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Melamine content in some milk-based products

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Abstract: This work reports the analysis of melamine in forty samples (22 of powdered milk and 18 chocolate) purchased from local market in Romania, in order to examine the presence of melamine over baseline level in products. The European Union has a clear legislation establishing a standard limit of the melamine content in milk powder for children. An analytical method including liquid extraction followed by mixed mode ion exchange/reversed phase solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used. The method is sensitive (limit of detection and quantification are 0.05 mg/kg and 0.150mg/kg), precise (RSDs<6.5%) and accurate, with recoveries averaged between 82.68 - 100.55%, for all fortification levels and matrices. Melamine was detected in 32 out of 40 samples, at concentrations ranging from 0.064 to 0.326 mg/kg. The low levels of melamine in analyzed samples indicated that these do not represent a health risk.

Keywords: melamine, LC-ESI-MS/MS, milk-based products.

INTRODUCTION

Milk and milk products are essential components of a human healthy diet for all age group, especially for children, because they provide high quality protein and represent a good source of vitamins and minerals[1]. Proteins, as important component of milk, are quantified to establish the quality of products. Some compounds, rich in nitrogen, can mimic a high protein concentration, leading to their use as adulteration[2]. Melamine, an organic compound containing 66% nitrogen, was reported as a major component used to adulterate milk-based products[3-5]. The sources of melamine contamination have been classified as base-line, which refers to levels that result from plastic migration and as adulteration, which results from the intentional addition of melamine to food or feed[6].

Although there are no approved uses for the direct addition of melamine to food, humans are exposed to melamine from different source such as: vegetable crops (via cyromazine used as fertilizer), animal origin food, when animals are fed with contaminated crops or their food contains feed additives[7,8].

The ingestion of melamine at levels above the safety limit can induce renal failure and death in children. To prevent further contamination, the melamine limit in food was established. For dairy products, the standard limits for melamine in children formula at 1 mg/kg and in other dairy products at 2.5 mg/kg were introduced by many countries[9,10].

In the recent years, melamine has received global attention due to a series of melamine-contaminated foods; therefore, it is very important to monitor the presence of melamine in foods, including milk, because of its use as an ingredient in many products: instant coffee, yogurt, chocolates, biscuits, protein ingredients[11].

LC-MS/MS was selected as the method of detection for the analysis of melamine, because it is very selective and sensitive and could reach the low requested detection limits. Milk products (such as milk powder, chocolate) are complex matrixes containing interfering compounds, such as protein, sugar or fat. These compounds may interfere with the targeted ones or may contaminate the analytical system, if the sample is introduced in the LC without selective sample preparation. However, in the analysis of the complex matrixes, it is necessary to obtain clean extracts in order to eliminate the co-extracted interferences before the next step of the analysis.

Mixed mode cation exchange/reversed phase extraction (SPE) was used for clean-up step. The paper aims to continue the researches related to the quality of the milk- based products on the Romanian food market. Until now, there are concerns regarding the organic pollutant and metal content[12,13] in the milk powder marketed in Romania, but there are no studies on the melamine content in these basic products. Therefore, our study was performed in order to show whether Romanian milk products, namely powdered milk and chocolates, are exposed over baseline levels of melamine.

EXPERIMENTAL

Reagents and chemicals: The standard of melamine was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (ACN), methanol, ammonium hydroxide, trichloroacetic acid were purchased from Merck (Darmstadt, Germany). For clean-up step, a cartridge (SampliQ SCX SPE cartridge) supplied by Agilent Technologies (USA) was used. Aminated methanol was prepared by a mixture of 5ml

ammonia solution and 95 ml methanol. De-ionized water used for the mobile phase preparation and dilution was prepared from a Milli-Q Plus system (Merck-Millipore, Darmstadt, Germany). Stock standard solution of melamine was prepared by dissolving approximately 10mg of powder, accuracy weighted in 100 ml of 5% aminated methanol, obtaining a final concentration of about 100µg/ml and stored at 4^0C in a dark glass bottle. Working standard solutions used for LC-MS/MS analysis or for sample fortification, were freshly prepared by appropriate dilutions of the stock solution, using deionized water.

Instrumentation: The LC-MS/MS system from Agilent Technologies consisted of a 1200 Series liquid chromatography pump coupled to a triple quadrupole mass spectrometer (6464 Triple Quad). The separation of melamine was performed on a ZORBAX Rx-SIL column, 2.1mm x 150mm, 5 µm (Agilent) using a binary mobile phase of (A) aqueous solution of 0.5mM ammonium formate and 0.01% (v/v) formic acid and (B) 0.01 % formic acid (v/v) in acetonitrile. The flow rate was 0.25ml/min and the injection volume was 15 µL.

The LC-MS/MS with electrospray ionization (ESI) was operated in the positive mode. Multiple-reaction monitoring (MRM) was performed on the protonated molecule for melamine using the following general parameters: gas temperature 350^0C, gas flow (N_2) 8L/min, nebulizer pressure 35psi, fragmentor 110V. The MRM transitions for melamine were *m/z* 127→85, (collision energy 20 eV) 127→68 (collision energy 30 eV) and 127→43 (collision energy 30 eV). A single MRM was used to perform quantification and the quantitative MRM transition was *m/z* 127→85.

Analytical procedure: The sample preparation procedure was based on the modified method, previously described by Wu[14]. Both powdered milk and chocolate samples were prepared in the same manner. 1g of solid samples, previously grounded and homogenized was transferred into a 50 ml centrifuge tube. Samples were extracted with 15ml of 5% tricholoacetic acid water solution, and 5ml of acetonitrile. The extract was sonicated for 10 min and then centrifuged at 4000 rpm for 10 min. The supernatant was transferred into a 25 ml volumetric flask and bring to volume with 5% trichloroacetic acid solution. A 5 mL aliquot of the extract was transferred into a glass tube and diluted with 5 mL HPLC-grade water.

All samples were solid phase extracted with cartridges, which were conditioned by passing 6 mL of methanol and 6 mL water. The samples were loaded at a flow rate of 1.0 mL/min. After pre-concentration, the sorbent was rinsed with water (2 x 20 mL) and vacuum dried. Trapped compound were desorbed using 6 ml methanol. After rota-vaporization to near dryness, the extracts were reconstituted with ACN/ water of 90:10 (v/v) to a final volume of 1 mL and filtered through a 0.2 µm regenerated cellulose membrane filter into a glass LC vial. Finally, 15µL was injected in the LC-ESI-MS/MS.

Application to samples: Forty products from Romanian markets were selected on the basis of their immediate availability, in the year 2012. Samples include powdered milk recommended for different stage of baby growing (n = 22) and different types of chocolate (n = 18), as follows **(Table 1).**

Table 1: Summary of numbers of milk-based products collected from local market

Number	milk powder					
	0-6 months M1	6-9 months M2	1 year M3	1-3 years M4	with hydrolysed proteins M5	'integral" M6
Total 22	4	3	3	4	4	4
Chocolates						
	milk chocolate C1	chocolate yogurt C2	white chocolate C3	chocolate with fruits C4	dark chocolate C5	chocolate with peanuts C6
Total 18	4	4	3	3	2	2

All numeric values represent mean values of 3 measurements ± standard deviation (SD).

RESULTS AND DISCUSSION

Validation of the method: Method validation, including linearity and limit of detection (LOD), was carried out before application to sample analysis. The linearity was measured by injecting in triplicate the standard calibration solutions of six different concentrations al levels ranging from 0.010 to 1.000µg/mL. Calibration curves, based on the peak area ratio of the analyte to the concentrations, were linear over the calibration range with a correlation coefficient of $R^2 > 0.999$. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentration with a signal to noise ratio (S/N) of 3 and 10, respectively. Under the concentrations specified in the method, the LODs and LOQs in powdered milk were 0.05 mg/kg 0.150 mg/kg, respectively. A recovery study was carried out to evaluate the method's accuracy and precision. Fortified samples at different concentrations were prepared with five replicates. Satisfactory recoveries (82.68 - 100.55 %) were obtained for all matrices. Precision, expressed as repeatability of the method, was estimated in terms of relative standard deviation (RSD, %) from the recovery experiments (n=4) at each fortification levels. Good precision (RSDs<6.5%) was obtained for all matrices.

In our study, melamine was present in the majority of samples, at concentrations <0.300mg/kg, which is according to the baseline criteria of melamine in food (< 1 mg/kg) recommended by WHO (2009); these levels are not considered to be a health concern.

Melamine was detected in 17 out of 22 powdered milk samples at concentrations ranging from 0.064 to 0.297 mg/kg, while five samples had melamine below the detection limit (0.05 mg/kg) of the assay method (**Table 2).** The lower concentrations were found in samples M1 and M2 (0-6 months and 6-9 months, respectively), and the highest concentrations of melamine were detected in the samples M4 and M6, which also indicates a higher content of total protein as indicated in the manufacturing. In addition, there is an apparent relationship between melamine concentrations and protein content in the case of these two types of milk, as shown by the coefficient of correlation ($r^2 = 0.9316$).

Table 2: Melamine in Infant Formula Samples Sold in Romania

milk powder	N	melamine (mg/kg) ± standard deviation			
M1	4	0.064±0.006	<LOD	<LOD	<LOD
M2	3	0.169±0.007	0.094±0.006	<LOD	-
M3	3	0.179±0.010	0.191±0.011	0.194±0.020	-
M4	4	0.210±0.034	0.244±0.036	0.264±0.017	0.184±0.005
M5	4	0.164±0.025	0.097±0.011	0.179±0.014	0.190±0.015
M6	4	0.249±0,016	0.297±0.021	0.268±0.015	<LOD

In majority of chocolate samples, melamine was detected at concentrations ranging from 0,094 to 0.279 mg/kg **(Table 3).** The maximum concentration observed was 0.326 mg/kg in white chocolate formula. The lower concentrations were detected in chocolates with fruits and with peanuts, while in dark chocolate it was not detected.

Table 3: Melamine in some Chocolate Samples Sold in Romania

chocolate sample	N	melamine (mg/kg) ± standard deviation			
C1	4	0.205±0.006	0.200±0.014	0.170±0.014	0.094±0.009
C2	4	0.279±0.016	0.264±0.031	0.272±0.013	0.194±0.012
C3	3	0.326±0,017	0.213±0.011	0.308±0.012	-
C4	3	0.142±0.018	0.074±0.009	<LOD	-
C5	2	<LOD	<LOD	-	-
C6	2	0.098±0.005	0.150±0.017	-	-

Fig. 1 shows the detailed distribution of analyzed samples based on melamine contents. It can be seen that approximately 23% of milk and 17% of chocolate samples were not contaminated by melamine, 36.3% of powdered milk had a melamine content of 0.1-0.2 mg/kg, while 33.3% of chocolates had a melamine concentration from 0.2 to 0.3 mg/kg. In addition, 2 white chocolates had more than 0.3 mg/kg melamine.

The relatively low concentrations of melamine, observed in the analyzed products, indicate that they did not have as a source of melamine intentional adulteration or migration from the packaging. Studies have shown that melamine can migrate into food samples from melamine-formaldehyde plastic ware[15]. Thus, Ishiwata showed that melamine migrates from melamine-formaldehyde resin cups into coffee and juice beverages, at concentrations ranging from 0.45 to 3.24µg/g[16].

The analyzed products could supply melamine from vegetable crops as a result of melamine from environment, from residues of the legal use of triazine pesticide or from the legal use of melamine in fertilizers. In this way, melamine may be present in foods of animal origin when animals are fed with crops. Qin showed that melamine is present in vegetable crops, at baseline criteria of melamine in foods, for the majority of analyzed samples[17]. The breakdown of melamine from cyromazine has been studied,

and melamine residues in milk are about 10% from this insecticide[18]. It seems possible that milk from cattle exposed to cyromazine may contain melamine, and if such milk is used to prepare powdered milk or chocolates, then the melamine may be incorporated into the final product.

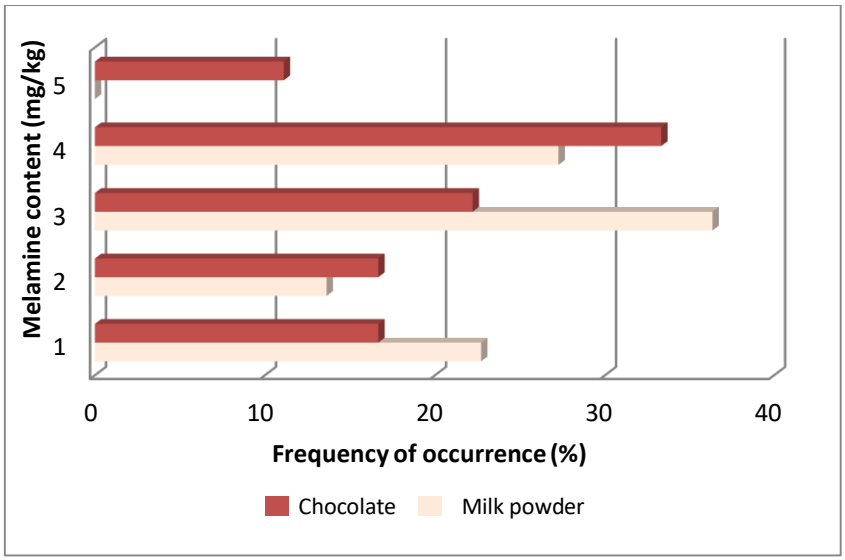

Figure 1: Frequency distribution of melamine concentration detected in milk products: (1=not detected; 2 = 0-0.100mg/kg; 3=0.100-0.200mg/kg; 4=0.200-0.300mg/kg; 5>0.300mg/kg)

Another possible source of melamine is the potential long-term exposure of animals to the baseline level of melamine or adulteration level. However, a recent study proved that low content of melamine was stored in animal tissues, and a higher quantity of melamine was excreted into the environment[17]. This indicates that these residues are considered to be a potential source of melamine exposure for environment.

The concentrations observed in our study are generally similar with baseline levels of melamine in infant formula samples, at concentrations ranging from 4.31 to 346µg/kg, reported by Tittlemier[19]. Residues of melamine at levels of 0.08 - 0.62 mg/kg have been reported in 30 milk products by Xiao-Dong[20]. Ibáñez et al., have analyzed 40 milk-based foods and beverages, finding residues of melamine in two of them (powdered milk and baking mix) as part of an EU proficiency test, in which highly satisfactory results were obtained[21]. The low levels of melamine in analyzed milk products indicated that these do not represent a health risk for humans.

As a conclusion, the present study shows that melamine content in analyzed samples do not represent a health risk for humans.

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Conflict of interest: The authors declare no conflict of interest.

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