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ORIGINAL PAPER

Assessment of residue melamine in dairy products exhibited in Zanjan market, Iran by high-performance liquid chromatography method

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Abstract An increasing number of infants are being made sick by dairy products contaminated with melamine. As a result, many countries have adopted additional requirements for milk-based food products. The scope of the present study was to assess amounts of melamine in some dairy products from Zanjan market, Iran. Amounts of melamine in samples of milk, yoghurt, infant formula, coffee mate and cheese were determined in five different brands by the high-performance liquid chromatography with ultraviolet detector system. In total, 225 samples were determined (five products × five brands × three samples × three repeated measurements). Amounts of melamine in samples (in μg/g) were determined in the range of 0.20–0.26 for milk, 0.57–0.99 for yoghurt, 0.35–3.40 for infant formula, 0.09–1.23 for coffee mate and 0.30–2.50 for cheese, and the mean values of melamine in products were evaluated as 0.24, 0.76, 1.38, 0.56 and 1.16, respectively. The minimum and maximum values for amounts of melamine were recorded in milk and infant formula, respectively. Comparison of test results with the standards set by the European Union and the US Food and Drug Administration for maximum residue-level values for dairy products contaminated with melamine showed that levels determined by tests had higher health risks than the regulations allowed. Iran has not yet adopted regulations to control levels of melamine in food products. The results show that maximum residue-level values need to be determined for melamine in Iranian dairy products, and foods should be monitored daily for levels of melamine contamination.

Keywords Food · Contamination · Protection · Safety · Assessment

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Introduction

Melamine (2,4,6-triamino-1,3,5-triazine, C3H6N6) (MM) is an industrial organic chemical compound that is very rich in nitrogen (66.6 % by mass). It usually occurs in the form of white crystals. The structure of MM and its analogues is shown in Fig. 1. This compound was first synthesized by the German chemist Justus von Liebig in 1834 and was subsequently used to make plastics and laminates (Zakeel 2008; Hong et al. 2009). It has been used in the synthesis of melamine–formaldehyde resins, manufacturing of laminates, plastics, coatings, commercial filters, glues and adhesives, dishware, kitchenware and flame retardant materials (Filazi et al. 2012; Wang et al. 2010; WHO 2008; Cheng 1994). MM has also been used as a fertilizer because it has high nitrogen content. Other applications include that of metabolite cyromazine, an approved insecticide that is used on a broad range of vegetable crops (Wang et al. 2010). This widespread use of MM and its derivatives means that it has penetrated the soil and thereby affects the human food chain. It can be found in the bodies of mammals that have ingested cyromazine (Haughey et al. 2013; Ge et al. 2011; Qin et al. 2010; Afoakwa 2008). The combination of melamine and cyanuric acid was thought to be responsible for renal

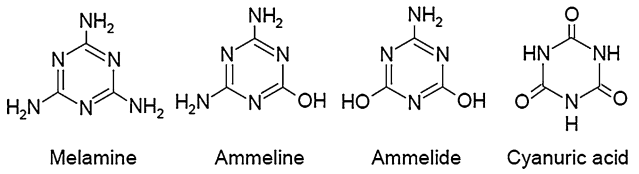

Fig. 1 Structures of melamine, ammeline, ammelide and cyanuric acid

impairment in mammals (Yin et al. 2012). MM concentrations contaminate a variety of foods notably dairy products. Contamination occurs in food from the materials that have contact with food during production processes, and dairy products are contaminated by animals that have been fed with MM or its analogues. Furthermore, the high content of nitrogen in MM means that it is sometimes added to food illegally in order to show an apparent increase in protein content (Filazi et al. 2012; Feng et al. 2012; Heintzelman 2011; Venkatasami and Sowa 2010).

Melamine can be very toxic at high-dose exposure (Langman 2009). The formation of an insoluble melamine–cyanurate complex can be fatal for babies and infants (Salman et al. 2012). MM in the human body can cause health problems such as kidney stones, renal failure, urinary problems and hyperplasia in the bladder (Filazi et al. 2012; Chang et al. 2012; Hau et al. 2009; WHO 2008). It has also been reported that MM was carcinogenic for male rats (Wang et al. 2010; WHO 2008). Its median lethal dose (LD50) was determined as 3,161 mg/Kg body weight for male rodents and 3,828 mg/Kg weight for females (Tian et al. 2012; Reimschuessel et al. 2009; WHO 2008).

It is very important to establish amounts of melamine in milk products, some foods, animal feed and sediment in order to monitor food contamination, and this has been the main consideration in studies related to the subject in recent years (Tian et al. 2012; Salman et al. 2012; Wang et al. 2009, 2010; Li et al. 2009; Afoakwa 2008).

Attention to the potential risks of melamine began in March 2007 when pet food ingredients were found contaminated with MM and its analogues, and the incident caused death in many pets (Salman et al. 2012; Tyan et al. 2009). MM has recently been found in infant formula in China and in some food categories that use milk powder as an ingredient, such as chocolate, biscuits, candy, ice cream and eggs. Up to September 22, 2008, a total of 52,857 cases of nephrolithiasis have been reported; in some cases, renal failure, 12,900 hospitalization and 4–6 deaths from various regions in China have been reported, all of which were traced back to consumption of melamine-contaminated powdered milk products. Test results conducted in China on samples of powdered infant formula showed that samples contained a wide range of concentrations (0.1–>2,500 ppm) melamine powder (Xu et al. 2009; Afoakwa 2008). Soon after these tests, various dairy and nondairy products originating from China were found contaminated with melamine (Gossner et al. 2009).

Many countries have adopted maximum residue level (MRL) for melamine in various products to regulate food safety and for public health protection. Regulations in many countries now stipulate that infant formula must be free of melamine (Filazi et al. 2012).

Several methods have been developed to detect melamine, such as GC, liquid chromatography, immunoassay, GC–MS, liquid chromatography–MS electrokinetic capillary chromatography (Vachirapatama and Maitresorsun 2013; Liu et al. 2012; Filazi et al. 2012; Wang et al. 2010), chemiluminescence (Zeng et al. 2011), fluorescent (Zhang et al. 2012), Raman (Cheng et al. 2010; Qin et al. 2013) and visual detection using gold nanoparticles (Li et al. 2010). Liu et al. (2012) review recent developments for detecting melamine and discuss future directions. This article discusses about confirmation and screening methods for determination of melamine.

Tian et al. (2012) measured MM concentrations in the compost and soil samples by high-performance liquid chromatography (HPLC) with UV detection method, an enzyme-linked immunosorbent assay (ELISA) test kit and an enzyme-linked rapid colorimetric assay (RCA) test kit. These methods range from sensitive liquid chromatographic-tandem mass spectrometric techniques to less sensitive immune selective assays such as ELISA.

Difficulties in analyzing melamine may include contamination, matrix effects and analyte instability. The effect of these difficulties generally depends on the method used, the food matrices involved and the analyte examined (Filazi et al. 2012).

Reversed-phase HPLC–UV can be used to determine melamine amounts, it is claimed, because this method is simple, highly sensitive and highly specific. This method has a wide linearity range and a low detection limit. The simple pretreatment of the sample makes the method independent of a matrix or any type of contamination. Furthermore, the easy sample pretreatment and short runtime make it an economical method for the analysis of melamine in milk and dairy products and for use as a routine monitoring program (Filazi et al. 2012; Tian et al. 2012).

Iran imports a large quantity of foodstuff from China so this study aimed to determine amounts of melamine in dairy products purchased from supermarkets in Zanjan

province. Food was tested with high-performance liquid chromatography–UV detector (HPLC–UV). It should be suitable for a variety of complex foods or environmental materials, has high sensitivity, high specificity, short detection time and low cost and requires minimal sample preparation (Salman et al. 2012). This study was conducted in the Environmental Sciences Research Laboratory of faculty of science, University of Zanjan, Iran, in 2012–2013.

Materials and methods

Reagent

All chemicals used in this study were reagents of the highest grade and used without further treatment. Melamine (99 % purity) was purchased from Sigma-Aldrich. Acetonitrile (HPLC grade, Romil) and ultra-pure water were used in the mobile phase. Ultra-pure water was obtained from a Millipore system.

Preparation of standard solutions of Melamine

A 100 μg/mL melamine stock standard was prepared by adding 10 mg accurately weighing of melamine into a 100-mL volumetric flask. Melamine was dissolved with ultra-pure water. The stock standard was diluted to prepare working standards with concentrations of 0.01, 0.05, 1.00, 2.50, 5.00 and 10.00 ppm for the calibration curve (Fig. 2). The calibration curve confirms ability of method in determination of melamine. Due to sample matrix contribute to analytical signals, standard addition method is used in determination of melamine in dairy samples. The limit of detection (LOD) for this method was defined as the concentration at which signal-to-noise ratio was 3:1 (Table 2).

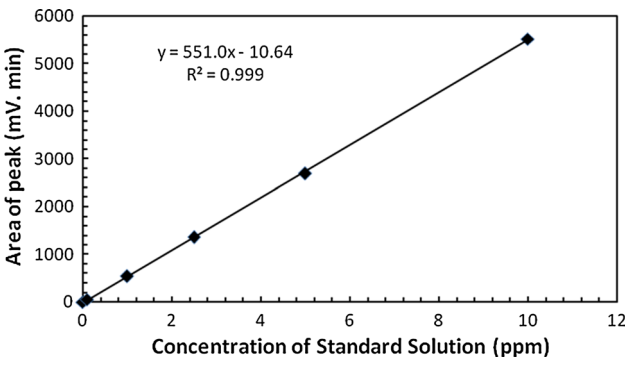

Fig. 2 Calibration curve in determination of melamine

HPLC system of analysis

Melamine composition was determined by HPLC Shimudzu system equipped with pump (LC-10AD Shimadzu), stainless steel filter, guard column C18 (Hector, 5 μm, 250 × 4.6 mm) and C18 column (Hector, 5 μm, 10 × 4.6 mm). The oven temperature (CTO-10AC Shimadzu) was set at 40 °C. A UV–Visible instrument (YL 9120) was used as the detector. YL clarity software was used to control instruments, for data collection, processing and joining the pump, oven and detector. YL clarity software was applied to run and control all calculations for the instrument. The injection loop was set up to 20 μL with manual Rheodyne injector (7725 i). The mobile phase was made by mixing 95 % of acetonitrile (Romil) and 5 % of deionized water. All solvents were filtered through a 0.45-μm Millipore filter before use and degassed in an ultrasonic bath. The HPLC system operated at a flow rate of 1.0 mL/min, the injection volume was 20 μL and the external temperature control column oven was set at 40 °C. The detection wavelength was 240 nm.

Sample collection and storage

Dairy products used in this study were purchased from supermarkets in Zanjan (Zanjan, Iran). Sampling was done in the winter of 2012 and the spring of 2013. Five types of dairy product were selected for sampling: milk, yoghurt, infant formula, coffee mate and cheese. Sampling was done on five brands of each product. For each product, three repetitions of samples were prepared and three different measurements were taken for each sample. Samples were stored in refrigerator conditions pending tests. The total number of samples in these tests was 225 (five products × five brands × three samples × three repeated measurements).

Sample digestion

Protein and carbohydrate contents are typically caused by complex matrices of food that can make analysis difficult. Isolation and extraction of melamine and its analogues need to be made from complex matrices prior to determining melamine concentrations (Salman et al. 2012). In this order, 1 ± 0.01 g samples of milk and yoghurt and 0.5 ± 0.01 g samples of cheese, infant formula and coffee

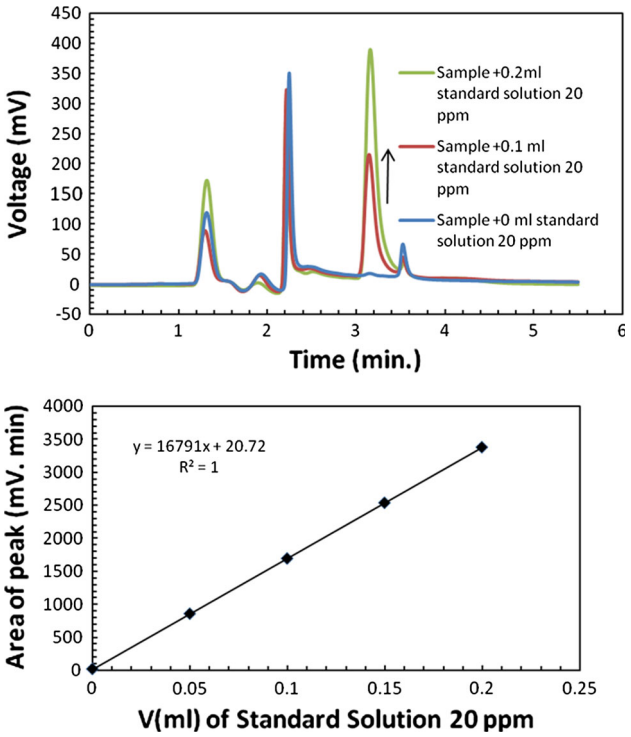

Fig. 3 Chromatogram and standard addition plot for identification and determination of melamine

mate were weighed into plastic tubes. An Ohaus balance analytical model GA200D (±0.00001 g) was used for weighing. To each 5 mL sample, a mixture of acetonitrile:water (50:50, vol/vol) was added. The tube was mixed for 1 min and sonicated for 30 min in an ultrasonic cleaning bath (Power sonic 405). The homogenate was centrifuged (Sigma 3–30 K) at 25,000×*g* for 15 min at room temperature, and the supernatant was filtered through a 0.45-µm syringe filter into a 2 mL vial and injected into the HPLC system. Disposable syringe filters (Chromafil Xtra PVDF-45/25 pore size 0.45 µm, membrane diameter of 25 mm) were purchased from Teknokroma. Total of this procedure was done for blank test. In this test, any peak does not appear in the retention time of melamine.

Sample analysis

The amount of melamine in each dairy product sample was analyzed with HPLC–UV system by the standard addition method. The melamine signal was clearly distinguished at 3 min (Fig. 3). The minor peak around 3.6 min was unknown but had no effect on the determination of melamine in the studied range. The selectivity factor between this peak and melamine peak was 1.47.

Table 1 Amount of melamine (µg/g) in various dairy products

Product	Brand	Sample			Average
		1	2	3	
Yoghurt	A	1.10[a]	0.97	0.77	0.95
	B	0.83	0.81	0.20	0.61
	C	0.25	1.12	0.34	0.57
	D	0.49	0.91	0.62	0.68
	E	1.93	0.40	0.64	0.99
Milk	F	0.23	0.29	0.25	0.26
	G	0.39	0.12	0.15	0.22
	E	0.21	0.41	0.18	0.27
	A	0.33	0.30	0.15	0.26
	H	0.28	0.13	0.17	0.19
Coffee mate	I	0.14	0.05	0.09	0.09
	J	0.18	0.66	0.19	0.34
	K	0.30	0.64	0.37	0.72
	L	1.08	1.52	1.06	1.22
	M	0.35	0.23	0.73	0.44
Infant formula	N	1.60	1.45	1.28	1.44
	O	0.34	ND[b]	1.63	0.98
	P	0.67	0.94	0.51	0.71
	Q	0.39	0.31	ND	0.35
	R	4.52	3.09	2.59	3.40
Cheese	S	0.85	0.97	1.56	1.15
	T	0.21	0.74	0.14	0.36
	U	ND	1.61	1.38	1.50
	V	1.86	ND	3.16	2.51
	F	ND	0.28	0.33	0.30

[a] Average of determinations in three repeated samples

[b] Not detected

Statistical analysis

SPSS statistical package (Window version 18) and Excel 2007 software were used for data analysis. The probability value of $p < 0.05$ was considered as statistically significant in this research.

Results and discussion

Extent of melamine concentration in dairy products

Data in Table 1 show the measured values of melamine in the various dairy products. These results show that mela-

Table 2 Descriptive statistics about the amount of melamine (μg/g) in different dairy products

	Dairy product				
	Yoghurt	Milk	Coffee mate	Infant formula	Cheese
Detected (%)	100	100	100	96	93
Min of the detected concentration	0.20	0.12	0.05	0.31	0.14
Max of the detected concentration	1.12	0.41	1.52	4.52	3.16
Mean of the detected concentration	0.69	0.23	0.51	1.49	1.09
Standard deviation[a]	0.30	0.09	0.43	1.25	0.88
MRL in the USA[b]	0.25	0.25	0.25	0.25	0.25
Percentage of samples containing melamine > US	100	60	80	100	100
MRL in European Union (EU), China, Australia and New Zealand	2.5	2.5	2.5	1.0	2.5
Percentage of samples containing melamine > EU, China, Australia and New Zealand	0	0	0	40	20
MRL in Canada	1	1	1	1	1
Percentage of samples containing melamine > Canada	0	0	20	40	60
Taiwan	All food products are prohibited from containing melamine. Any product with detectable melamine must be removed from the shelves				

[a] Standard deviation for melamine concentration in all samples

[b] Maximum residue level is given from Salman et al. 2012; WHO 2009a, b; Liu et al. 2012; Veritas 2008

mine determinations were 100 % in yoghurt, milk, and coffee mate, 96 % in infant formula and 93 % in cheese samples (Table 2). Amounts of melamine (in μg of melamine per g of sample) in the samples were found in the range 0.20–0.26 for milk, 0.57–0.99 for yoghurt, 0.35–3.40 for infant formula, 0.09–1.22 for coffee mate and 0.30–2.50 for cheese. Descriptive statistics on amounts of melamine in the tested products are shown in Table 2. Averages for melamine content (in ppm) were as follows: infant formula was highly contaminated (1.49), ranked after that was cheese (1.09), then yoghurt (0.69) and coffee mate (0.51). The lowest average level was recorded for milk (0.23).

The Institute of Standards and Industrial Research of Iran (ISIRI) has no regulations for melamine contamination in food products. Therefore, these results were compared with maximum residue levels (MRLs) specified by some other countries. Table 3 shows a simple comprehensive interpretation of the obtained data and a comparison of melamine concentrations in the tested samples to MRL values. European Union (EU), China, Australia and New Zealand have all recommended the same MRL values (Salman et al. 2012; WHO 2009a, b; Liu et al. 2012; Veritas 2008).

According to the results mentioned in Table 2, all of the yoghurt, milk and coffee mate samples contained melamine levels lower than the values specified by related MRLs in EU. In contrast to those results, infant formula and cheese samples showed percentages of 40.0 and 20.0 %, respectively, and amounts of melamine were above the levels recommended in the EU-MRLs.

In comparison with the published MRLs for melamine in the USA, amounts of melamine in 100 % of the yoghurt, cheese, infant formula, 60 % of milk and 80 % coffee mate samples were evaluated as not permissible. It is noteworthy that the Department of health of Taiwan banned all food products containing melamine (Veritas 2008). Thus, if melamine is detected in samples of dairy products, these products should be withdrawn from the market.

Comparison of melamine content among dairy products

Average amounts of melamine in samples show that the various products had different amounts of melamine. The Kruskal–Wallis method was used in the same way as that of ANOVA and allowed testing significant difference among means. The latter is a nonparametric test without the requirements necessary for the ANOVA test (Zamani et al. 2012). The Kruskal–Wallis test showed that there was significant difference in terms of amounts of melamine in the different dairy product samples.

Table 3 Homogeneous subsets of dairy products with regard of melamine content

Dairy products	Subset for $p = 0.05$		
	1	2	3
Milk	0.24[a]		
Coffee mate	0.51	0.51	
Yoghurt	0.69	0.69	
Cheese		1.09	1.09
Infant formula			1.48
Sig.	0.43	0.19	0.57

[a] Means melamine content for groups in homogeneous subsets is displayed

Table 4 Homogeneous subsets of dairy producers with regard of melamine content

Producer	Subset for $p = 0.05$	
	1	2
Coffee mate		
I	0.90[a]	
J	0.35	
K	0.43	
L	0.44	
M		1.22
Cheese		
S	0.30	
T	0.37	
U	1.12	1.12
V	1.49	1.49
F		2.51

[a] Means melamine content for groups in homogeneous subsets is displayed. Producer is base of classification

Post hoc tests are designed for situations in which the researcher has already obtained a significant difference among three or more means, and additional exploration of the differences among means is needed to provide specific information. In this study, Tukey's HSD test was used for these multiple comparisons as mentioned above. This method confirmed that melamine mean values of samples showed three homogenous subset groups. Table 3 shows the dairy product groups that were significantly similar in terms of melamine content. This means that the studied dairy products were classified according to three groups: (1) milk, coffee mate and yoghurt, (2) coffee mate, yoghurt and cheese and (3) cheese and infant formula.

Comparison of melamine content among dairy producers

Production processes can cause food contamination in dairy products; the Kruskal–Wallis test was used to compare mean values of melamine content among dairy products categorized according to producer or brand. This statistic analysis confirmed that in two of the products, coffee mate and cheese, the factor of producer caused significant difference in terms of melamine contamination. According to scientific ethics, publication of the names of specific companies is prohibited. As shown by results in Table 4, the five producers of coffee mate and cheese were classified into two groups according to spatial similarities and dissimilarities based on melamine content. Melamine contamination in coffee mate and cheese products for M and F producers was significant. This means that the production process of these two companies was the main cause of melamine contamination in their products.

Despite evidence that amounts of melamine in yoghurt, milk and infant formula from different producers had no significant difference, the production process in these companies was evaluated as similar in terms of melamine contamination levels in yoghurt, milk and infant formula products.

Melamine was present in all samples analyzed. The amount of melamine in dairy products exhibited concentrations in the following increasing order: milk < yoghurt < coffee mate < cheese < infant formula. We assume that the lower amounts were the result of contamination during the preparation of dairy products and that the higher amounts were the result of deliberate additions. Consumption of food containing these low levels of melamine does not constitute a health risk of consumers. According to the obtained data, only at higher concentrations would the tested products present a risk to human health from melamine contamination. Also, only in two samples of milk and one sample of cheese, there were evaluations much higher than the EU standards. There is no codification for amounts of melamine in different dairy products in Iran. It is necessary that MRL values for melamine in Iranian dairy products be defined and dairy produce be monitored daily. High-performance liquid chromatography presents a suitable method for this purpose.

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