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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

AURÉLIEN DESMARCHELIER, MIRIAM GUILLAMON CUADRA, THIERRY DELATOUR, AND PASCAL MOTTIER*

Nestlé Research Centre, Nestec Ltd., Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

An isotope dilution liquid chromatography−electrospray ionization tandem mass spectrometry method for the simultaneous determination of melamine and cyanuric acid in cow's milk (range of 0−0.3 mg/kg) and milk-based infant formulas (ranges of 0−0.3 and 0−2.0 mg/kg) is described. This quantitative method entails simple sample preparation, limited to a protein precipitation in acetonitrile/water followed by a centrifugation and direct injection of the supernatant. Selected reaction monitoring of two diagnostic transition reactions for each analyte and each corresponding ($^{13}C_3$,$^{15}N_3$)-labeled compound enables selective and confirmatory detection. Acquisition was performed sequentially in the negative ion mode for cyanuric acid, while in the positive mode for melamine within the same run. Validation of the method was conducted according to European Union criteria (CD 2002/657/EC). Internal standard-corrected recoveries were within the 99−116% range for both analytes in the two matrix types, along with repeatability and intermediate reproducibility values of ≤12.3 and ≤31.2%, respectively. LODs were 0.025 and 0.050 mg/kg for melamine and cyanuric acid, respectively, whereas LOQs, set arbitrarily at the lowest fortification level, were 0.05 and 0.10 mg/kg for melamine and cyanuric acid, respectively. CCα and CCβ, at the 1 mg/kg maximum limit (ML) for infant formula powder endorsed by WHO, were respectively 1.03 and 1.05 mg/kg for melamine and 1.04 and 1.09 mg/kg for cyanuric acid.

KEYWORDS: Melamine; cyanuric acid; milk; liquid chromatography tandem mass spectrometry; LC-MS/MS

INTRODUCTION

Melamine (MEL), chemically known as 2,4,6-triamino-1,3,5-triazine, is produced in large amounts (1.2 million tons in 2007) (*1*) primarily for use in the synthesis of melamine formaldehyde resins for the fabrication of laminates, plastics, coatings, commercial filters, glues or adhesives, as well as for dishware and kitchenware (*2*−*4*). Cyanuric acid (CA, 1,3,5-triazine-2,4,6-triol) can be produced either as a byproduct during the manufacturing process of melamine or by bacteria-mediated metabolism of MEL (*1*). CA is commonly used as a disinfectant, particularly for the treatment of water (*5*).

These two compounds have been considered to be relatively nontoxic when administrated separately, although chronic administration of high concentrations can induce renal pathology (*6*). However, the strong affinity between MEL and CA for one another was described to form the barely soluble melamine−cyanurate complex through hydrogen bonding (*7*, *8*). Several events triggered the role of these chemicals and their resulting complex in the death or illness of animals, most notably cats, fed contaminated feed (*9*, *10*) and of Chinese infants fed infant formulas and other milk powders tainted with MEL (*1*).

On the basis of a risk assessment, the WHO/FAO Expert Meeting 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 (*1*). The tolerable daily intake derived by WHO for CA indicates that it is less toxic than MEL. On the other hand, available data indicate that simultaneous exposure to MEL and CA is more toxic than exposures to each compound individually (*1*). To ensure the safety of milk-based infant formulas with regard to MEL, a ML at 1 mg/kg has been adopted in many countries and, as a provisional precautionary measure, the same ML has been applied to CA in several countries including Puerto Rico and the United States (*11*).

Hence, there is a need for effective and reliable methods to monitor MEL and CA in milk-based infant formula and control raw cow's milk. Several HPLC-UV methods have been proposed for the quantitative determination of MEL (*12*−*14*) and CA (*5*). However, the UV spectra of both MEL and CA exhibit absorption bands below 250 nm (*15*, *16*), which may lead to erroneous quantification if insufficient care is paid to chromatographic conditions and/or optimization of sample preparation. In complex matrices such as food, the baseline of HPLC-UV analysis

*Corresponding author [e-mail pascal.mottier@rdls.nestle.com; telephone (+41/21) 785 8231; fax (+41/21) 785 8553].

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recorded below 250 nm is severely disrupted by compounds present in the extract. The signal of interest may be significantly overestimated if a compound interferes chromatographically with the analyte to be monitored (MEL or CA in the present case). Liquid chromatography−tandem mass spectrometry (LC-MS/MS) prevents such misquantification as the selectivity of the detection is ensured by selected reaction monitoring (SRM)-based acquisition.

The present paper describes a fully validated method based on LC-MS/MS for the simultaneous monitoring of MEL and CA in cow's milk and milk-based infant formula. The method entails a precipitation of proteins in acetonitrile/water prior to centrifugation and direct injection of the supernatant into the LC-MS/MS system. No cleanup by solid phase extraction was applied to avoid any plastic-derived contamination of the analytes during the sample preparation. The validation was carried out according to the criteria of European Commission CD 2002/657/EC (*17*), and decision limits (CCα) and detection capabilities (CCβ) were calculated in milk-based infant formula assuming a ML at 1 mg/kg.

MATERIALS AND METHODS

Chemicals and Reagents. Melamine (2,4,6-triamino-1,3,5-triazine) and cyanuric acid (2,4,6-triol-1,3,5-triazine) were obtained from Sigma (Buchs, Switzerland). Their respective isotopically labeled homologues, that is, ($^{13}C_3$,$^{15}N_3$)-melamine (isotopic purity, $^{13}C_3$, 99%; amino-$^{15}N_3$, 98%; chemical purity, ≥98%, ($^{13}C_3$,$^{15}N_3$)-MEL) and ($^{13}C_3$,$^{15}N_3$)-cyanuric acid (isotopic purity, $^{13}C_3$, 99%; $^{15}N_3$, >98%; chemical purity, 90%, ($^{13}C_3$,$^{15}N_3$)-CA) were supplied by Cambridge Isotope Laboratories (Andover, MA). Ammonium acetate, acetonitrile, and LiChrosolv water were from Merck (Darmstadt, Germany).

Standard Solutions. Stock solutions (250 μg/mL) of the unlabeled analytes were prepared separately by dissolving each compound in water by means of an ultrasonic bath for 15 min. Further separate working standard solutions in water (at 20 and 2 μg/mL and 200 ng/mL) were then obtained by successive dilutions. Working standard solutions of the labeled homologues (used as internal standards, IS) at the same concentrations were obtained similarly from a 100 μg/mL ready-to-use ampule.

Food Samples. Raw cow's milk samples were obtained from local dairies and stored at 4 °C until use. To avoid separation between the oily and aqueous phases, the sample was rehomogenized before weighing the test portion. Similarly, milk-based infant formulas were collected from several local supermarkets, and homogenization was ensured by transferring the whole content of each package into a sample container of capacity about twice that of the laboratory sample volume and the container was repeatedly shaken and inverted.

Sample Preparation. Two separate concentration ranges were considered during this validation: (a) from 0 to 0.3 mg/kg and dedicated to the analysis of both cow's milk (because this raw material undergoes an 8-fold concentration process to give milk powder) and milk-based infant formula (to efficiently quantify MEL and CA at low doses in case the WHO limit is lowered) and (b) from 0 to 2.0 mg/kg in milk-based infant formula (to have a methodology adapted to the quantification of the actual 1 mg/kg WHO limit).

Cow's Milk and Milk-Based Infant Formula (for Analysis over a 0−0.3 mg/kg Concentration Range). A well-homogenized test portion (1.0 g) was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France) and fortified with a 50 μL aliquot of each of the IS working standard solutions (2 μg/mL, corresponding to 0.10 mg/kg equivalent in sample concentration). The sample was mixed and allowed to stand for 10 min. Water (5 mL) and then acetonitrile (5 mL) were added successively, and the resulting slurry was thoroughly mixed after each solvent addition, ensuring there was no aggregate in the sample. The slurry was further diluted with acetonitrile (30 mL) and water (10 mL) and placed onto an automated shaker for 5 min (IKA KS 501D, 200 rpm, IKA-Werke, Staufen, Germany). The tube was then centrifuged at 4000*g* at room temperature for 10 min (centrifuge Multifuge 3s, Heraeus, Geneva, Switzerland). When the resulting supernatant was no longer limpid, an aliquot was transferred into a 2 mL Eppendorf tube and further centrifuged at 8500*g* for 10 min (centrifuge Eppendorf 5415C, Hamburg, Germany). The supernatant (ca. 1 mL) was then transferred into a HPLC vial for further LC-MS/MS analysis.

Milk-Based Infant Formula (for Analysis over a 0−2.0 mg/kg Concentration Range). The procedure was identical to that described above except that a 50 μL aliquot of each of the IS working standard solutions (20 μg/mL, corresponding to 1.0 mg/kg equivalent in sample concentration) was used.

LC-MS/MS. HPLC analysis was performed on a hydrophilic−lipophilic (HILIC) TSKgel Amide-80 column (2.0 × 250 mm, 5 μm, Tosoh Bioscience GmbH, Stuttgart, Germany) using an Agilent 1100 binary pump system (Geneva, Switzerland). 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 1 min; 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. The HPLC flow was directed into the MS detector between 3 and 13.5 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX).

Detection was performed on a QTrap 4000 (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source. MS tuning was performed in positive electrospray ionization (ESI) for MEL and in negative ESI for CA by infusing separately a solution of each analyte (at a concentration of 10 μg/mL) at a flow rate of 10 μL/min mixed with a HPLC flow made of solvents A and B (50:50, v/v; 0.25 mL/min) using a T-connector. The block source temperature was maintained at 500 °C, and the gas set values were as follows: curtain gas, 10 psi; nebulizer gas, 30 psi; turbo gas, 30 psi; collision gas, 1.2×10^{-4} psi. Other parameters having values common to both analytes were the entrance potential (10 V) and the collision exit potential (15 V). 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. The electrospray capillary voltage was set at −3.5 kV for CA and at 3.5 kV for MEL. 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**). Data processing was carried out using Analyst software 1.4.2.

Quantification. MEL and CA were quantified by means of external calibration curves (analyte/IS area ratio (= y) vs analyte/IS concentration ratio (= x)). Six calibration levels, each containing both labeled and unlabeled analytes, constructed in acetonitrile/water (70:30, v/v) were considered for (a) cow's milk and milk-based infant formula (low range): from 0 to 30 pg injected on column (covering thus a 0−0.3 mg/kg range, concentrations equivalent in sample) with the concentration of ISs fixed at 10 pg injected on column (0.1 mg/kg in sample) and (b) milk-based infant formula (high range): from 0 to 200 pg injected on column (covering thus a 0−2.0 mg/kg range, concentrations equivalent in sample) with the concentration of ISs fixed at 100 pg injected (1.0 mg/kg in sample). The linearity of the response was checked by calculating the relative standard deviation (RSD) of the average of response factors (RF, RF = y/x), which should be $RSD_{RF} < 15\%$ (*18*). Calibration curves were also constructed in various milk-based infant formulas of different compositions to check whether any potential matrix effects could be found.

Confirmation Criteria. The analytes were considered to be positively identified when the following criteria were met simultaneously: (a) the ratio of the chromatographic retention time of the analyte to that of the corresponding IS, that is, the relative retention time of the analyte, corresponded to that of the averaged relative retention time of the calibration solutions within a ±2.5% tolerance; (b) the peak area ratios from the different transition reactions recorded for both unlabeled and labeled species were within the tolerances fixed by the EU criteria (*17*), as shown in **Table 1**.

Method Validation. Recovery and within-day (SD(r)) and within-laboratory (SD(iR)) precisions were calculated according to ref *19* from the analysis of blank matrices spiked with each analyte at three fortification levels: (a) 0.05, 0.10, and 0.15 mg/kg for MEL and 0.10, 0.15, and 0.20 mg/kg for CA in cow's milk and milk-based infant formula (low

Table 1. Transition Reactions Monitored by LC-MS/MS for the Analysis of Melamine and Cyanuric Acid and Their Corresponding Isotopically Labeled Homologue (Collision Energies in Electronvolts Are Given in Parentheses) and Peak Area Ratios along with Their Limit of Acceptance according to Reference *17*

	transition reactions (*m/z*) used for		
	quantification	analyte confirmation	peak area ratio ±limit (%)
MEL	127.0 → 85.1 (26)	127.0 → 68.0 (45)	0.28 ± 25
($^{13}C_3$,$^{15}N_3$)-MEL (IS)	133.0 → 89.1 (26)	133.0 → 71.1 (45)	0.19 ± 25
CA	128.0 → 42.1 (30)	128.0 → 85.2 (14)	0.55 ± 20
($^{13}C_3$,$^{15}N_3$)-CA (IS)	134.0 → 44.1 (30)	134.0 → 88.9 (14)	0.52 ± 20

range) and (b) 0.50, 1.00, and 1.50 mg/kg for both compounds in milk-based infant formula (high range). Three operators were involved in these experiments, each performing two replicates of each fortification level on two occasions over a two-week period (thus, a total of 12 separate experiments for each fortification level). Repeatability (r) and intermediate reproducibility (iR) limits at the 95% confidence level were then deduced from the within-day and within-laboratory precisions (expressed as RSD(r) and RSD(iR), respectively) using an expansion factor of 2.77 and were thus r = 2.77 × RSD(r) and iR = 2.77 × RSD(iR) (*20*).

Decision Limits (CCα) and Detection Capabilities (CCβ). CCα and CCβ were calculated at a maximum limit (ML) of 1.0 mg/kg in milk-based infant formula, using the calibration curve procedure (ISO 11843) described in ref *17*.

Measurement Uncertainties. Measurement uncertainties were estimated using the simplified approach based on existing validation data proposed by Barwick and Ellison (*21*) and concerning mainly precision and recovery studies. This approach, if properly planned to cover as many of the uncertainty sources previously identified, provides the necessary data required to calculate measurement uncertainty. Precision and recovery contributions were combined together as follows to obtain the overall uncertainty at each fortification level:

$$U\,(\%) = 2\times \mathrm{RSD(u)} = 2\times\sqrt{\mathrm{RSD(iR)}^2 + \mathrm{RSD(Rec)}^2}$$

U (%) = expanded uncertainty at the 95% confidence interval, RSD(u) = relative standard deviation of uncertainty, RSD(iR) = relative standard deviation of intermediate reproducibility, and RSD(Rec) = relative standard deviation of recovery.

Proficiency Tests. With regard to MEL, performance of the method was evaluated within the frame of two proficiency tests (P tests). One P test was organized internally in November−December 2008, whereas the second one was coordinated by the European Commission Joint Research Centre/Institute for Reference Materials and Measurements (JRC/IRMM) and executed in January−February 2009. The internal P test encompassed four milk-based infant formula samples (samples A−D) prepared in a Nestlé Product Technology Centre in Switzerland. Sample A was a nonspiked MEL-free milk-based infant formula, whereas samples B−D were supplemented with MEL at different levels. Analyses were carried out in Nestlé Research Centre (NRC), Nestlé Quality Assurance Laboratories (NQAL), and external laboratories working in partnership with Nestlé. The assigned values (general medians) were calculated on the basis of 27 participants using various LC-MS/MS methods. Only NRC and two NQALs used the method currently reported.

For the European P test, one milk powder sample and one baking mix sample were obtained from JRC/IRMM, and the analyses were performed at NRC. The number of participants was 114 from 31 countries, and any quantitative method was accepted for the P test.

The z score was calculated according to equation z score = $(M_{lab} - \mathrm{value}_{assigned})/\mathrm{SD}_{robust}(R)$, with M_{lab} for the laboratory mean, $\mathrm{value}_{assigned}$ the assigned value, and $\mathrm{SD}_{robust}(R)$ the robust standard deviation of reproducibility. Performance criteria were as follows: $|z| < 2$, satisfactory result; $2 < |z| < 3$, questionable result; $|z| > 3$, unacceptable result.

RESULTS AND DISCUSSION

Collision-Induced Dissociation-Mediated Fragmentation of MEL and CA. *Melamine*. Fragmentation by collision-induced dissociation (CID) was carried out on both MEL and ($^{13}C_3$,$^{15}N_3$)-MEL to obtain valuable information regarding the fragmentation pathway of MEL. The full-scan spectrum of MEL, recorded in positive mode, exhibits a prominent ion at m/z 127, whereas it is shifted to ion m/z 133 with ($^{13}C_3$,$^{15}N_3$)-MEL. These ions are assigned to the protonated molecules $[M + H]^+$. The product ion scan of MEL exhibits a low intense fragment ion at m/z 110 ($\Delta M = -17$ Da), which suggests the loss of ammonia (**Figure 1**). This observation is supported by the CID spectrum of ($^{13}C_3$,$^{15}N_3$)-MEL, which shows a transition m/z 133 → 115 corresponding to the elimination of $^{15}NH_3$ (M = 18 Da). The presence of a fragment ion at m/z 85 (m/z 89, respectively) in the CID spectrum of MEL (($^{13}C_3$,$^{15}N_3$)-MEL, respectively) is rationalized in terms of elimination of HN=C=NH (H^{15}N=^{13}C=NH, respectively). The four-member ring structure of the fragment ion at m/z 85 proposed by Vail et al. (*22*) was not adopted in the present study as it is probably unstable and therefore does not fit with the high intensity of m/z 85. On the other hand, the structure of the fragment ion at m/z 85 proposed in the current work strongly supports the presence of fragment ion at m/z 68 in the CID spectrum of MEL and the doublet at m/z 71 and 72 in the case of ($^{13}C_3$,$^{15}N_3$)-MEL. Depending on the position of the charge, the cleavage of the fragment ion at m/z 89 in the CID spectrum of ($^{13}C_3$,$^{15}N_3$)-MEL drives elimination of either $^{14}NH_3$ (giving rise to m/z 72) or $^{15}NH_3$ (formation of m/z 71). In the case of MEL, these eliminations are obviously not distinguished, and only the fragment ion at m/z 68 is observed. The fragment ion at m/z 60 in the CID spectrum of MEL is formed by cleavage of the triazine ring giving rise to the guanidinium ion. This assignment is consistent with the presence of a fragment ion at m/z 62 in the CID spectrum of ($^{13}C_3$,$^{15}N_3$)-MEL; the +2 Da upmass shift is due to the presence of one ^{13}C and one ^{15}N in the structure of the guanidinium ion. The guanidinium ion is further decomposed into the fragment ion at m/z 43 (for MEL) or m/z 45 (for ($^{13}C_3$,$^{15}N_3$)-MEL) by the elimination of ammonia.

Cyanuric Acid. A similar study was conducted with CA and ($^{13}C_3$,$^{15}N_3$)-CA to comprehensively elucidate the mechanism of fragmentation of CA by CID in negative mode. The full-scan spectra of CA and ($^{13}C_3$,$^{15}N_3$)-CA exhibit prominent ions at m/z 128 and 134, respectively, corresponding to the deprotonated ion molecules. The deprotonation of an alcohol function in CA can induce an electronic rearrangement leading to the cleavage of a C−N bond with a concomitant transfer of the negative charge on the nitrogen atom (**Figure 1**). This form undergoes HO−CN elimination by electron transfer to give rise to a fragment ion at m/z 85. This assignment is confirmed by the CID spectrum of ($^{13}C_3$,$^{15}N_3$)-CA, which shows a fragment ion at m/z 89; the upmass shift of ΔM = +4 Da (compared to nonlabeled CA) is rationalized in terms of the presence of two ^{15}N and two ^{13}C atoms in the fragment. A similar subsequent loss of HO−CN leads to the formation of fragment ions at m/z 42 for CA and at m/z 44 for ($^{13}C_3$,$^{15}N_3$)-CA.

Method Development. Sample preparation was limited to weighing the sample in an acetonitrile/water medium. Precipitation of the proteins and further dilution were then performed within a single container, avoiding any solid phase extraction (SPE) for cleanup/enrichment steps and unexpected cross-contamination of the extract from diverse contact materials. Optimization trials were focused on evaluating the test portion size (from 0.5 to 2.0 g) and changing the acetonitrile/water proportions, that is, 100:0 (v/v, 15 mL), 66:33 (v/v, 30 mL), and

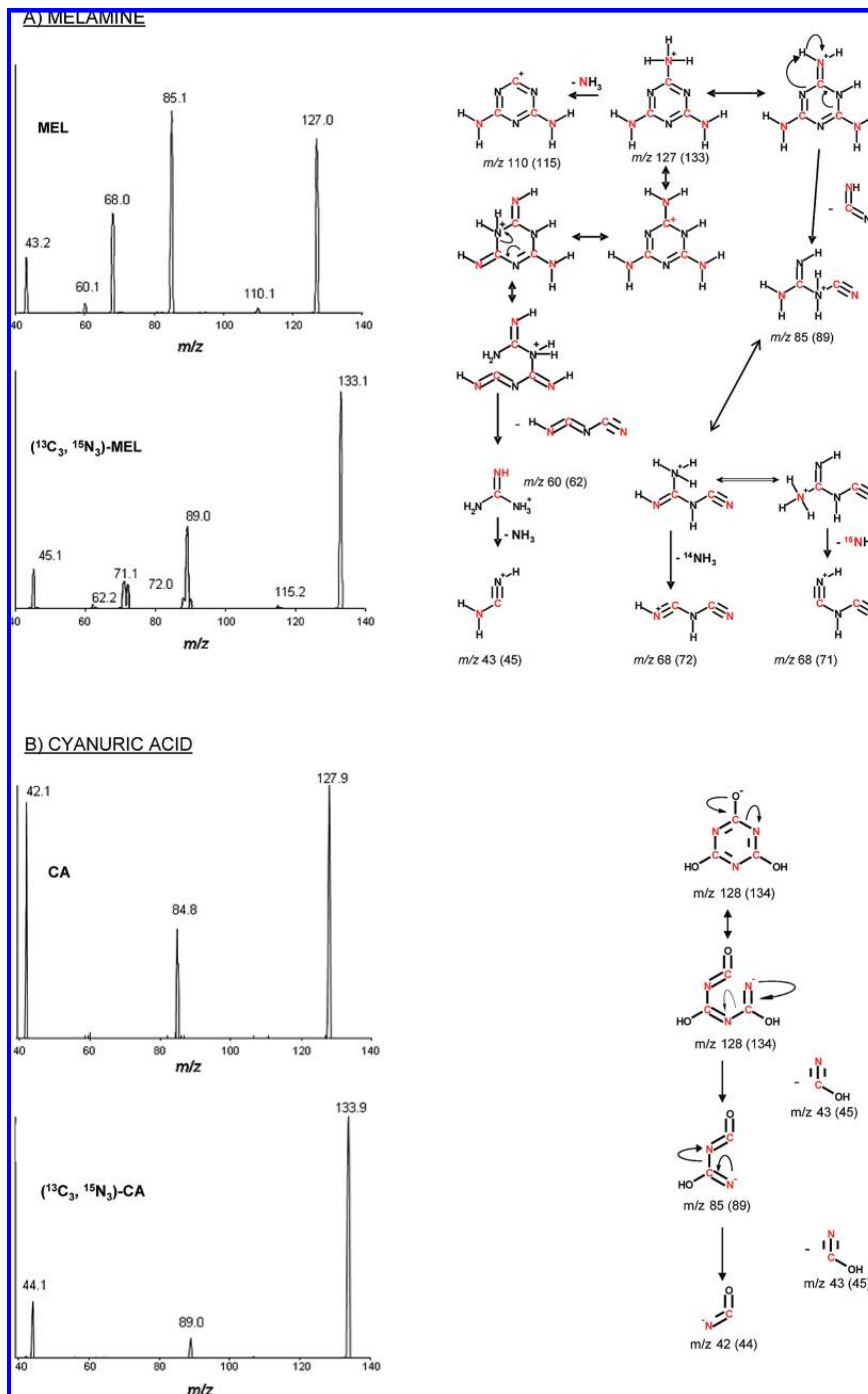

Figure 1. CID spectra of (**A**) MEL and ($^{13}C_3$,$^{15}N_3$)-MEL and (**B**) CA and ($^{13}C_3$,$^{15}N_3$)-CA and their respective postulated fragmentation pathway.

70:30 (v/v, 50 mL). The optimal conditions in terms of cleanliness of the extracts, analyte response at the lowest fortification levels, and minimal ion suppression due to matrix effects were finally obtained by considering 1 g of sample in 50 mL of the acetonitrile/water (70:30, v/v) medium.

Chromatographic separation of polar and hydrophilic compounds has long been problematic for reasons related to retention,

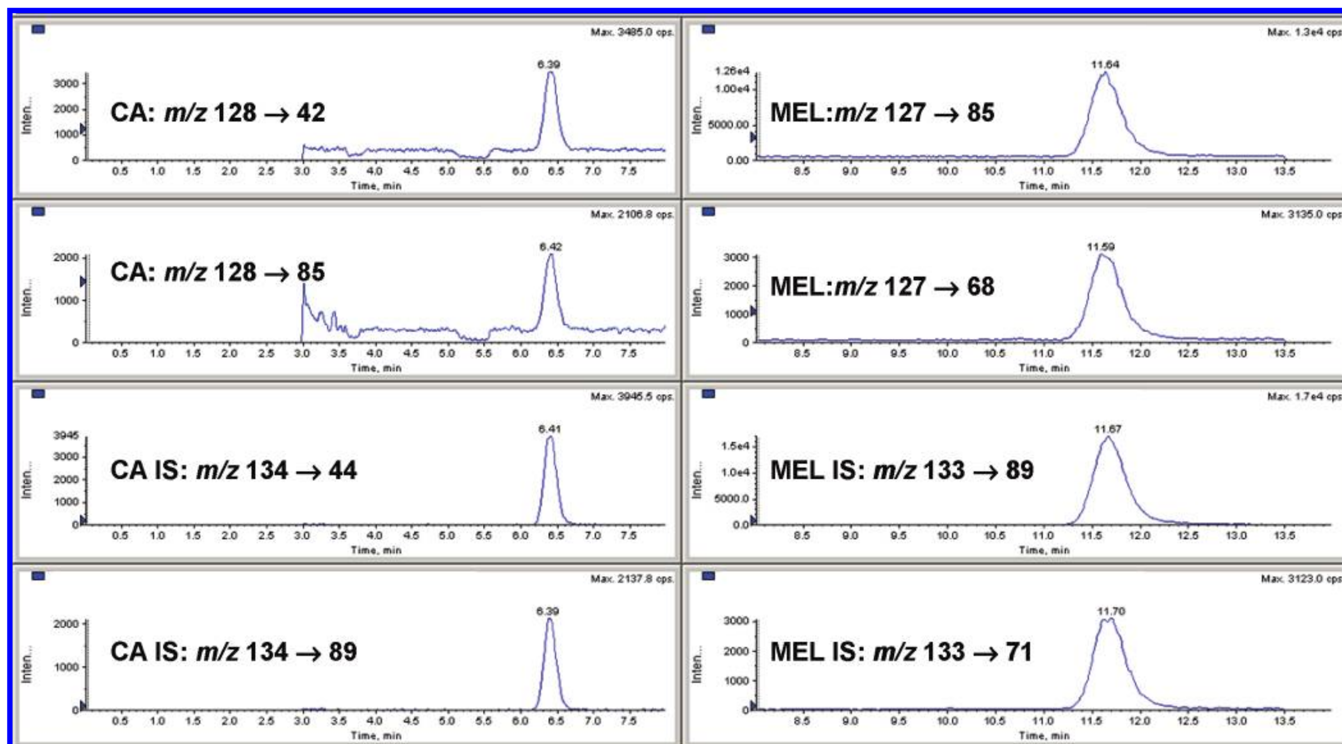

Figure 2. LC-MS/MS chromatograms of a cow's milk extract spiked with 0.05 mg/kg melamine (MEL) (MEL IS = 0.1 mg/kg) and 0.1 mg/kg cyanuric acid (CA) (CA IS = 0.1 mg/kg).

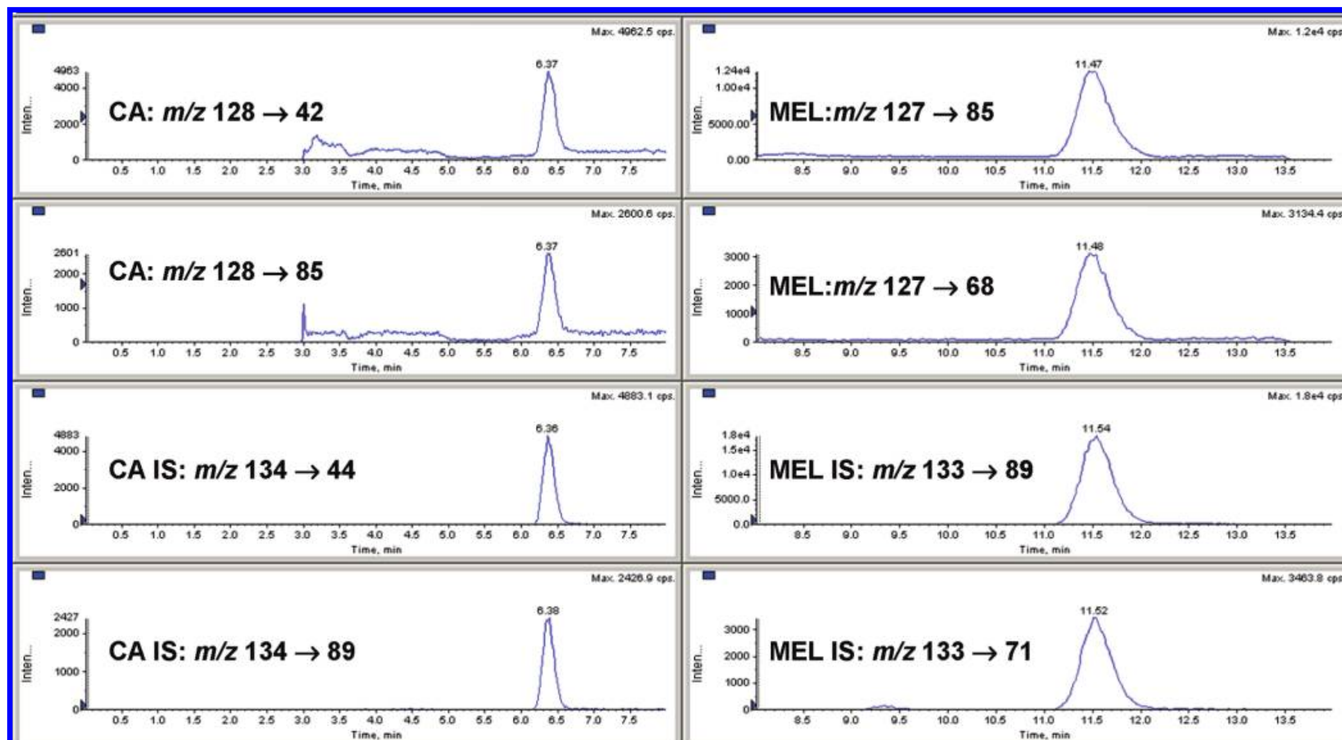

Figure 3. LC-MS/MS chromatograms of a milk-based infant formula extract spiked with 0.05 mg/kg melamine (MEL) (MEL IS = 0.1 mg/kg) and 0.1 mg/kg cyanuric acid (CA) (CA IS = 0.1 mg/kg).

peak shape, and reproducibility. Over the past few years, the need to analyze polar compounds in complex mixtures and the widespread use of MS coupled to LC have seen an increase in popularity of the HILIC mode of chromatography (*23*). In our work, several HILIC columns from different suppliers were tested with the ultimate goals of (a) having a retention time (RT) of each analyte at least twice that of the void volume of the column (*17*) and (b) enabling a sufficiently large ΔRT and being repeatable enough to allow both compounds to be detected within the same run using sequentially the negative and positive ionization modes. The LC columns considered were Acquity UPLC BEH HILIC (Waters Corp., Milford, MA, not used in UPLC conditions),

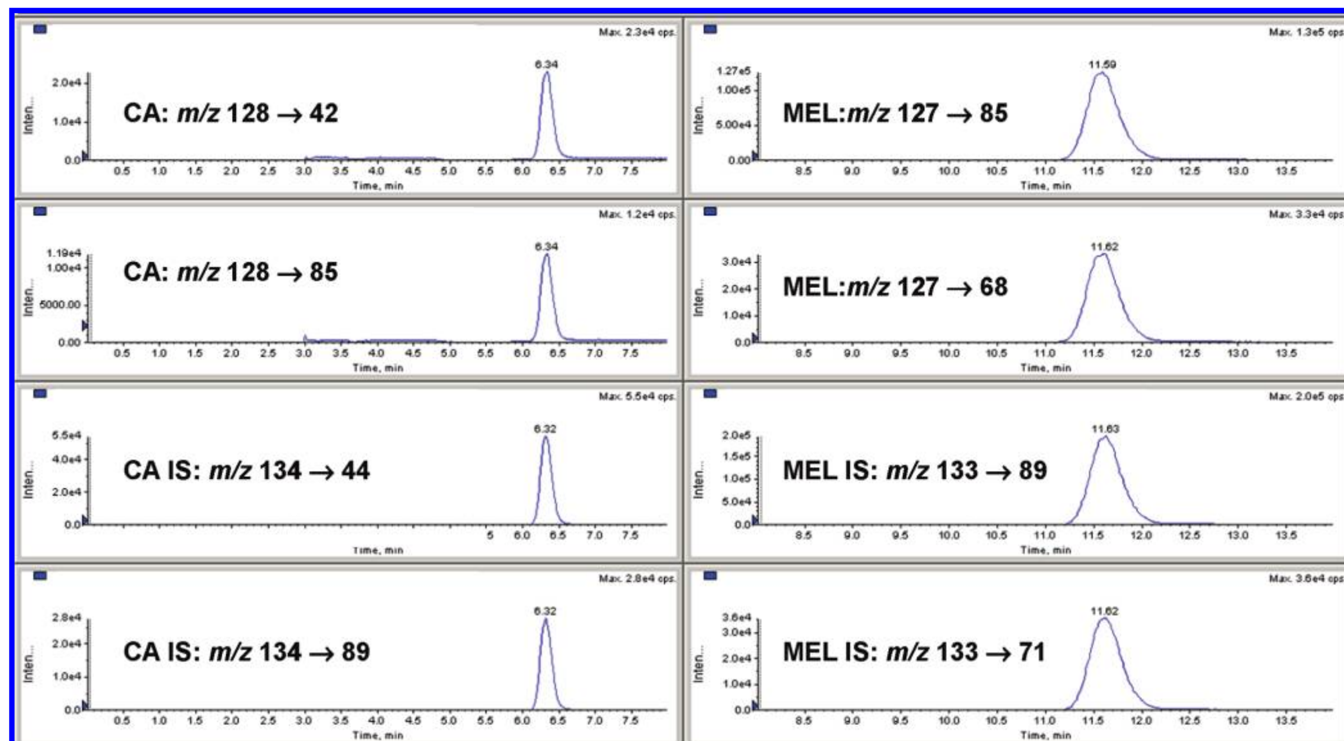

Figure 4. LC-MS/MS chromatograms of a milk-based infant formula extract spiked with both melamine (MEL) and cyanuric acid (CA) at the 1.0 mg/kg limit set by WHO (*1*). MEL IS and CA IS were also added at the 1 mg/kg concentration level.

Table 2. Method Performance Data for (**A**) Cow's Milk and (**B**) Milk-Based Infant Formula

(**A**) Cow's Milk

	melamine			cyanuric acid		
fortification level (mg/kg)	0.05	0.10	0.15	0.10	0.15	0.20
IS-corrected recovery (%)	107	102	103	99	101	102
repeatability[a] (%)	3.0	6.1	3.6	4.6	8.1	4.2
intermediate reproducibility[a] (%)	12.6	6.3	6.4	10.2	9.0	7.2
expanded uncertainty[a] (%)	11.6	7.1	5.5	7.7	6.9	5.6
LOD (mg/kg)		0.025			0.05	
LOQ[b] (mg/kg)		0.05			0.10	

(**B**) Milk-Based Infant Formula

	melamine						cyanuric acid					
fortification levels (mg/kg)	0.05	0.10	0.15	0.50	1.00	1.50	0.10	0.15	0.20	0.50	1.00	1.50
IS-corrected recovery (%)	111	109	107	102	102	102	116	111	111	105	103	103
repeatability[a] (%)	12.3	4.6	2.0	4.0	3.4	1.4	8.9	10.7	9.8	1.1	3.4	5.0
intermediate reproducibility[a] (%)	14.7	13.3	3.8	5.8	3.8	5.3	31.2	16.4	22.2	13.2	13.1	6.4
expanded uncertainty[a] (%)	15.1	13.3	7.0	4.8	3.6	4.2	27.7	15.9	20.0	10.3	10.2	6.0
LOD (mg/kg)			0.025						0.05			
LOQ[b] (mg/kg)			0.05						0.10			
$CC\alpha$[c] (mg/kg)			1.03						1.04			
$CC\beta$[c] (mg/kg)			1.05						1.09			

[a] Repeatability, intermediate reproducibility and expanded uncertainty limits are given at the 95% confidence interval level. [b] Limits of quantification were arbitrarily set at the lowest validated fortification levels. [c] $CC\alpha$ and $CC\beta$ were calculated by assuming a maximum limit of 1.0 mg/kg in milk powder (*1*).

Inertsil HILIC (GL Sciences, Torrance, CA), ZIC-HILIC (Merck, Darmstadt, Germany), and TSKgel Amide-80 (i.e., the one used in this paper). In our hands, the Acquity UPLC column did not provide any retention for CA, and unstable retention times were observed when using the Inertsil HILIC column after a few injections. Only the TSKgel Amide-80 and the ZIC-HILIC columns fulfilled the above-mentioned prerequisites over a long period of time, as demonstrated during the analysis of >1400 routine samples. However, even with these selected LC columns, any attempts to shorten the overall run time (25 min) by reducing

the reconditioning step led to rapid column clogging, variable RTs, soiling of the MS interface, and, consequently, significant reductions in the detector responses. **Figures 2− 4** show SRM chromatograms of cow's milk and milk-based infant formula extracts spiked at the lowest fortification levels, with typical RT of 6.3 min for CA (obtained in isocratic condition with a 90% percentage of acetonitrile) and 11.6 min for MEL (obtained in gradient condition).

Matrix effects were evaluated by building matrix-matched calibration curves from blank milk powders of different formulations (obtained by spiking the analytes and their corresponding ISs at the beginning of the sample workup) and comparing their slopes with those of solvent-based curves. Negligible slope deviations (< 10%) were observed with low impact on the final results, meaning a good compensation of matrix effects by the ISs. Therefore, only solvent-based curves were considered for convenience, and MS responses were linear over the two ranges of concentrations considered ($RSD_{RF} < 15\%$ and $r^2 > 0.997$). No interference at the expected retention times was noted when several cow's milk samples and milk-based infant formulas of different compositions were analyzed. The low concentration levels of each analyte (< 500 ng/mL) in the standard solutions and in the final extracts prevented the formation and precipitation of the melamine−cyanuric acid complex (melamine cyanurate) as already demonstrated by Heller et al. (*24*) and Filigenzi et al. (*25*). This stability of MEL and CA in the final extracts was further demonstrated by re-injecting a series of processed samples left at room temperature onto the autosampler for a 48 h period. No significant difference (peak area and results comparison) was observed. The same conclusion was also made for extracts stored at 4 °C for 1 month.

Method Performance Characteristics. The overall performance data of this LC-MS/MS procedure in cow's milk and milk-based infant formula are summarized in **Table 2**. Internal standard-corrected recoveries were within the 99−116% range for both analytes in the two matrix types surveyed, along with repeatability and intermediate reproducibility values (at the 95% confidence interval level) of ≤12.3% and ≤31.2%, respectively. The highest intermediate reproducibility value (31.2%) concerned CA in milk-based infant formula at the lowest fortification level (0.100 mg/kg). Expanded uncertainties were often (15 of 18 cases) inferior or equal to the intermediate reproducibility values, supporting the fact that the intermediate reproducibility, in this validation scheme, is a more realistic indicator of the "true" uncertainty than that obtained by calculation. From the analysis of 2 cow's milk samples and 15 different milk-based infant formulas, spiked at 0.05 mg/kg MEL and 0.10 mg/kg CA, the limits of detection (LODs, signal-to-noise ratio of 3) were broadly estimated to be within 0.005−0.025 mg/kg for MEL and within 0.02−0.05 mg/kg for CA. These variations of LODs were imputed to the different matrix effects (signal suppression) encountered during the analysis of this set of samples when very low doses were considered. On the other hand, limits of quantification (LOQs) were arbitrarily set at the lowest fortification levels, that is, 0.05 and 0.10 mg/kg for MEL and CA, respectively, as the detection/quantification at these levels were easily achievable irrespective of the sample under survey.

WHO has established tolerable daily intakes (TDIs) of 0.2 and 1.5 mg/kg of body weight for MEL and CA, respectively, and has stated that limits for MEL in milk-based infant formula (1 mg/kg) and other foods (2.5 mg/kg) would provide a sufficient margin of safety for dietary exposure relative to the TDI (*1*). Due to a lack of data, the same 1 mg/kg ML was considered for CA in milk-based infant formula. CCα (concentration at which there is a 5% error probability of declaring the sample as containing less than the ML) and CCβ (concentration at which the method is able to detect the permitted limit concentration with a statistical certainty of 95%), calculated as described under Materials and Methods, were thus respectively 1.03 and 1.05 mg/kg for MEL and 1.04 and 1.09 mg/kg for CA.

Table 3. Results of a Nestlé Proficiency Test for the Analysis of Melamine in Milk-Based Infant Formulas (Values in Milligrams per Kilogram)

	sample A[a]	sample B	sample C	sample D
assigned value[b] ± SD(R)		0.235 ± 0.062	1.264 ± 0.104	2.785 ± 0.220
Laboratories:				
NRC				
results	<LOD[c]	0.239	1.316	2.957
z score	na	0.06	0.5	0.78
NQAL1				
results	<LOD[c]	0.157	1.275	2.725
z score	na	−1.28	0.11	−0.27
NQAL2				
results	<LOD[c]	0.200	1.057	2.512
z score	na	−0.57	−1.98	−1.24

[a] Nonspiked melamine free sample. [b] Assigned values were derived from the data of 27 laboratories. [c] LOD = 0.025 mg/kg.

Table 4. Results of IRMM[a] Proficiency Test for the Analysis of Melamine (Values in Milligrams per Kilogram)

	milk powder	backing mix
assigned value[b] ± SD(R)	10.0 ± 1.12	3.18 ± 0.43
result	10.5	3.21
z score	0.4	0.0

[a] Institute for Reference Materials and Measurements (IRMM). [b] Assigned values were derived from the data of 144 laboratories from 31 countries.

Internal and European Proficiency Tests. The data of the internal P test indicate that the method described here is suitable for the quantitative determination of MEL in milk-based powdered infant formulas. All *z* scores obtained by NRC and the two NQALs are $|z| < 2$, leading to the conclusion that all results are satisfactory (**Table 3**). The method was developed at NRC and transferred in the NQALs without any physical intervention of NRC experts; this demonstrates the fitness-for-purpose and the easiness-to-handle of the method. The results obtained by NRC with the European P test (**Table 4**) are also satisfactory as the *z* score was calculated at 0.4 for the milk powder sample and at 0.0 for the baking mix. Interestingly, the excellent performance recorded for the baking mix suggests that the method is applicable not only to dairy products but also to other food matrices, that is, those for which the 2.5 mg/kg limit set by WHO (*1*) has to be applied.

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