

Introduction

Humans are exposed to melamine (MEL) and its analogues like cyanuric acid (CA) from different sources and these two compounds have been considered relatively non-toxic when administrated separately. However, increased incidence of renal pathologies in Chinese infants have been attributed to the ingestion of infant formulas and other milk powders tainted with MEL and CA. Transmission of melamine from adulterated feed to milk appeared to be an other source of contamination [1]. On the basis of a risk assessment, the WHO/FAO Expert Meeting [2] concluded that, for powdered infant formulas, a maximum limit (ML) for MEL at 1 mg/kg would provide a sufficient margin of safety for dietary exposure. Hence, there is a need for effective and reliable methods to monitor MEL and CA in milk-based infant formula and to control raw cow's milk.

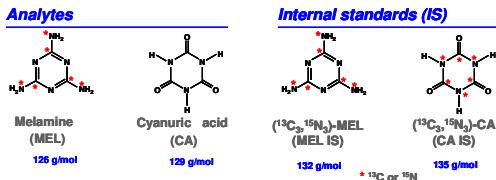
✓ An isotope dilution LC-ESI-MS/MS is described for the simultaneous determination of MEL and CA in raw cow's milk and infant formula within the ranges:

Infant formula: MEL: 0.5 – 1.5 mg/kg & CA: 0.5 – 1.5 mg/kg **Raw Cow's Milk:** MEL: 0.05 – 0.15 mg/kg & CA: 0.1 – 0.2 mg/kg .
Range of validation was lower for raw milk than for milk powder, given the fact that this raw material undergoes an 8-fold concentration process to give milk powder.

✓ Selected reaction monitoring of two diagnostic transition reactions for each analyte and each corresponding (¹³C₃, ¹⁵N₃)-labeled compounds enables selective and confirmatory detection. Quantification was performed based on the isotope dilution approach.

✓ Validation of the method was conducted according to the European Union criteria (2002/657/EC) [3].

Figure 1: Chemical structures of Melamine, Cyanuric acid and their (¹³C₃, ¹⁵N₃)-internal standards



Material and Method

Sample preparation

Test portion (1.0 g) was weighed into a 50 mL Falcon polypropylene tube and fortified with the internal standards (IS) to reach either 0.1 mg/kg for raw milk or 1 mg/kg for milk powder. Water (5 mL) and then acetonitrile (5 mL) were added successively, and the resulting slurry was thoroughly mixed after each solvent addition. The slurry was further diluted with acetonitrile (30 mL) and water (10 mL) and placed onto an automated shaker for 5 min. The tube was then centrifuged at 4000 x g at room temperature for 10 min. The supernatant (ca. 1 mL) was then transferred into a HPLC vial for further LC-MS/MS analysis.

Chromatography

HPLC analysis was performed on a hydrophilic-lipophilic (HILIC) Tosoh Bioscience TSKgel Amide-80 column (2.0 x 250 mm, 5 µm). The mobile phase was constituted of solvent A, water containing 10 mM ammonium acetate, and solvent B, acetonitrile. A linear gradient program was set up with 0-8 min, 10% A; 8-13 min, 65% A; 13-14 min, 90% A; hold at 90% A for 1min; return to 10 % A in 0.5 min (the HPLC column was reconditioned at 10% A for an additional 9.5 min). The flow rate was 0.25 mL/min, and 5 µL of the extract was injected onto the column.

Mass spectrometry

Detection was performed on an Applied Biosystems QTrap 4000 equipped with a TurbolonSpray ionization source. MS tuning was performed in positive electrospray ionization (ESI) for MEL and in negative ESI for CA. Both compounds were analyzed within the same HPLC run by switching from the negative ionization mode to the positive one at time t = 8 min. Quantitative analysis was performed using tandem MS in selected reaction monitoring (SRM) mode alternating two transition reactions for each compound and their corresponding IS with a dwell time of 100 ms (Table 1).

Table 1: Transition reactions monitored by LC-MS/MS for the analysis of melamine and cyanuric acid and their corresponding isotopically labeled homologue and peak area ratios along with their limit of acceptance according to [3]

	Transition reactions (m/z) used for:		Peak area ratio ± limit (%)
	Quantification	Analyte confirmation	
Melamine	127.0 → 85.1	127.0 → 68.0	0.28 ± 25
(¹³ C ₃ , ¹⁵ N ₃)-melamine (IS)	133.0 → 89.1	127.0 → 71.1	0.19 ± 25
Cyanuric acid	128.0 → 42.1	128.0 → 85.2	0.55 ± 20
(¹³ C ₃ , ¹⁵ N ₃)-cyanuric acid (IS)	134.0 → 44.1	134.0 → 88.9	0.52 ± 20

Results and Discussion

MEL and CA were quantified by means of external calibration curves in acetonitrile:water (70:30, v:v) containing both labeled and unlabeled analytes (Figure 1). Recovery, repeatability and intermediate reproductibility precisions were calculated from the analysis of blank matrices spiked with each analyte at three fortification levels (Table 2). Three operators were involved in these experiments, each performing 2 replicates of each fortification level on two occasions. LODs/LOQs were 0.025/0.050 mg/kg for MEL and 0.050/0.10 mg/kg for CA (Figure 2). CCα and CCβ, at the 1 mg/kg ML for infant formula powder endorsed by WHO, were respectively 1.03 and 1.05 mg/kg for MEL and 1.04 and 1.09 mg/kg for CA. The accuracy of the method was proved for: 1) MEL on 6 samples in the frame of 2 proficiency tests (Table 3) and 2) MEL and CA on 2 samples of raw material with high risk of adulteration, i.e. soja powder and egg powder in 1 proficiency test (Table 4). The method was developed at NRC and transferred in Nestlé Quality Assurance Laboratories and external

Laboratories working in partnership with Nestlé (Tables 3 and 4) without any physical intervention. This demonstrates the fitness-for-purpose and the easiness-to-handle of the method.

Figure 2: LC-ESI-MS/MS chromatograms of Melamine (MEL) and Cyanuric Acid (CA) from an extract of spiked Raw Milk at the LOQ. Spiking levels: MEL 0.05 mg/kg (IS 0.1 mg/kg); CA 0.1 mg/kg (IS 0.1 mg/kg).

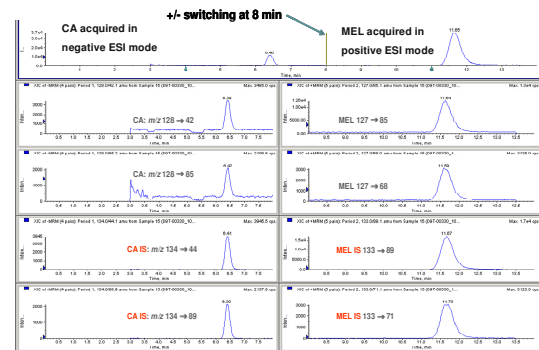


Table 2: Method Performance Data for Raw milk and Milk Powder

	Raw Milk				Milk Powder			
	Fortification level	Rec (%)	r (%)	IR (%)	Fortification level	Rec (%)	r (%)	IR (%)
Melamine	0.05	107	3	13	0.5	102	4	6
	0.1	102	6	9	1	102	3	4
	0.15	103	4	6	1.5	102	1	5
Cyanuric Acid	0.1	99	5	10	0.5	105	1	13
	0.15	101	8	9	1	103	3	13
	0.2	102	4	7	1.5	103	5	16

Fortification level are given in mg/kg; Rec: Recovery; r: Repeatability; IR: Intermediate Repeatability

Table 3: Results of Nestlé internal P-test and Institute for Reference Materials and Measurements (IRMM) P-test for the analysis of Melamine. (Results of laboratories using the presented method are listed below).

Sample type	Nestlé internal P-test ^{a)}				IRMM P-test ^{b)}	
	Milk powder 1	Milk powder 2	Milk powder 3	Milk powder 4	Skimmed milk powder	Backing Mix
Assigned value ^{c)}	0.1	0.235	1.264	2.785	10.0	3.2
Blank sample						
Result ^{d)} and z-score ^{e)} values						
NRC	< LOD	0.239	1.316	2.957	10.5	3.2
z-score	na	0.060	0.5	0.78	0.4	0.0
Lab 1	< LOD	0.157	1.275	2.725		
z-score	na	-1.28	0.11	-0.27		
Lab 2	< LOD	0.200	1.057	2.512		
z-score	na	-0.57	-1.98	-1.24		

^{a)} 27 laboratories took part in the Nestlé internal P-test
^{c)} Assigned values and result values are given in mg/kg

^{b)} 144 laboratories from 31 countries took part in the IRMM P-test
^{e)} |z| < 2: good result; 2 < |z| < 3: questionable result; |z| > 3: unsatisfactory result

Table 4: Result of laboratories using the presented method in the frame of a Nestlé P-test for the analysis of Melamine and Cyanuric Acid in raw material

Sample type	Egg powder		Soja powder	
	MEL	CA	MEL	CA
Number of participants	12	11	19	18
Assigned value ^{a)}	0.100	0.190	0.496	1.585
Result ^{b)} and z-score ^{c)} values				
NRC	0.115	0.178	0.485	1.439
z-score	0.7	-0.3	-0.1	-0.7
Lab 1	0.101	0.156	0.440	1.49
z-score	0.0	-0.9	-0.6	-0.4
Lab 2	0.100	0.190	0.430	1.52
z-score	0.0	0.0	-0.7	-0.3
Lab 3	0.096	0.182	0.496	1.555
z-score	-0.2	-0.2	0.0	-0.1
Lab 4	0.093	0.186	0.483	1.585
z-score	-0.3	-0.1	-0.1	0.0

^{a)} Assigned values and result values are given in mg/kg

^{b)} |z| < 2: good result; 2 < |z| < 3: questionable result; |z| > 3: unsatisfactory result

Conclusion

This quantitative method entails a simple sample preparation, limited to a protein precipitation in acetonitrile:water followed by a centrifugation and direct injection of the supernatant. No clean-up by solid phase extraction was applied to avoid any plastic-derived contamination of the analytes during the sample preparation. Performance evaluation based on 3 proficiency tests (including matrices like milk powder, backing mix, egg powder and soja powder) indicates that the method is suitable (|z| < 2) for the quantitative determination of MEL and CA for a broad variety of food matrices. Robustness of the method was demonstrated during the analysis of >6000 routine samples. The method has been published recently [4].

References:

- [1] Pathway confirmed for the transmission of melamine from feed to cow's milk. C.W. Cruywagen, M. A. Stander, M. Adonis, and T. Calitz. *J. Dairy Sci.* 92:2046–2050 doi:10.3168/jds.2009-2081
- [2] World Health Organization/Food and Agriculture Organization of the United Nations. *Expert Meeting to Review Toxicological Aspects of Melamine and Cyanuric Acid*, 2008; pp 1–10.
- [3] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities* 2002, L221, 8–36.
- [4] Simultaneous Quantitative determination of Melamine and cyanuric Acid in Cow's Milk and milk-Based Infant Formula by Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry. A. Desmarchelier, M. Guillamon Cuadra, T. Delatour and P. Mottier. *J. Agric. Food Chem.* 2009, 57, 7186-7193. DOI: 10.1021/j991355v.