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ORIGINAL ARTICLE

Investigation of melamine and cyanuric acid concentration in several brands of liquid milk and its non-carcinogenic risk assessment in adults and infants

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Abstract In this study, the melamine and cyanuric acid concentration of widely used milk brands and the probability of non-carcinogenic risk of the brands for adults and infants were investigated. These values were 1.37 mg/L, 1.10 mg/L, and 1.09 mg/L, which corresponded to creamy sterilized sample, high-fat (creamy) pasteurized sample, and low-fat (less-creamy) pasteurized sample, respectively. Similarly, the highest amount of cyanuric acid occurred in brand A with the values of 0.79 mg/L, 0.65 mg/L, and 0.64 mg/L, which was reported in the same samples mentioned for melamine. The HQ (Hazard Quotient) of melamine in the brands of A, B, C and D for adults was 0.0025, 0.0011, 0.0006 and 0.0008 respectively. These values for infants were reported as 2.2280, 0.9444, 0.5714 and 0.6714 respectively. The risk probability of melamine for adults was less than 1. However, the HQ in brand A for infants was greater than 1 (2.380), which indicate the high probability of non-carcinogenic risk. Furthermore, the HI (Hazard Index) values of the brands of A, B, C and D for infants were 2.7913, 1.1737, 0.7067 and 0.838, respectively. The simultaneous melamine and cyanuric acid in the brands A and B in for infants increase the non-carcinogenic risk probability by approximately 2.8 and 1.2 times, respectively. The results revealed that the melamine and cyanuric acid concentrations in creamy milk samples (0.5%) were higher than in less-creamy milk samples (2.5%). Moreover, the amount of the compounds in sterilized milk samples was higher than pasteurized. In this study, a conversion factor (0.7) was proposed in order to find out the concentration of cyanuric acid in milk sample with the amount of melamine is known but the cyanuric acid concentration is unknown.

Keywords Melamine · Cyanuric acid · Liquid milk · Risk assessment · Hazard quotient · Hazard index

Introduction

Melamine is a nitrogen compound that is added to milk to increase the apparent protein content (Abedini et al. 2021). According to two pet food scandals in North America in 2007 and a milk scandal in China in 2008, melamine has been highlighted as a major health concern. Melamine can produce ammeline, ammelide, cyanuric acid in milk by hydrolysis in a strong acid or strong base solution (Zhu et al. 2019). This compound has a high absorption rate (more than 90%) through the digestive system and is also excreted through the renal system. In the United States and Taiwan, a significant association between the presence of melamine in urine and complaints related to kidney stones and kidney failure has been reported (Wang and Wu 2018). Vachirapatama et al. carried out a study on simultaneous determination of melamine and cyanuric acid in liquid milk. Their

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results indicated that the concentration of the compounds were 0.10 mg/L and 0.08 mg/L, respectively (Vachirapatama and Maitresorasun 2013). Similarly, Xia et al. reported the concentration of the compounds in liquid milk 0.05 mg/L and 0.02 mg/L (Xia et al. 2010). Concomitant oral exposure to melamine and cyanuric acid causes much more severe kidney damage than exposure to either melamine or cyanuric acid alone. In mice exposed orally to melamine and cyanuric acid 50 mg/kg body weight per day along with the same dose of cyanuric acid for 3 days, an increase in creatinine and urea nitrogen, as well as an increase in kidney weight and the formation of kidney crystals was reported (Naderi and Nasseri 2020). The risk assessment of melamine and its derivatives (cyanuric acid) in milk and dairy products can be a solution to estimate the cumulative risk of these compounds in adults and infants (Abad, Pirkharrati, Mojarrad 2021). The risk assessment of the process is the probability of an adverse health outcome as a result of exposure to risks. According to the US EPA, there are 4 main stages in each risk assessment: (1) Hazard Identification, (2) Dose–Response Assessment, (3) Exposure Assessment, (4) Risk Characterization. In order to obtain the cancer risk, HI and HQ calculate the final step of the non-carcinogenic and carcinogenic health risks of the considered compound (Zio 2018). Many studies have evaluated the health risk of melamine and its derivatives according to the level of external exposure (Zhu and Kannan 2020). For example, Zhu and Kannan identified the concentrations of melamine and its derivatives in indoor dust and foodstuffs to assess the risk of human exposure (Zhu and Kannan 2018). The health risk from exposure to melamine and its derivatives may be underestimated by evaluating only one compound (Zhu and Kannan 2019). So far, no study has been conducted to perform a cumulative risk assessment (CRA) of melamine and its derivatives in humans (Zhou et al. 2015). In general, the CRA of melamine and its derivatives can be calculated by summing the hazard quotient (HQ) of each substance, which has been proposed as a potential health risk assessment method. The exposure of people to melamine and its derivatives simultaneously with these compounds shows more toxicity than exposure to one of those compounds alone (Sathyanarayana et al. 2019; Shi et al. 2020). Consequently, performing CRA to assess the non-carcinogenic risk of melamine will improve the risk management and control of this compound in milk. Melamine and cyanuric acid are known kidney toxins at high concentrations, but little is known about environmental exposure in the general population. Most of the relevant studies were conducted on children, while there are few studies on the consumption of melamine in adults. Melamine and cyanuric acid are currently used in a variety of common consumer products and are found in foods such as milk, which in concentrations higher than the set standard, causes kidney stones and acute kidney damage in children and adults. Milk production in the world increased from 522 million tons in 1987 to 843 million tons in 2018, which shows an increase of more than 61%. The Iran's contribution in milk production in 2018 was about 1% of the total milk production in the world (Abedini et al. 2021). Therefore, this study focused on identifying the concentrations of melamine and cyanuric acid present in the most widely consumed brands of milk, as well as evaluating the cumulative risk of these two compounds.

Materials and methods

Size sample

In the study, two aspects were examined to determine the sample size. One method was to refer to the studies and check the sample size in those studies. The second method was to calculate the sample size based on statistical indicators and using formulas. This calculation method depends on whether the size of the statistical population is known or not. Based on the conditions, a special formula is used to calculate the sample size. Assuming that this number is unknown and 95% confidence limits and 5% error and SP and P values of 0.5, the sample size of this study was 385. But this method is not used alone and it is necessary to consider a reasonable sample size based on the study design and the cost of analysis (the cost of measuring the concentration of melamine and cyanuric acid by HPLC). Finally, the average number of samples based on the published articles in this field was 16, the lowest of which was 6 and the highest was 26. Therefore, in this study, 16 samples were evaluated with three repetitions (48 samples).

The Milk samples collection

Four of the most widely used milk brands were purchased from local supermarkets in Tehran in August 2022. Each brand was considered two high-fat and low-fat samples, and each of these two samples was studied pasteurized and sterilized. Finally, by repeating the measuring of melamine concentration of the samples (3 repetitions), a total of 48 samples was obtained. The milk samples were collected from the whole city of Tehran from August to November 2022, and then transferred to the food laboratory of Tehran University of Medical Sciences. The milk samples were stored in the freezer at – 23 °C. The samples were defrosted right before the preparation and analysis.

Chemicals and reagents

Cleanert PCX-SPE cartridges (3 mL/60 mg) were obtained from Beijing Agela Technologies Company (Beijing, China). Sodium *n*-heptanesulfonate (chromatographic grade) was used as an ion pair reagent. Melamine (HPLC grade with 99% purity) was purchased from from Sigma-Aldrich, St. Bought Louis. Ammonia solution (25% v/v), acetonitrile (ACN, HPLC grade), methanol (HPLC grade), trichloroacetic acid (93% HPLC grade), sodium hexasulfonic acid, and citric acid were purchased from Merck (Darmstadt, Germany). An individual stock standard solution, 1000 µg/mL, was prepared by dissolving the melamine in a mixture of methanol and water (1:4, v/v), and was stable for at least 1 month if stored at 4 °C. All chemicals used in this study were top reagents and were used without further treatment. Additionally, ionized water was obtained from a Milli-Q Plus system at 18.2 MH (MilliPore, Bedford, MA, USA).

Preparation of standard solutions

The stock solution of melamine or CYA (1000 mg/L) was prepared by dissolving 10.0 mg melamine (or CYA) into 10 mL of acetonitrile and water (1:1, V/V) and then stored at – 20 °C. To prepare standard solution (1000.0 µg/mL), 100.0 mg of melamine and cyanuric acid was separately weighed into a 100-mL volumetric flask, dissolved with methanol: water solution (50:50 v/v) and sonicated. Subsequently, the solution was diluted with the mobile phase into a series of standard working solutions (from 0.2 to 1.2 µg/mL) and injected into HPLC and then, calibration curve with $r^2 = 0.99$ was created.

Preparation of samples

Analytical grade HNO_3 purchased from Merck was used in this work. Deionized water was used to prepare the solution and also for dilution purposes. All the glass containers were washed and dried in the oven at 105 degrees Celsius. Sample bottles were cleaned by washing in metal-free soap and then soaking in 10% HNO_3 before sampling. Finally, the bottles were washed with deionized water. Then, 15 mL of 1% trichloroacetic acid and 5 ml of acetonitrile were added to 2 mL of milk in a 50 mL centrifuge tube and the mixture was sonicated for 30 min. The sample was shaken at 70 rpm for 30 min. Then it was centrifuged at 15,000 rpm for 15 min using filter paper. The filtered solution was triturated with 1% to 25 mL trichloroacetic acid and centrifuged for 5 min. The supernatant was diluted with 5 mL of distilled water. After that, the sample purification method was performed using solid phase extraction (SPE). The cleaned sample was dried under a stream of dry nitrogen gas and dissolved in 1 mL of mobile phase. The final solution was filtered through a 0.45 micron syringe filter (Furusawa 2023).

Sample extraction

The extraction method was based on the US FDA method (GC–MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric Acid) (FDA 2008). In the procedure, 20 mL of a solution of acetonitrile, water and diethylamine (5:4:1, V/V/V) was added to each 50 mL polypropylene (PP) centrifuge tube containing 0.5 mL of milk sample. In each tube, 50 µL of DACP (internal standard, 5.0 mg/L) was added. Then, the sample was shaken manually (30 s) and extracted for 7 min with 10 mL of methanol–5.0% formic acid mixture (50:50, v/v) by sonication (for 10 min and frequency of > 20 kHz). The sample was then centrifuged at 4000 rpm for 5 min at 5 °C. A 5 mL aliquot of supernatant was removed to a 15 mL polypropylene centrifuge tube. The remaining portion in the 50 mL tube was discarded. Dichloromethane (10 mL) was added to the contents of the 15 mL tube, and the sample was shaken for two minutes. The sample was centrifuged at 4000 rpm for 5 min at 5 °C. A portion (2.5 mL) of the upper aqueous layer was carefully removed to a glass culture tube. Water (2.5 mL) was added to the dichloromethane layer and that sample was re-extracted by shaking for 1 min. The polypropylene tube was again centrifuged at 4000 rpm for 5 min at 5 °C, and the entire upper aqueous layer was removed and combined with the first aqueous extract in the glass culture tube. This extract was vortex mixed for 5 s. An Oasis MCX SPE cartridge was used to clean-up sample extracts. All SPE elution steps including conditioning, sample application, washing, and the final elution were performed without the application of vacuum. Vacuum was only applied to dry the cartridges. The SPE cartridge was conditioned with 5 mL of methanol followed by 5 mL of water. The sample was applied to the conditioned cartridge and allowed to elute by gravity. The column was washed with 5 mL of 0.1 N HCl, followed by 2 mL of methanol. The cartridge was dried by applying vacuum for 1 min. The column was eluted into a glass culture tube using 5 mL of 5% ammonium hydroxide in methanol. The eluate was evaporated to dryness at 55 °C under flowing nitrogen at 15 psi for 20 min. The dried extract was reconstituted in 1.0 mL of 95:5 acetonitrile:ammonium formate (20 mM), vortex mixed for 10 s, and filtered through a 0.2 µm nylon syringe filter into a glass HPLC syringe (EPA 2007; Furusawa 2023).

HPLC parameters

High pressure liquid chromatography (HPLC) KNAUER 0000 (Germany) equipped with C18 column 4.6 × 250 mm (ODS-3) and UV detector at 242 nm was used. The mobile phase was applied at a specified ratio (450 mL of buffer solution and 50 mL of methanol suitable for liquid chromatography) at a flow rate of 1 mL/min.

Data analysis

For find out the normality of the data distribution, the *Kolmogorov-Smironov test* was used, which was chosen because of the small number of samples (48 samples). The data distribution of the milk samples followed a normal distribution. Then, *Levene's test* that is an inferential statistic was used to assess the equality of variances for a variable calculated for two or more groups. In order to analyze the data and find out the difference in the average concentrations of melamine and cyanuric acid of the samples in different brands, the *multi-way ANOVA test* was used and for multiple comparisons the *Tukey's HSD (honestly significant difference) test* was considered. Data analysis was performed using *SPSS software version 21* and two-sided P values < 0.05 were considered statistically significant.

Human risk assessment steps

Human health risk assessment involves determining the nature and extent of adverse health effects in humans who may be exposed to toxic substances in a contaminated environment. In the present study, exposure and risk assessment was performed according to the USEPA methodology.

The standard limit and TDI of Melamine and cyanuric acid

In order to assess the risk of melamine and cyanuric acid, the standard limits of these compounds in milk were extracted from the world's most reliable organizations. For this, the standard limit in milk and also the tolerable daily intake (TDI) or reference dose (RfD) of the compounds was considered. For melamine, the standards of the Food and Drug Administration (FDA) were considered, which the standard limit for adults, the standard limit for infants, the TDI for adults and the TDI for infants are 2.5 mg/L (mg melamine per liter of milk), 1 mg/L, 0.63 mg/kg/day (mg melamine per kg of body weight per day) and 0.063 mg/kg/day, respectively. Similarly, for cyanuric acid these values based on Word Health Organization (WHO) are 40 mg/L (mg cyanuric acid per liter of milk), 16 mg/L, 1.5 mg/kg/day and 0.15 mg/kg/day (mg cyanuric acid per kg of body weight per day), respectively. It should be noted that no standard has been established for cyanuric acid in milk so far, and as a result, in order to determine a confidence limit, the standard limit of this compound in drinking water, which is 40 mg/l, was used in this study (Hua et al. 2015).

Average daily potential dose (ADD)

In order to assess the risk and calculate the risk probability of melamine and cyanuric acid present in the milk samples, the ADD of the ingestion route for adults and infants was calculated for each of the brands based on the following formula (EPA 2011);

$$\text{ADD} = \frac{\text{Cmedium} \cdot \text{IngR} \cdot \text{EF} \cdot \text{ED}}{\text{BW} \cdot \text{AT}} \quad (1)$$

where:

ADD = Average daily potential dose (mg/kg day)

Cmedium = Concentration of contaminant in medium (mg/L)

IngR = Ingestion rate (L/day)

F = Exposure frequency (days/year)

ED = Exposure duration (years)

BW = Body weight (kg)

AT = Averaging time (days)

The input assumptions and their values for computing the chronic daily intake via oral ingestion have been summarized in Table 1.

Hazard Quotient (HQ)

The potential health risk of melamine and cyanuric acid was assessed as a hazard quotient (HQ), which was obtained as the ratio of melamine or cyanuric acid exposure to the tolerable daily intake (TDI) or reference dose (RfD) value. The HQ for each sample was calculated using the ratio of computed ADD of melamine or cyanuric acid ingested with contaminated milk to the reference oral dose (RfD) via oral ingestion for adults and infants (Bamuwamye et al. 2015; WHO 2009).

Table 1 Input parameters and abbreviations for exposure assessment of melamine or cyanuric acid

Input parameters	Units	Values	References
Concentration of Contaminant in medium (C_{medium})	mg/L	See Table 1	This study
Ingestion rate (IngR)	L/day	For adults 0.13 L/day and for infants 0.47 L/day	USEPA
Exposure frequency (EF)	Days/year	365	USEPA
Exposure duration (ED)	Years	For adults 70 years and for infants 1 years	USEPA
Body weight (BW)	kg	For adults 70 kg and for infants 3 kg	USEPA
Averaging lifetime (AT)	Days	70 × 365	USEPA

$$HQ = \frac{ADD}{RfD} \quad (2)$$

where ADD and RfD (or TDI) are expressed in mg/kg-day. In this study, the RfD of melamine and cyanuric acid was considered according to the standards of the FDA (EPA 2011).

Hazard Index (HI)

To estimate the total potential non-carcinogenic health impacts caused by exposure to a mixture of the melamine and cyanuric acid of milk, the HI or Cumulative Risk (CR) of these compounds was computed according to the EPA guidelines for health risk assessment. The sum of all HQs gives an estimation of total potential health risks or HI. The computed HI is compared to standard values: there is the possibility that non-carcinogenic impacts may occur in the residents when HI > 1, while the exposed person is unexpected to experience evident harmful health impacts when HI < 1. The calculation of the HI caused by the melamine and cyanuric acid of milk in each brand was presented in following equation (EPA 2011, WHO 2009):

$$HI = \sum_{k=1}^{n} HQ = HQmel + HQcyan \quad (3)$$

Results and discussion

The concentration of melamine and cyanuric acid in the most widely used milk brands were studied and the individual and simultaneous non-carcinogenic risk assessment of these two compounds was investigated (Fig. 1).

The investigation of the milk samples adulteration

Currently, according to the report of the Iranian Statistics Center, milk consumption for Iranian adults and infants (0–1 years and 3 kg in weight) per capita in 2022 is about 50 L and 175 L, respectively. This amount per day has been estimated 0.13 and 0.47 L. In this study, the variables of brand type, disinfection type, fat content on the concentration of melamine and cyanuric acid of milk samples were investigated.

The concentration of melamine in milk samples

The results of this study showed that the highest amount of melamine was related to the brand A. These values were 1.37 mg/L, 1.10 mg/L, and 1.09 mg/L, which corresponded to high-fat sterilized sample, high-fat pasteurized sample, and low-fat pasteurized sample, respectively. Although the concentration of melamine in all samples compared to WHO standard (2.5 mg/L) and FDA standard (2.5 mg/L) were found acceptable for adults, the amounts was not permissible for infants base on FDA standard (1 mg/L). In addition, the results of the present study showed that the highest

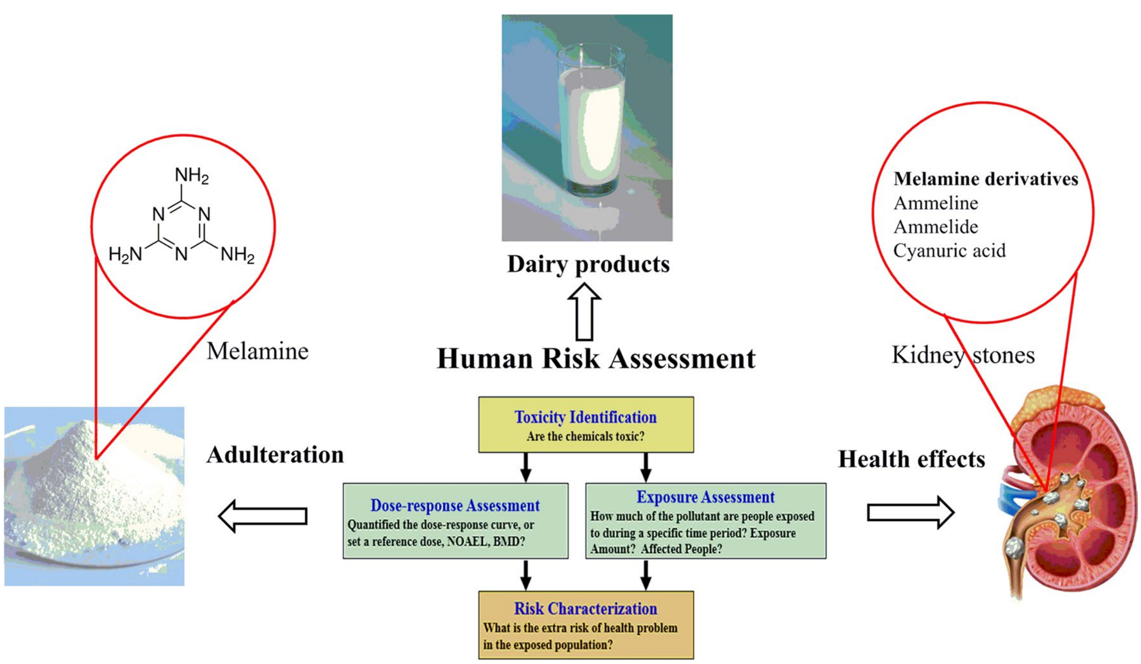

Fig. 1 Schematic illustration of the relationship between milk, melamine and kidney disorders and non-carcinogenic risk analysis

concentration of melamine was reported in brand A and the lowest concentration was reported in brand D (Fig. 2). Many organizations have established acceptable concentrations of melamine in formula and liquid milk for adults and infants. The FDA has specified a maximum level of 2.5 ppm for melamine and its analogs in foods other than infant formula (WHO 2009). The European Food Safety Authority has set a standard of 2.5 mg per kilogram (ppm) for melamine for all foods containing milk. The results of this study showed that the concentration in high-fat milk samples was higher than in low-fat milk samples (Fig. 2). This could be because fatty acids can make melamine more stable in milk. Melamine is a heterocyclic molecule and consists of a triazine ring that has three amine groups at positions 2, 4, and 6. Melamine works well for intermolecular hydrogen bonds: the amine groups can act as proton donors while the nitrogen atoms in the triazine ring can be proton acceptors through their lone pair electrons. Besides, its planar geometry and aromatic system can increase surface adsorption. Melamine shows high affinity with other organic compounds such as fatty acids (Walch et al. 2009). Walch et al. studied the formation of bimolecular isotopological monolayers composed of melamine and members of the homologous series of fatty acids extending from pentanoic to tridecanoic acid at the liquid interface. The results of their study revealed that the basic unit consists of a melamine hexamer stabilized by internal N–H-N hydrogen bonds and radially surrounded by 12 fatty acid molecules (Walch et al. 2009). Furthermore, the results related to the disinfection process showed that the amount of melamine in sterilized milk samples was higher than pasteurized. In the pasteurization process, milk is heated at 62 °C for at least 30 min or at 72 °C for at least 15 s, while in the process of sterilization, the milk is heated at a temperature of 138–145 °C for a period of 2 s. Melamine is commonly considered as a fire retardant additive in the manufacture of polymeric materials with a stable heterocyclic structure (triazine) (Yu et al. 2008). Therefore, the reason for the lower concentration of melamine in pasteurized milk compared to sterilized milk cannot be the higher temperature of the sterilization process because this compound is resistant to high temperatures. The higher amount of melamine in sterilized milk can be due to the fact that the temperature in the sterilization process is higher than in the pasteurization process, and as a result, more protein is destroyed. Thus, milk producing companies add more melamine to sterilized milk in order to compensate for the protein content of milk.

The concentration of cyanuric acid in milk samples

The results of this study showed that the highest amount of cyanuric acid was related to brand A. These values were 0.79 mg/L, 0.65 mg/L, and 0.64 mg/L, which corresponded to high-fat sterilized sample, high-fat pasteurized sample, and low-fat pasteurized sample, respectively (Table 2). Although the concentration of melamine in all samples compared to WHO standard were acceptable

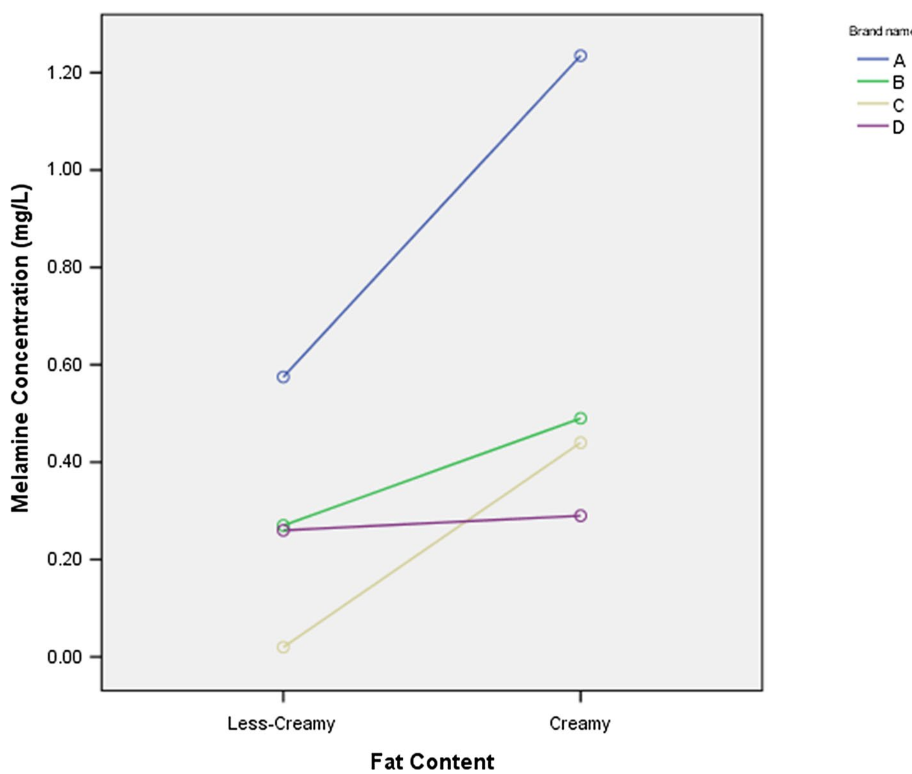

Fig. 2 Comparison of the mean concentration of melamine in different brands of milk versus the fat content

Table 2 The concentration of melamine and cyanuric acid present in a sample of milk of different brands in the present study

Brand	Disinfection process	Fat content	Melamine Co. (mg/l) (Mean ± SD)	Cyanuric acid Co. (mg/L) (Mean ± SD)	Melamine/Cyanuric acid Ratio
A	Pasteurized	Low	1.09 ± 0.09	0.64 ± 0.08	1.44
		High	1.10 ± 0.09	0.65 ± 0.08	1.45
	Sterilized	Low	0.06 ± 0.02	0.04 ± 0.01	1.16
		High	1.37 ± 0.27	0.79 ± 0.15	1.31
B	Pasteurized	Low	0.01 ± 0.01	0.01 ± 0.01	0.79
		High	0.27 ± 0.09	0.16 ± 0.05	1.49
	Sterilized	Low	0.53 ± 0.23	0.31 ± 0.14	1.26
		High	0.71 ± 0.40	0.43 ± 0.25	0.98
C	Pasteurized	Low	0.04 ± 0.05	0.03 ± 0.03	0.59
		High	0.02 ± 0.01	0.01 ± 0.01	0.98
	Sterilized	Low	0.00 ± 0.00	0.00 ± 0.00	0.55
		High	0.86 ± 0.15	0.50 ± 0.12	1.26
D	Pasteurized	Low	0.51 ± 0.08	0.30 ± 0.06	1.29
		High	0.44 ± 0.05	0.26 ± 0.04	1.33
	Sterilized	Low	0.01 ± 0.01	0.01 ± 0.01	0.82
		High	0.14 ± 0.09	0.08 ± 0.05	0.94
Total mean			*0.45	*0.26	1.10

*0.45/0.26 = 1.73

for adults and infants, which the standard limits were 40 mg/L and 16 mg/L, respectively. Furthermore, the results of the present study showed that the highest concentration of cyanuric acid was related to brand A and the lowest concentration was reported in brand D (Fig. 3). The results of this study showed that the cyanuric acid concentration in high-fat milk samples was higher than in low-fat milk samples (Fig. 3). Because cyanuric acid is a derivative

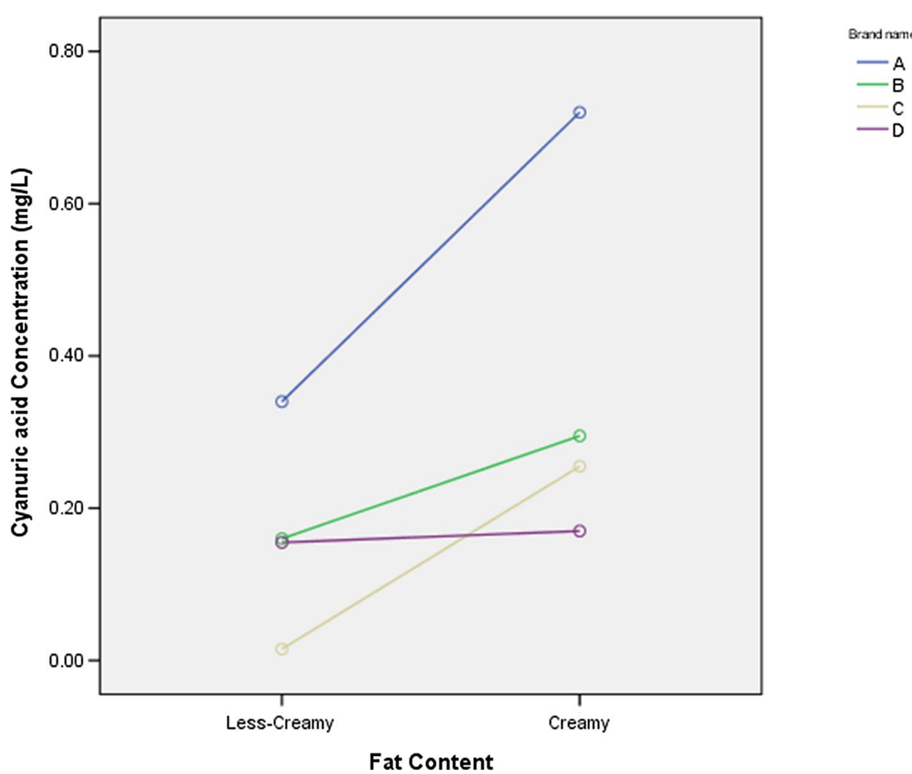

Fig. 3 Comparison of the mean concentration of cyanuric acid in different brands of milk versus the fat content

of melamine, the increase of melamine in milk increases the concentration of cyanuric acid. Like melamine, cyanuric acid forms a complex with fatty acids and this action makes this compound stable (Walch et al. 2009). Moreover, the results related to the disinfection process showed that the amount of cyanuric acid in sterilized milk samples was higher than pasteurized. In the sterilization process, milk is subjected to more heat compared to the pasteurization process, and as a result, the proteins undergo more changes and are denatured in the sterilization process. Therefore, some industries producing sterilized milk add melamine to milk in order to compensate for protein, and since cyanuric acid is a derivative of melamine, this compound is also found in sterilized milk (Deeth and Lewis 2016).

Non-carcinogenic risk assessment of melamine and cyanuric acid

The ADD of melamine of the milk brands via ingestion

The mean of the melamine concentrations in terms of the brands of A, B, C and D of milk were reported 0.90, 0.38, 0.23 and 0.27 mg/L, respectively (Table 3). The ADD values of melamine in the brands A, B, C and D of milk for adults were 0.001, 0.0007, 0.0004 and 0.0005 mg/kg day, respectively. Moreover, these values were reported as 0.410, 0.0565, 0.0360 and 0.0423 mg/kg day for infants (Table 3). The ADD values for adults and infants were computed and are given in the following calculations:

For adults:

$$\mathrm{ADDmel,\,brandA,\,adult} = \frac{0.90\frac{\mathrm{mg}}{\mathrm{L}}.0.13\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.70\mathrm{years}}{70\mathrm{kg}.(70*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandA,\,adult} = 0.0016\ \mathrm{mg/kg\ day}.$$

For infants:

$$\mathrm{ADDmel,\,brandA,\,infant} = \frac{0.90\frac{\mathrm{mg}}{\mathrm{L}}.0.47\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.1\mathrm{years}}{3\mathrm{kg}.(1*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandA,\,infant} = 0.1410\ \mathrm{mg/kg\ day}.$$

For adults:

$$\mathrm{ADDmel,\,brandB,\,adult} = \frac{0.38\frac{\mathrm{mg}}{\mathrm{L}}.0.13\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.70\mathrm{years}}{70\mathrm{kg}.(70*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandB,\,adult} = 0.0007\ \mathrm{mg/kg\ day}.$$

For infants:

$$\mathrm{ADDmel,\,brandB,\,infant} = \frac{0.38\frac{\mathrm{mg}}{\mathrm{L}}.0.47\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.1\mathrm{years}}{3\mathrm{kg}.(1*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandB,\,infant} = 0.0595\ \mathrm{mg/kg\ day}.$$

For adults:

$$\mathrm{ADDmel,\,brandC,\,adult} = \frac{0.23\frac{\mathrm{mg}}{\mathrm{L}}.0.13\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.70\mathrm{years}}{70\mathrm{kg}.(70*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandC,\,adult} = 0.0004\ \mathrm{mg/kg\ day}.$$

For infants:

$$\mathrm{ADDmel,\,brandC,\,infant} = \frac{0.23\frac{\mathrm{mg}}{\mathrm{L}}.0.47\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.1\mathrm{years}}{3\mathrm{kg}.(1*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandC,\,infant} = 0.0360\ \mathrm{mg/kg\ day}.$$

For adults:

$$\mathrm{ADDmel,\,brandD,\,adult} = \frac{0.27\frac{\mathrm{mg}}{\mathrm{L}}.0.13\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.70\mathrm{years}}{70\mathrm{kg}.(70*365\mathrm{days})}$$

$$\mathrm{ADDmel,\,brandD,\,adult} = 0.0005\ \mathrm{mg/kg\ day}.$$

For infants:

$$\mathrm{ADDmel,\,brandD,\,infant} = \frac{0.27\frac{\mathrm{mg}}{\mathrm{L}}.0.47\frac{\mathrm{L}}{\mathrm{day}}.365\mathrm{years}.1\mathrm{years}}{3\mathrm{kg}.(1*365\mathrm{days})}$$

Table 3 The calculated parameters of non-carcinogenic risk assessment of the melamine and cyanuric acid of different brands of liquid milk in the present study

Contaminant	Brand	Mean Concentration (mg/L)	Average daily potential dose (ADD) (mg/kg-day)		Hazard Quotient (HQ)	
			Adults	Infants	Adults	Infants
Melamine	A	0.90	0.0016	0.1410	0.0025	2.2380
	B	0.38	0.0007	0.0595	0.0011	0.9444
	C	0.23	0.0004	0.0360	0.0006	0.5714
	D	0.27	0.0005	0.0423	0.0008	0.6714
Cyanuric acid	A	0.53	0.0009	0.0830	0.0004	0.5533
	B	0.22	0.0004	0.0344	0.0003	0.2293
	C	0.13	0.0002	0.0203	0.0001	0.1353
	D	0.16	0.0003	0.0250	0.0002	0.1666

$$ADDmel, brandD, infant = 0.0423 \text{ mg/kg day.}$$

The health Canada scientists established a toxicological reference dose (TRD) for melamine of 0.35 mg/kg body weight per day to ensure that all age groups are protected (WHO 2009). This means that a person can consume up to 0.35 mg of melamine per kilogram of body weight per day with reasonable assurance of no adverse health effects.

The ADD of cyanuric acid via ingestion

Similarly, the mean of the cyanuric acid concentrations of the milk brands of A, B, C and D were 0.53, 0.22, 0.13 and 0.16 mg/L, respectively (Table 3). The ADD values of cyanuric acid in the brands of A, B, C and D of milk for adults were 0.0009, 0.0004, 0.0002 and 0.0003 mg/kg-day, respectively. Moreover, these values were reported as 0.0830, 0.0344, 0.0203 and 0.0250 mg/kg-day for infants (Table 3). The ADD values for adults and infants were calculated and are given below:

For adults:

$$ADDcyan, brandA, adult = \frac{0.53\frac{mg}{L}.0.13\frac{L}{day}.365years.70years}{70kg.(70 * 365days)}$$

$$ADDmel, brandA, adult = 0.0009 \text{ mg/kg day.}$$

For infants:

$$ADDcyan, brandA, infant = \frac{0.53\frac{mg}{L}.0.47\frac{L}{day}.365years.1years}{3kg.(1 * 365days)}$$

$$ADDmel, brandA, infant = 0.0830 \text{ mg/kg day.}$$

For adults:

$$ADDcyan, brandB, adult = \frac{0.22\frac{mg}{L}.0.13\frac{L}{day}.365years.70years}{70kg.(70 * 365days)}$$

$$ADDmel, brandB, adult = 0.0004 \text{ mg/kg day.}$$

For infants:

$$ADDcyan, brandB, infant = \frac{0.22\frac{mg}{L}.0.47\frac{L}{day}.365years.1years}{3kg.(1 * 365days)}$$

$$ADDmel, brandB, infant = 0.0344 \text{ mg/kg day.}$$

For adults:

$$ADDcyan, brandC, adult = \frac{0.13\frac{mg}{L}.0.13\frac{L}{day}.365years.70years}{70kg.(70 * 365days)}$$

$$ADDmel, brandC, adult = 0.0002 \text{ mg/kg day.}$$

For infants:

$$ADDcyan, brandC, infant = \frac{0.13\frac{mg}{L}.0.47\frac{L}{day}.365years.1years}{3kg.(1 * 365days)}$$

$$ADDmel, brandC, infant = 0.0203 \text{ mg/kg day.}$$

For adults:

$$ADDcyan, brandD, adult = \frac{0.16\frac{mg}{L}.0.13\frac{L}{day}.365years.70years}{70kg.(70 * 365days)}$$

$$ADDmel, brandD, adult = 0.0003 \text{ mg/kg day.}$$

For infants:

$$ADDcyan, brandD, infant = \frac{0.16\frac{mg}{L}.0.47\frac{L}{day}.365years.1years}{3kg.(1 * 365days)}$$

$$ADDmel, brandD, infant = 0.0250 \text{ mg/kg day.}$$

While the common risk characterization is specific to the toxic effects caused by melamine alone, this position will be given renewed attention when the combined results of melamine and cyanuric acid are presented. This approach is according to the WHO recommendation to use toxicological reference values derived based on melamine toxicity. Because there is no study that has evaluated the combined toxicity of melamine and cyanuric acid together. It provides the possibility of extracting the acceptable consumption amount for the presence of both compounds (WHO 2009).

Hazard Quotient (HQ) of melamine and cyanuric acid of milk brands

Melamine The HQ of melamine in the brands of A, B, C and D for adults was 0.0025, 0.0011, 0.0006 and 0.0008 respectively. These values for infants were reported as 2.2280, 0.9444, 0.5714 and 0.6714 respectively. The results showed that the risk probability of melamine for adults was less than 1, so the possibility of non-carcinogenic risk for the adult group is very low. However, the HQ in brand A for infants was greater than 1 (2.380), which indicate the high probability of non-carcinogenic risk. The HQ values for adults and infants were obtained and have been given below:

Brand A:

$$HQmel, brandA, adult = \frac{0.0016}{0.63} = 0.0025$$

$$HQmel, brandA, infant = \frac{0.1410}{0.063} = 2.2380$$

Brand B:

$$HQmel, brandB, adult = \frac{0.0007}{0.63} = 0.0011$$

$$HQmel, brandB, infant = \frac{0.0595}{0.063} = 0.9444$$

Brand C:

$$\mathrm{HQmel, brandC, adult} = \frac{0.0004}{0.63} = 0.0006$$

$$\mathrm{Qmel, brandC, infant} = \frac{0.0360}{0.063} = 0.5714$$

Brand D:

$$\mathrm{HQmel, brandD, adult} = \frac{0.0005}{0.63} = 0.0008$$

$$\mathrm{HQmel, brandD, infant} = \frac{0.0423}{0.063} = 0.6714$$

Tittlemier et al. carried out a study on the occurrence and risk assessment of melamine in powdered milk consumed in Canada (Tittlemier et al. 2009). The results of their study showed that the average possible daily intake of melamine (PDIs) for these infants ranged from 0.57 to 2.4 µg/kg body weight per day. In general, the relatively low concentrations observed in their study showed that the presence of melamine in infant formula did not pose a health risk (Tittlemier et al. 2009). However, in the present study, which was conducted on liquid milk, melamine in one brand showed a high probability of non-carcinogenic risk for infants.

Cyanuric acid The HQ of cyanuric acid of the brands of A, B, C and D for adults was 0.0004, 0.0003, 0.0001 and 0.0002, respectively. The values for infants were reported as 0.5533, 0.2293, 0.1353 and 0.1666, respectively. The results obtained from this study showed that the HQ of cyanuric acid of all analyzed milk samples was less than 1, which indicated the low probability of non-carcinogenicity of the samples. The HQ values of the adults and infants were calculated and have been given below:

Brand A:

$$\mathrm{HQcyan, brandA, adult} = \frac{0.0009}{1.5} = 0.0004$$

$$\mathrm{HQcyan, brandA, infant} = \frac{0.0830}{0.15} = 0.5533$$

Brand B:

$$\mathrm{HQcyan, brandB, adult} = \frac{0.0004}{1.5} = 0.0003$$

$$\mathrm{HQcyan, brandB, infant} = \frac{0.0344}{0.15} = 0.2293$$

Brand C:

$$\mathrm{HQcyan, brandC, adult} = \frac{0.0002}{1.5} = 0.0001$$

$$\mathrm{HQcyan, brandC, infant} = \frac{0.0203}{0.15} = 0.1353$$

Brand D:

$$\mathrm{HQcyan, brandD, adult} = \frac{0.0003}{1.5} = 0.0002$$

$$\mathrm{HQcyan, brandD, infant} = \frac{0.0250}{0.15} = 0.1666$$

The HQ of cyanuric acid in dairy and milk products has not been calculated and reported in any study so far.

Hazard Index (HI) of the melamine and cyanuric acid of milk brands

For evaluate the cumulative non-carcinogenic risk of melamine and cyanuric acid, the Hazard Index (HI) of the different brands was calculated. The HI values the brands of A, B, C and D for adults was 0.003, 0.0014, 0.0007 and 0.001, respectively. The results showed that the cumulative risk of melamine and cyanuric acid of the brands is very low for adults. Additionally, the HI values of the brands of A, B, C and D for infants was 2.7913, 1.1737, 0.7067 and 0.838, respectively. The calculations related to the cumulative HI of these two compounds for adults and infants based on the different brands of milk are given below:

For the brand A:

Adults:

$$\mathrm{HI} = 0.0025 + 0.0004 = 0.003$$

Infants:

$$\mathrm{HI} = 2.2380 + 0.5533 = 2.7913$$

For the brand B:

Adults:

$$\mathrm{HI} = 0.0011 + 0.0003 = 0.0014$$

Infants:

$$\mathrm{HI} = 0.9444 + 0.2293 = 1.1737$$

For the brand C:

Adults:

$$\mathrm{HI} = 0.0006 + 0.0001 = 0.0007$$

Infants:

$$\mathrm{HI} = 0.5714 + 0.1353 = 0.7067$$

For brand D:
Adults:

$$HI = 0.0008 + 0.0002 = 0.001$$

Infants:

$$HI = 0.6714 + 0.1666 = 0.838$$

Various studies have been conducted to identify and determine the concentration of melamine and cyanuric acid, as well as evaluate the non-carcinogenic risk of melamine. However, no study has yet been presented to assess the cumulative risk of melamine and its derivatives such as cyanuric acid in humans (Guo et al. 2020). In the present study, the CRA of melamine its derivatives were calculated by summing the hazard quotient (HQ) of each compound, which was considered as a potential health risk assessment tool (Shi et al. 2020). People are simultaneously exposed to melamine and its derivatives, and simultaneous exposure to melamine and its derivatives shows more toxicity than individual exposure (Sathyanarayana et al. 2019). Thus, it is important to conduct CRA exposure to melamine and its derivatives. The results revealed that the HI of brands A (2.7913) and B (1.1737) for infants up to one year and with a weight of 3 kg was higher than 1. The simultaneous melamine and cyanuric acid in the brands A and B in for infants increase the non-carcinogenic risk probability by approximately 2.8 and 1.2 times, respectively. Dorne et al. conducted a study on recent advances in the risk assessment of melamine and cyanuric acid in animal feed (Dorne et al. 2013). Experimental evidence and pathological signs of animals fed fake food showed that simultaneous exposure to melamine and cyanuric acid has more kidney damage than exposure to each of these compounds alone. Very limited reports on the toxicity of melamine simultaneously with amelide and amelin also show higher toxicity (Dorne et al. 2013). For exposure with melamine and its analogues such as cyanuric acid, a TDI cannot be derived and a TDI for melamine alone is inappropriate because of the greater toxicity resulting from such co-exposures and the lack of complete exposure dose–response findings (Dorne et al. 2013). Moreover, melamine and its derivatives, ammeline, ammelide, cyanuric acid are widely present in environmental. CRA in animals have reported co-exposure to melamine and its derivatives and investigated the relationship between exposure and routine blood parameters. Such information is largely unknown in human studies and has not yet been rigorously evaluated (Liu et al. 2022). In a study conducted by Liu et al., the urinary concentration of melamine and its derivatives was detected in 239 Chinese adults who underwent CRA by evaluating HQ and HI. Their results indicated that the concentration of melamine and its derivatives in urine was positively associated with normal blood parameters (Liu et al. 2022).

The conversion factor of melamine to cyanuric acid

Unfortunately, no standard has been established for cyanuric acid in dairy products and milk. As a result, in this study, in order to compare the cyanuric acid concentration of milk samples with an acceptable value, the standard established for drinking water was used. The standard limit of cyanuric acid in drinking water is 40, and because the ratio of melamine standard for adults to melamine standard for babies is 2.5, the standard of cyanuric acid in this study was considered 16 for infants. The Figures of the melamine and cyanuric acid show that the increase in concentration of these two compounds in milk samples is almost similar (Figs. 2 and 3). But, this occurred in different concentration ranges; because the concentration of melamine is always higher than the concentration of cyanuric acid. In the current study, the ratio of the average melamine concentrations of all milk samples to the average cyanuric acid concentrations of all samples was calculated and this value was 1.73 (Table 2).

$$0.45/0.26 = 1.73$$

Therefore, a conversion factor (0.7) was proposed in order to find out the concentration of cyanuric acid in milk sample with the amount of melamine is known but the cyanuric acid concentration is unknown.

$$C_{\text{cyanuric acid}} = C_{\text{melamine}} \times 0.7 \tag{4}$$

This equation can be used to estimate the concentration of cyanuric acid in milk and other dairy products.

Conclusion

The presence of melamine along with one of its derivatives called cyanuric acid in milk increases the toxicity and hazard risk. The results of the present study indicated that the concentration of melamine and cyanuric acid was higher in the sterilized and high-fat milk samples. Although the concentration of the compounds in all milk samples was lower than the standard set by WHO and FDA. Additionally, the non-carcinogenic risk assessment of melamine and cyanuric acid found in milk samples revealed that HQ of melamine in brand A for infants was greater than 1 (2.24), but this value for cyanuric acid was reported to be less than 1 in all samples. The HI related to the simultaneous melamine and cyanuric acid in the brands A and B for infants was higher than 1 and as a result the non-carcinogenic risk probability was approximately 2.8 and 1.2 times, respectively. Moreover, in this study, a conversion factor (0.7) was proposed and presented in order to convert the concentration of melamine to the concentration of cyanuric acid amount in liquid milk. According to the results obtained from this study, it is suggested that infants (0–1 years, 3 kg in weight) use less pasteurized and sterilized milk and it is better to use mother's milk. Moreover, it is recommended that adults consume more pasteurized and low-fat milk brands.

Author contributions RA conducted methodology, GJK carried out the conceptualization, MA conducted the supervision and EMA and PS performed the reviewing–revising and editing.

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Data availability All data generated or analyzed during this study are included in the manuscript and will be sent to the journal with a reasonable request.

Code availability For experimental design, statistical analysis of data, optimization for removal efficiency of the total and fecal coliforms and imaging of response surfaces, SPSS software version 21 was used. Moreover, the data were analyzed by ANOVA in order to obtain the interactions between the process variables.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval Not applicable.

Consent to participate The authors declare that they have no competing interests**.**

Consent to publication The authors declare that they have no competing interests.

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