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應用手冊

Analysis of Melamine and Its Degradation Products in Milk-Based Products Using GC-MS/MS

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Abstract

This application note describes the major advantages of analyzing melamine and its degradation products (ammeline, ammelide, and cyanuric acid) using GC-MS/MS with an isotopic dilution approach.

Introduction

Melamine is an organic chemical compound rich in nitrogen most commonly found in the form of white crystals. It is commercially used for whiteboards, floor tiles, kitchenware, fire retardant fabrics, and filters. In 2007, melamine was found in wheat gluten and rice protein concentrates used in the manufacture of pet food. This caused the deaths of a large number of dogs and cats due to renal failure. In September 2008, this compound was found in powdered infant formula and caused the deaths of four children, and hospitalization of about 13,000 children in China. It was found that melamine was added to raw milk that was previously diluted with water in order to increase its volume. Indeed, addition of this compound increases the nitrogen content of the milk and therefore its apparent protein content.

Data from animal studies revealed that melamine associated to cyanuric acid can form insoluble crystals that can give rise to kidney stones responsible for acute renal failure.[1]

The import of milk and milk products originating from China has been prohibited into the European Union since 2002. However, composite food products, such as biscuits and chocolate, which could be made from contaminated milk powder, may have reached the EU. Therefore, decision 2008/798/EC was enacted by the European Commission on 14 October 2008. This regulation imposes special conditions governing the import of products containing milk or milk products originating in, or consigned from China, and fixes a maximum concentration of 2.5 mg kg^{-1} of melamine in food products.[2] The concentration of melamine in food products needs to be monitored with an accurate and specific analytical technique so contaminated batches that do not meet the EU regulation can be destroyed. This application note describes the major advantages of analyzing melamine and its degradation products (ammeline, ammelide, and cyanuric acid) using GC-MS/MS with an isotopic dilution approach.

Experimental

Standards of melamine, ammeline, ammelide, and cyanuric acid, shown in Figure 1, were prepared by dissolving 10 mg in 10 mL of a water/diethylamine (80/20, v/v) mixture. Stable isotope labeled internal standards for

melamine ($^{13}C_3$ $^{15}N_3$ melamine) and cyanuric acid ($^{13}C_3$ $^{15}N_3$ cyanuric acid) were obtained from Cambridge Isotope Laboratories.

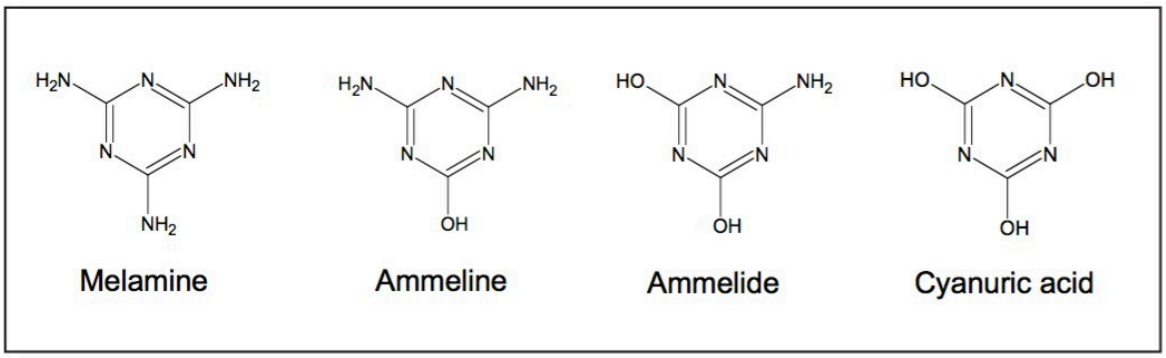

Figure 1. Structures of melamine, ammeline, ammelide, and cyanuric acid.

Extraction Procedure

The separation procedure was derived from a method previously described.[3] Samples were mixed in a blender. 100 mg sample was weighed into a 15-mL glass tube and spiked with 200 ng ^{13}C-^{15}N labeled internal standard. 4 mL acetonitrile/water mixture (50/50, v/v) was added, and the solution was shaken vigorously for 30 s and then sonicated for 30 min. The tubes were centrifuged at 2000 g and 5 °C for 10 min. Then the supernatant was filtered through a 0.45 µm filter. 50 µL filtrate was placed into a vial containing 2.5 ng benzoguanamine (external standard). The solution was evaporated to dryness under a gentle stream of nitrogen before being reconstituted with 50 µL MSTFA and derivatized for 40 min at 60 °C.

GC Conditions

GC system:	Agilent, 6890 series
Column:	Zebron ZB-5MS (30 m x 0.25 mm x 0.25 µm)
Injection mode:	Splitless
Injector temperature:	280 °C
Carrier gas:	Helium
Flow rate:	1.0 mL/min

Temperature program:	120 °C (1 min) to 320 °C (2 min) at 10 °C/min

MS Conditions

MS system:	Quattro Micro GC Tandem Quadrupole Detector
Ionization mode:	Electron ionization operated at 70 eV
Source temperature:	250 °C
Collision gas:	Argon at 3.10^{-4} mbar
Acquisition mode:	Multiple Reaction Monitoring (MRM)

Multiple Reaction Monitoring (MRM) transitions were monitored to meet relevant criteria for unambiguous identification and confirmation of the analytes. The MRM transitions and collision energies are listed in Table 1.

Compounds	Indicative RT (min)	Transition 1	Collision T1 (eV)	Transition 2	Collision T2 (eV)
Cyanuric acid $^{13}C_3$ $^{15}N_3$ (IS)	8.34	351 > 336	5		
Cyanuric acid	8.35	345 > 330	5	345 > 188	5
Ammelide	9.59	344 > 329	5	344 > 286	15
Ammeline	10.60	343 > 328	5	343 > 285	10
Melamine $^{13}C_3$ $^{15}N_3$ (IS)	11.35	348 > 173	20		
Melamine	11.36	342 > 327	5	342 >171	20
Benzoguanamine (ES)	14.54	331 > 316	5		

Table 1. Indicative retention times (min) and monitored transitions for GC-MS/MS analysis (IS: Internal Standard ; ES: External Standard).

Results and Discussion

High Specificity

Detection of melamine using GC-MS/MS was facilitated by a derivatization step with MSTFA that increased the mass of the different compounds, as shown in Figure 2, and multiple transitions of significance were obtained. As illustrated in Figure 3, melamine can be easily detected in a biscuit sample at 2.5 mg kg^{-1} with three specific

transitions, allowing unambiguous identification of the target analytes, especially with regard to the criteria of the Commission Decision 2002/657/EC.[4] Increasing the mass of the monitoring ions led to a very specific signal; this is not the case for LC-MS/MS analysis, a popular technique, but one that presents the drawback of detecting the pseudo-molecular ion $[M + H]^+$ of melamine, with more or less specificity.

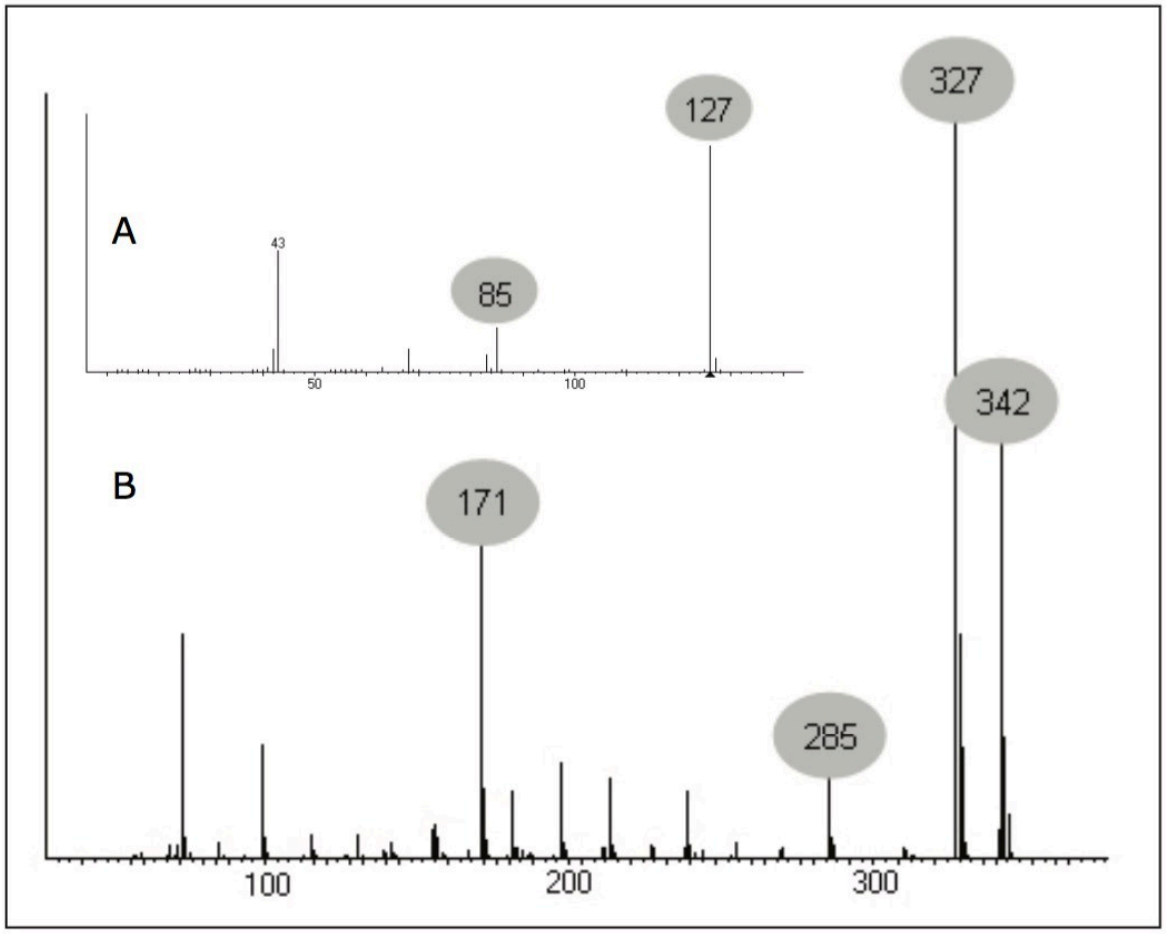

Figure 2. Mass spectrum of melamine (A) and tri-TMS melamine derivative (B).

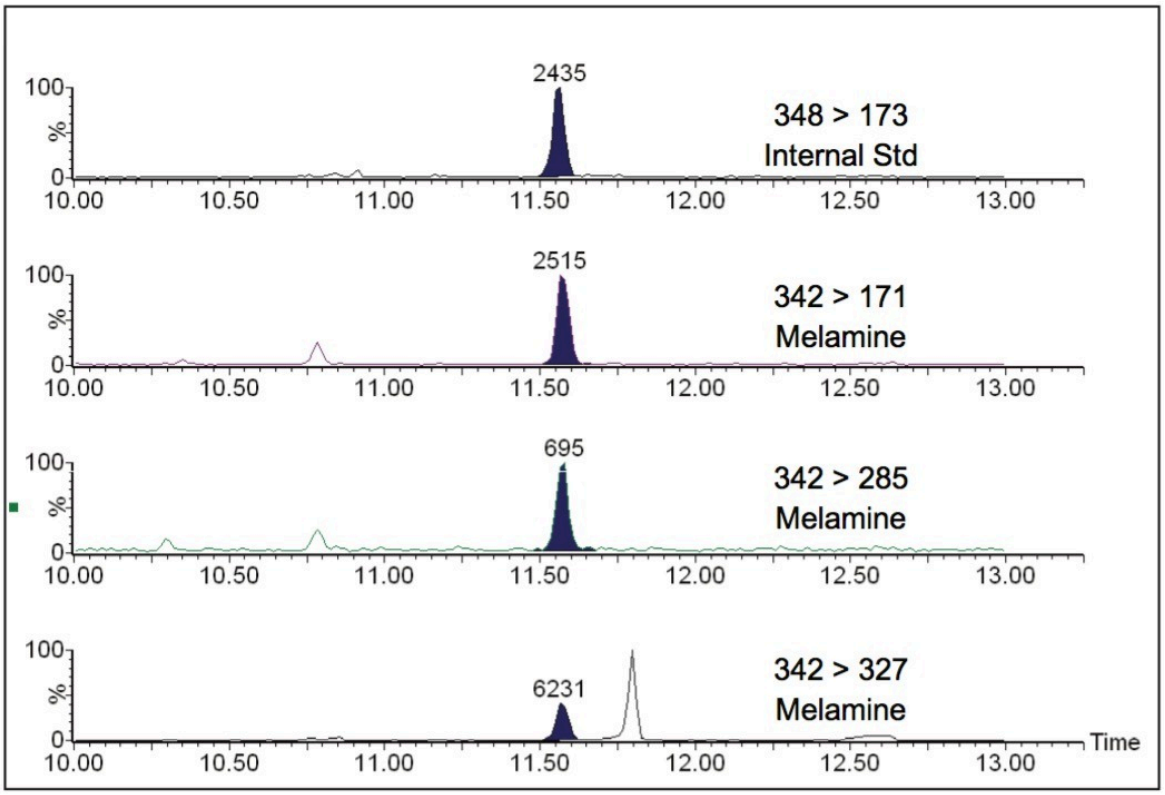

Figure 3. Chromatographic profiles of $^{13}C_3$-$^{15}N_3$-melamine (internal standard) and three specific transitions for melamine in biscuit at 2.5 mg kg^{-1}.

High Throughput Screening

The high specificity, combined with the good sensitivity of the tandem quadrupole detector allows for the identification and quantification of melamine at low levels in food samples with no clean-up. Application of the very fast procedure previously described enables detection of melamine at low concentration levels, with application of a large dilution factor, which is only possible with a very sensitive instrument. Therefore, application of this procedure allows analysis of melamine and its degradation products in food samples at low levels (0.1 mg kg^{-1} for melamine, in accordance with the 2008/757/EC regulation) in a very short time.

As illustrated in Figure 4 (porridge sample spiked with melamine at 0.12 mg kg^{-1}), detection of melamine is very easy, the "absence/presence" diagnostic is drastically facilitated, and the quantification is not affected by any interference.

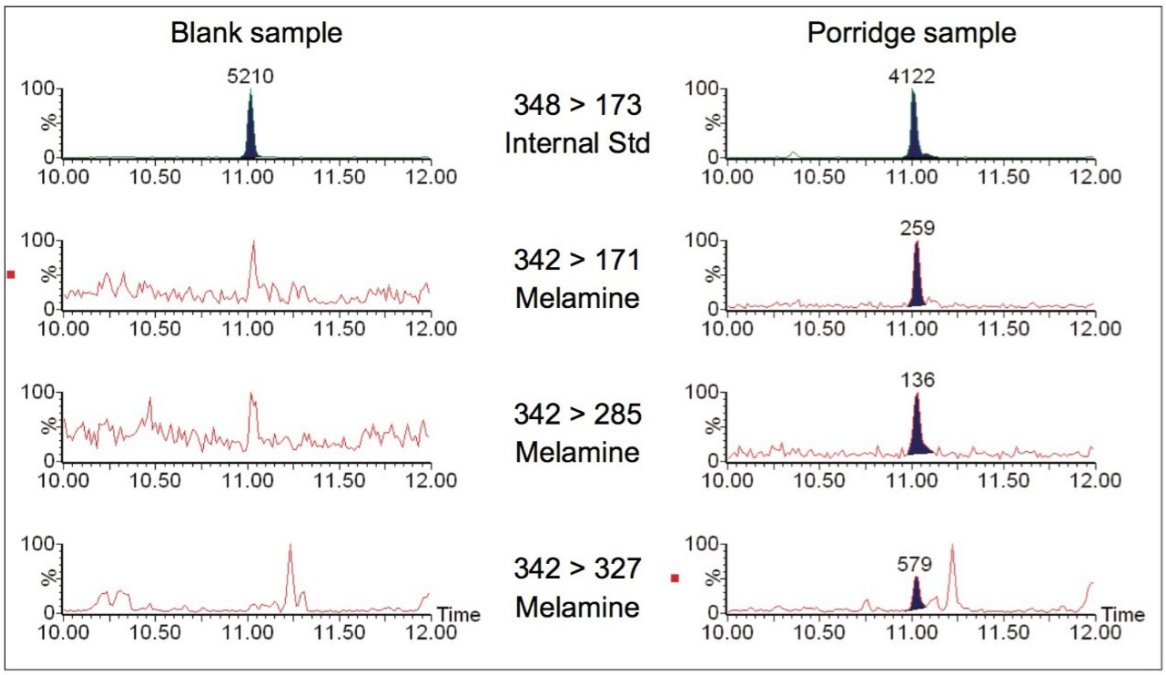

Figure 4: Ion chromatograms corresponding to $^{13}C_3$-$^{15}N_3$-melamine (internal standard) and melamine in a blank sample, and a porridge sample at 0.12 mg kg^{-1}.

Simultaneous Analysis of Degradation Products

Use of the described protocol, without the use of SPE clean-up, generally based on cation-exchange (for melamine analysis), oranion exchange (for cyanuric acid analysis), enables a simultaneous detection of all target compounds, as shown in Figure 5. The fact that no SPE clean-up step is needed is ideal for the determination of the contamination profile, since it has been proven that the toxicity of melamine is due to its combination with cyanuric acid.

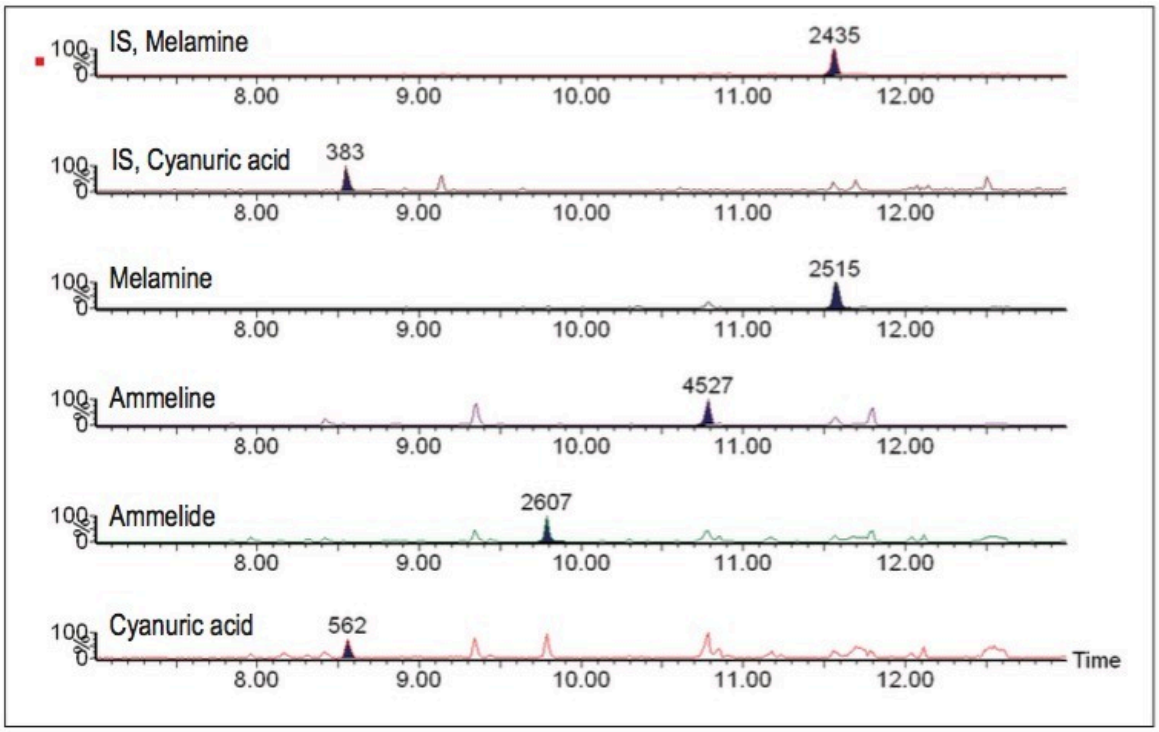

Figure 5: Ion chromatograms corresponding to $^{13}C_3$-$^{15}N_3$-melamine and $^{13}C_3$ $^{15}N_3$- cyanuric acid (internal standards) and cyanuric acid, ammelide, ammeline, and melamine in biscuit spiked at 2.5 mg kg^{-1}.

Performance

By including stable isotope-labeled internal standards in the extract, linearity of the analytes was achieved, from 0.1 to 10 mg kg^{-1} as shown in Figure 6. Limits of detection were between 0.1 to 0.2 mg kg^{-1}, except for cyanuric acid (1.1 mg kg^{-1}), which presented less sensitivity due to its lower response factor. Identification criteria were calculated and are in accordance with 2002/657/EC decision criteria, thanks to the MS/MS detection that provides at least two specific transitions for each compound, guaranteeing reliable analyte identification.

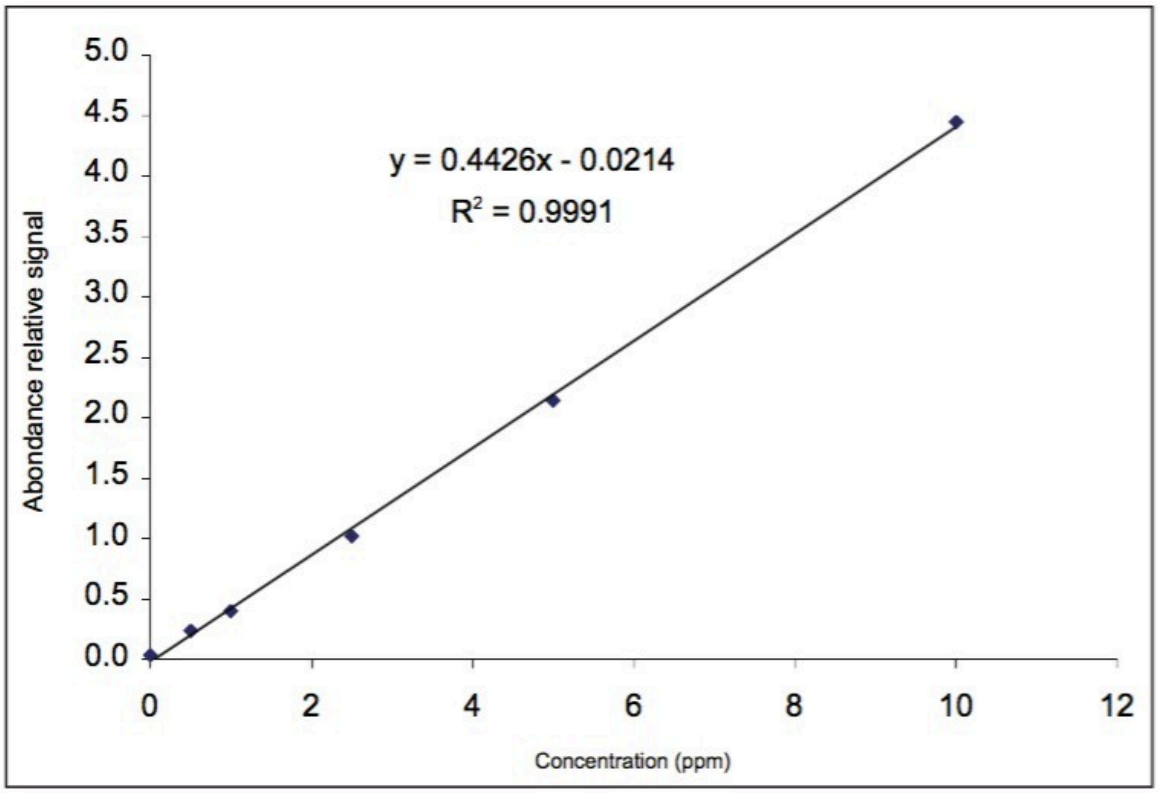

Figure 6: Calibration curve ranging from 0.1 to 10 mg kg^{-1} melamine.

Conclusion

There is a need for a rapid and sensitive method for the analysis of melamine and its degradation products in milk-based products, following the introduction of Commission Decision 2008/757/EC. This application note describes a method[5] that facilitates the identification and quantification of all the compounds of interest, without performing SPE ion-exchange clean-up. The absence of this purification step is only possible when using a very specific and sensitive detection method, such as tandem quadrupole MS/MS. Use of GC-MS/MS after a derivatization step allows for the simultaneous detection of melamine, ammelide, ammeline, and cyanuric acid with very good sensitivity, and a signal that is not affected by matrix effect (ion suppression), as sometimes observed in LC (ESI)/MS/MS.

With the desire of food producers worldwide to demonstrate due diligence regarding the safety and quality of products containing milk or milk products, the method described offers significant business advantages for meeting the challenge of timely, uninterrupted supply of product, while simultaneously and unequivocally ensuring the safety of consumers.

References

1. Puschner ***et al,*** Journal of Veterinary Diagnostic Investigation, Vol. 19 Issue 6, 616–624.
2. Commission Decision 2008/798/EC 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
3. "GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric Acid". (FDA method) – http://www.fda.gov/cvm/GCMSMelamine.htm
4. Commission Decision 2002/657/EC, Official ***J. European Communities,*** No. L221/8.
5. LABERCA method – http://www.laberca.org/newsletter/en/LABERCA08MEL-Al-7.pdf

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