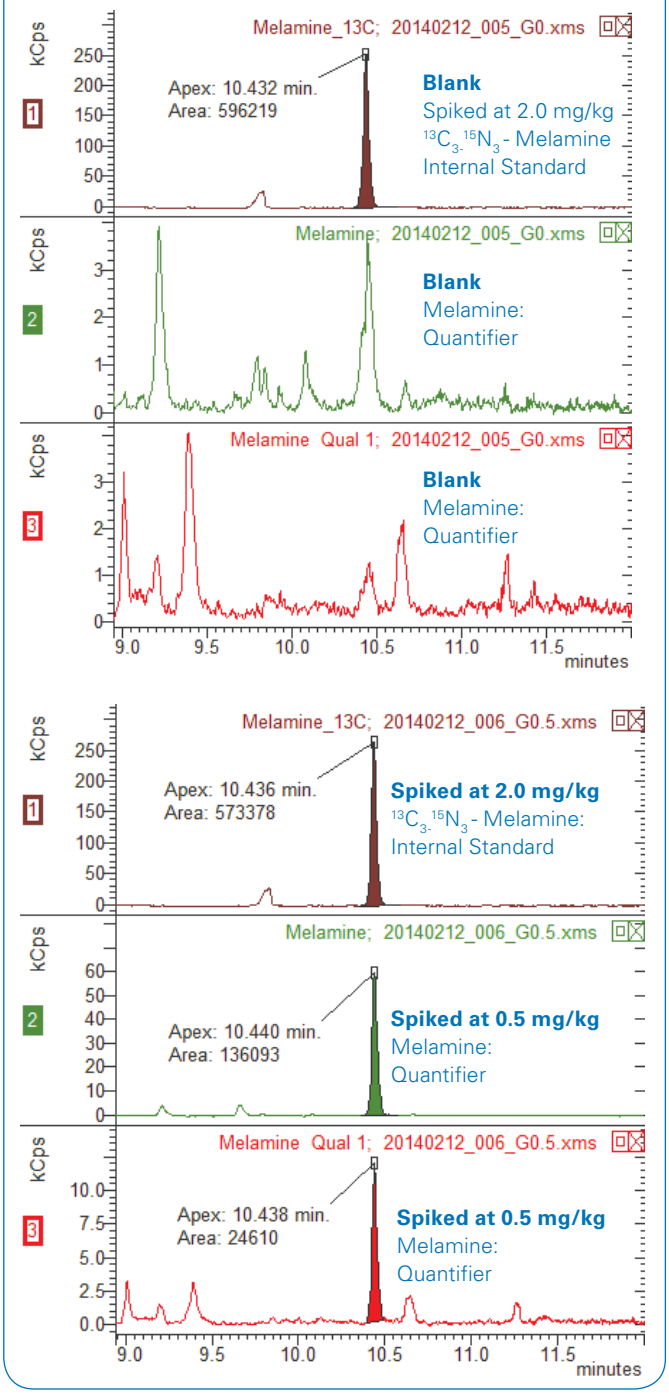


Figure 1: Blank (top) and spiked (bottom) sample of powdered milk. Blank was spiked only with $^{13}C_{3}$-$^{15}N_3$ – melamine, whereas spiked sample contained also melamine at 0.5 mg/kg.

In every sequence, five calibration solutions were injected plus a blank solution containing only external and internal standards on matrix. Quality control sample were also injected to ensure the best level of performance of the system. Samples were then injected for detection and quantification.

Prior to SRM analysis, full scan and product-scan analysis were performed on MSTFA-derivatized molecules to optimize SRM parameters.

Results

According to the analytical criteria described in Commission Decision 2002/657/EC, two diagnostic SRM transitions were recorded for each analyte, providing unambiguous identification of compounds.

In order to evaluate the instrument's sensitivity for melamine without SPE clean-up step during sample preparation, a blank and a spiked sample were analyzed. Both samples were infant formula (powdered milk) and the spiked sample contained 0.5 mg/kg melamine, as illustrated in Figure 1. The spiked sample at 0.5 mg/kg represents half of the maximum residue limit in infant formulae, and the melamine was easily identified.

Molecule	R^2
Cyanuric Acid	0.999492
Ammelide	0.999960
Ammeline	0.999958
Melamine	0.999804

Figure 2: linearity obtained for each target analyte in spiked infant formula samples.

Figure 2 illustrates the linearity achieved for all compounds (coefficient of determinations greater than 0.999). Isotope labeled cyanuric acid was used as the internal standard for ammelide, and labeled melamine was used as the internal standard for ammeline.

Figure 3 illustrates the calibration curve for melamine, with r^2 equal to 0.999804.

Linearities of melamine and analogs between 0.5 and 10 mg/kg were achieved on the same infant powdered formula, using isotope dilution.

Calibration curves for melamine and its analogs between 0.5 and 10 mg/kg were achieved on the same infant powdered formula using isotope dilution. The limits of quantification are 10 to 20 times lower than the regulation (Maximum Residue Limit of 1.00 mg/kg for infant formula). Cyanuric acid had less sensitivity than the other analytes. Despite the fact that the quantifier for this acid was easily observed at 0.5 mg/kg, the lack of specificity of the transitions lead to a limit of quantification of only 1.0 mg/kg.

Calibration curve for Melamine

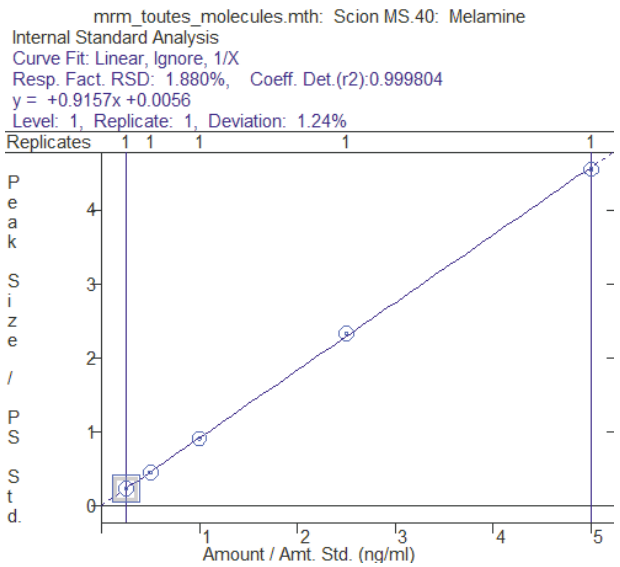


Figure 3: Example of calibration curve obtained for melamine

Chromatogram: spiked powdered milk at 0.5 mg/kg

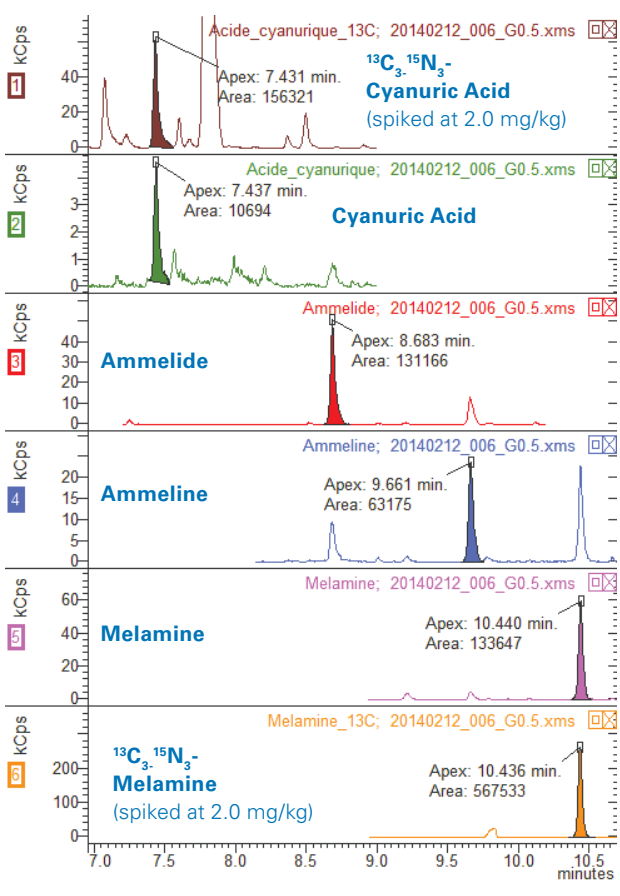


Figure 4: typical diagnostic ion chromatograms (quantifier SRM transition) obtained for a spiked powdered milk sample. $^{13}C_{3}$-$^{15}N_{3}$ – melamine and cyanuric acid (internal standards) were spiked at 2.0 mg/kg and native melamine and analogs at 0.5 mg/kg

These limits of quantification represent 10 to 20 times lower than the regulation (Maximum Residue Limit of 1.00 mg/kg for infant formula). Cyanuric acid presented less sensitivity. Despite the fact that the quantifier for this acid was well observed at 0.5 mg/kg, the lack of specificity of the transitions lead to a limit of quantification of only 1.00 mg/kg.

Since it was proven that the toxicity of melamine is increased in the presence of cyanuric acid, structural analogs were also analyzed. They were included at the same concentration as melamine, 0.5 mg/kg, and cyanuric acid had his own isotope labeled internal standard, $^{13}C_3$-$^{15}N_3$ cyanuric acid. Figure 4 shows the response of all compounds (quantifiers) from a spiked milk sample. Limits of quantification were determined to be between 0.050 and 0.100 mg/kg for melamine, ammelide and ammeline.

Conclusion

The sensitivity of the EVOQ GC-TQ system combined with the specificity of SRM analysis, allowed the efficient detection and quantification of melamine and its analogs even without SPE cleanup, which provides a high throughput workflow. Melamine could be quantified approximately 20 times lower than the Maximum Residue Limit for infant formulae.

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References

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- "Toxicological and Health Aspects of Melamine and Cyanuric Acid". Report of a WHO Expert Meeting in collaboration with FAO. Health Canada, Ottawa, Canada 1–4 December 2008. http://whqlibdoc.who.int/publications/2009/9789241597951_eng.pdf

- EU COMMISSION DECISION 2002/657/EC N° L221/8 of 12 August 2002, Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

- EU COMMISSION DECISION N° L273/18 of 14 October 2008 imposing special conditions governing the import of products containing milk or milk products originating in or consigned from China, and repealing Commission Decision 2008/757/EC.

- EU COMMISSION REGULATION 594/2012 N° 176/43 of 5 July 2012 amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs.

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