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Determination of melamine in milk and dairy products by high performance liquid chromatography

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ABSTRACT

A simple, precise, accurate, and validated reverse-phase HPLC method was developed for the determination of melamine in milk (pasteurized and UHT milk) and dairy products (powdered infant formula, fruit yogurt, soft cheese, and milk powder). Following extraction with acetonitrile:water (50:50, vol/vol), samples were purified by filter (0.45 μm), separated on a Nucleosil C8 column (4.6 mm × 250 mm, 3 μm) with acetonitrile:10 mmol/L sodium L-octane sulfonate (pH 3.1; 15:85, vol/vol) as mobile phase at a flow rate of 1 mL/min, and determined by a photodiode array detector. A linear calibration curve was obtained in the concentration range from 0.05 to 5 mg/kg. Milk and dairy products were fortified with melamine at 4 levels producing average recovery yields of 95 to 109%. The limits of detection and quantification of melamine were 35 to 110 and 105 to 340 μg/kg, respectively. The method was then used to analyze 300 samples of milk and dairy products purchased from major retailers in Turkey. Melamine was not found in infant formulas and pasteurized UHT milk, whereas 2% of cheese, 8% of milk powder, and 44% of yogurt samples contained melamine at the 121, 694 ± 146, and 294 ± 98 μg/kg levels, respectively. These findings were below the limits set by the Codex Alimentarius Commission and European Union legislation. This is the first study to confirm the existence of melamine in milk and dairy products in Turkey. Consumption of foods containing these low levels of melamine does not constitute a health risk for consumers.

Key words: melamine, dairy product, HPLC, milk

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine, $C_3H_6N_6$) is an organic base chemical most commonly found in the form of white crystals rich in nitrogen. It is produced in large amounts primarily for use in the synthesis of melamine formaldehyde resins for the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives, and dishware and kitchenware (WHO, 2009).

Aside from common commercial uses, melamine became a topic of discussion in 2007 when veterinary scientists determined that pet food contamination of melamine was the cause of hundreds of pet deaths (Tyan et al., 2009). Melamine was implicated in a major pet food recall throughout the United States and Canada. Certain imported cereal-based pet food ingredients (e.g., wheat flour, wheat gluten, corn flour, and rice protein concentrate were deliberately adulterated with melamine to boost their total nitrogen content (melamine contains 66.6% N by weight; Ehling et al., 2007). Ingestion of melamine may lead to kidney stones, renal failure, and other health problems (Chen and Yan, 2009). In 2008, high concentrations of melamine were reported in contaminated Chinese infant formula. The World Health Organization (**WHO**) reviewed the 2008 melamine contamination event of China. More than 51,900 infants and young children in China were hospitalized for urinary problems, possible renal tube blockages, and possible kidney stones related to the consumption of melamine-contaminated infant formula and related dairy products. Six deaths among infants were confirmed in China (Xu et al., 2009).

Soon after, melamine was found in liquid milk and yogurts, frozen desserts, powdered milk, cereal products, confectionaries, cakes and biscuits, protein powders, and some processed foodstuffs. Subsequently, various nondairy products originating from China were found to be contaminated with melamine. These products included ammonium bicarbonate, animal feed and animal

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Table 1. Analytical conditions for HPLC

Instrument	Shimadzu LC-20A system (Shimadzu, Tokyo, Japan)
Column	Nucleosil 120-3 C8 column; 4.6 mm (internal diameter) × 250 mm (length), 3 μm (Macherey-Nagel, Düren, Germany)
Mobile phase	A = 10 mmol/L sodium citrate buffer containing 10 mmol/L sodium L-octane sulfonate (pH 3.1); B = acetonitrile; A/B = 85/15
Flow rate	1.0 mL/min
Column temperature	40°C
Detector	SPD-M20A photodiode array UV-VIS spectrophotometric detector (Shimadzu, Tokyo, Japan)
Detection wavelength	240 nm

feed ingredients, dried whole egg, fresh hen eggs, and nondairy creamer (Gossner et al., 2009).

Melamine is not a natural product and is not approved for direct addition to food or feed; however, it is approved for use as part of certain food-contact substances (Tyan et al., 2009). To protect public health and food safety, many countries have established maximum residue limits (**MRL**) for melamine in various products. For example, the European Union (**EU**) set the MRL of melamine in dairy products and high-protein foods at 2.5 mg/kg, whereas the US FDA set the MRL of melamine in milk and dairy products and milk foods as 0.25 mg/kg, and stressed that infant formula sold to US consumers must be completely free of melamine. On April 22, 2010, the Ministry of Health of China published new dairy safety standards and emphasized that food should not be tainted with melamine. In other words, any act of adding melamine to dairy products artificially is illegal, even if the amount is much lower than the MRL (Guo et al., 2011).

Intensive controls on melamine by national safety authorities, importers, producers, and other parties of the food industry worldwide are being conducted to protect human health. Therefore, there is an increasing need to perform melamine testing (Chen and Yan, 2009).

Several detection methods for melamine have been developed, such as GC, liquid chromatography, immunoassay, GC-MS, and liquid chromatography-MS (Wu et al., 2009). Current methods range from sensitive liquid chromatographic-tandem mass spectrometric techniques to less sensitive immunoselective assays such as ELISA. Difficulties in analysis may include contamination, matrix effects, and analyte instability. The effect of these difficulties generally depends on the

Table 2. Recovery, LOD, LOQ, and RSD for the method validation of melamine in milk and dairy products[1]

Product	Spiked content (mg/kg)	Percentage recovery (CV)	Average percentage recovery (CV)	LOD (μg/kg)	LOQ (μg/kg)	RSD (%)
Fruit yogurt	0.1	104 (3.85)	95 (11.30)	110	330	1.5
	1	90 (1.02)				
	2.5	82 (0.89)				
	5	102 (0.91)				
Soft cheese	0.5	93 (0.49)	97 (11.44)	35	105	0.8
	1	85 (0.18)				
	2.5	112 (0.005)				
	5	98 (0.03)				
Pasteurized milk	0.05	88 (0.63)	95 (9.01)	70	200	1.5
	0.5	88 (0.1)				
	1	106 (0.05)				
	2.5	98 (0.005)				
UHT milk	0.1	116 (0.58)	103 (8.88)	110	320	1.5
	1	95 (0.16)				
	2.5	101 (0.13)				
	5	100 (0.02)				
Milk powder	0.1	110 (0.17)	101 (6.47)	110	340	2.0
	1	95 (0.01)				
	2.5	101 (0.01)				
	5	100 (0.01)				
Infant formula	0.05	122 (0.01)	109 (9.33)	90	280	1.0
	0.1	108 (0.01)				
	0.5	97 (0.1)				
	1	110 (0.01)				

[1]LOD = limit of detection; LOQ = limit of quantification; RSD = relative standard deviation.

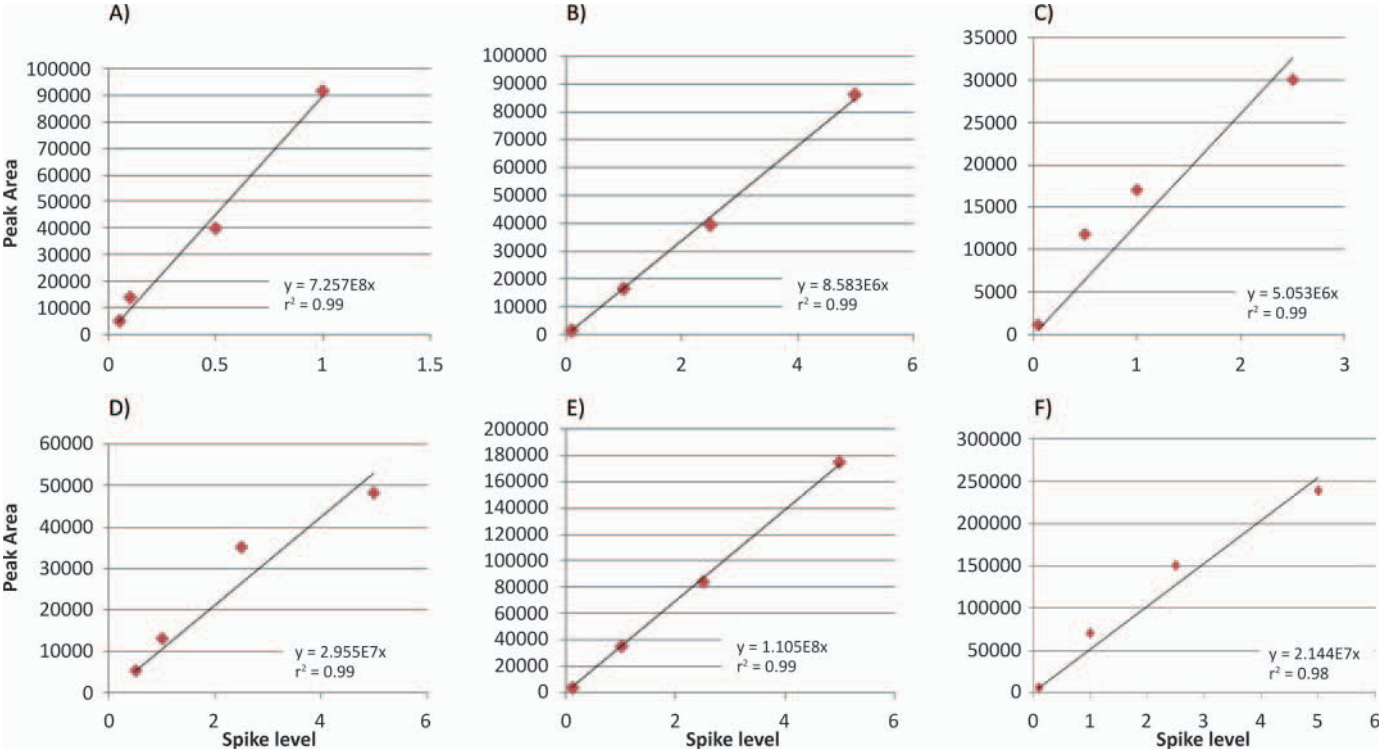

Figure 1. Calibration curves for melamine. Data obtained from the analysis of (A) infant formula, (B) yogurt, (C) pasteurized milk, (D) cheese, (E) milk powder, and (F) UHT milk fortified from 0.05 to 5 μg/g and then taken through the extraction procedure. Color version available in the online PDF.

method used, the food matrices involved, and the analyte examined (Tittlemier, 2010).

The present study proposes a simple, sensitive, and economical method for the analysis of melamine by reversed-phase HPLC in milk and dairy products and a routine monitoring program. The method was then used to analyze 300 samples of dairy milk purchased from major retailers in Turkey.

MATERIALS AND METHODS

Chemicals, Reagents, and Equipment

All reagents and solvents were analytical grade unless otherwise specified. Melamine (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile was HPLC grade and purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained from a Millipore system (Millipore, Molfheim, France). Disposable syringe filters (Chromafil Xtra PVDF-45/25 pore size 0.45 μm, membrane diameter of 25 mm) were purchased from Macherey-Nagel (Düren, Germany). An ultrasonic bath (Bandelin Sonorex, Berlin, Germany), centrifuge (Beckman Coulter, Krefeld, Germany), and HPLC (Shimadzu, Tokyo, Japan) were used in sample treatment. The HPLC analytical conditions are given in Table 1.

Preparation of Standards

A stock standard solution of melamine was prepared at concentration of 10 μg/mL in water, stored in a refrigerator at 4°C in the dark, and used to prepare working standard solutions by appropriate dilution with the mobile phase. Each standard solution was injected into the HPLC system 6 times. A certain average peak area was regressed with a certain level to calculate the calibration equation.

Sample Preparation

A portion of each homogenized milk or dairy product was weighed (1 ± 0.01 g) into a polypropylene centrifuge tube and 5 mL of acetonitrile:water (50:50, vol/vol) was added. The tube was mixed for 1 min, sonicated for 30 min in an ultrasonic cleaning bath, and mixed again on a Vortex mixer for 1 min. The homogenate was centrifuged at 13,200 × *g* for 5 min at room temperature, and the supernatant was filtered through a 0.45-μm syringe filter into a 2-mL autosampler vial. Then, 250 μL of

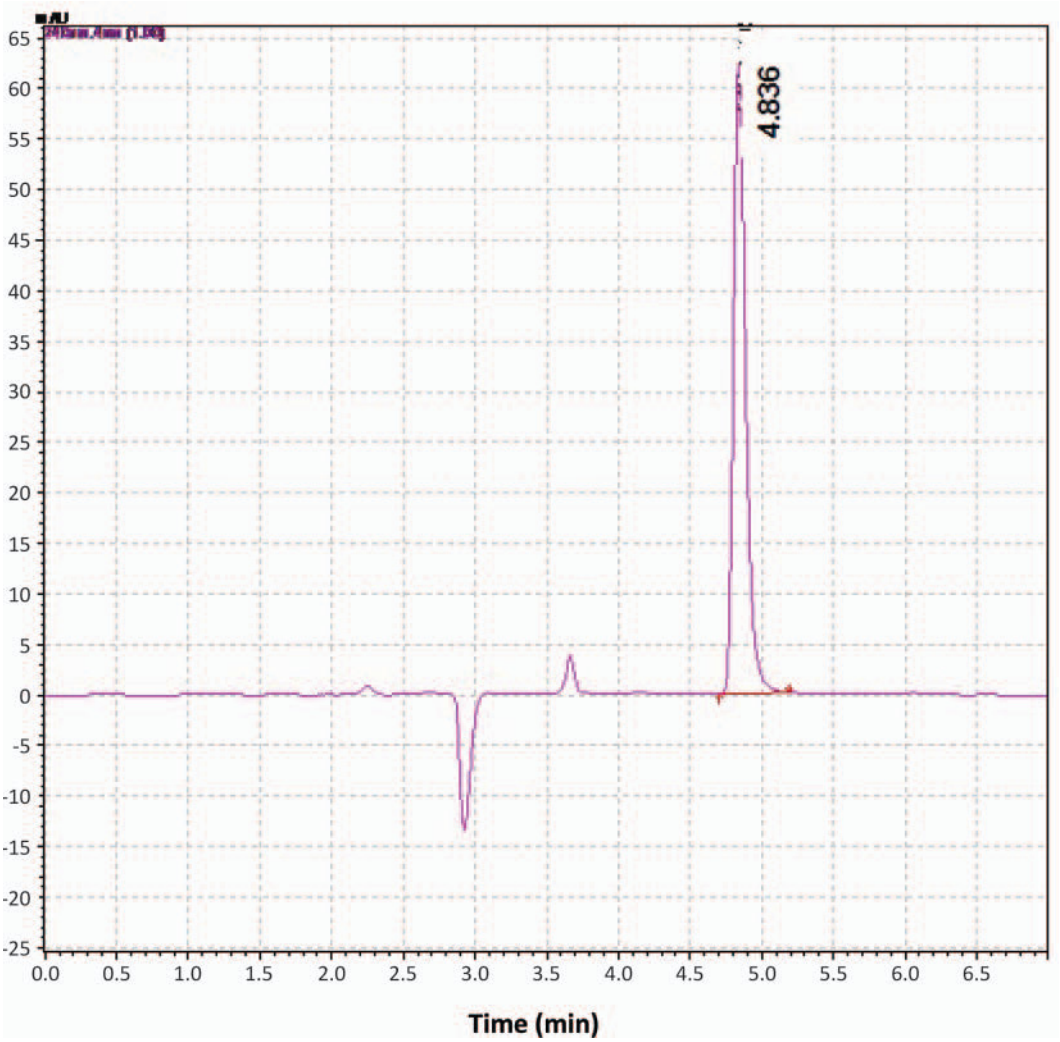

Figure 2. Chromatogram obtained by injecting 20 μL of 5 μg/mL melamine standard solution. Color version available in the online PDF.

this filtrate was added to 750 μL of water and injected into the HPLC system (Shimadzu, 2008).

Sample Preparation for Method Validation

A 6-point calibration curve was used to establish instrument response. Quantities of 0.05, 0.1, 0.5, 1, 2.5, and 5 μg of melamine were added to 1 g of milk or dairy product, and standard addition samples were treated as described above under Sample Preparation. For the assessment of precision and accuracy, 4 spiking levels were added to 1 g of sample (6 replicates were done for each level) as listed in Table 2. Fortified samples were treated as described above under Sample Preparation. The recoveries of melamine from milk and dairy products at each concentration were calculated by means of standard calibration curves with the peak area. The limit of detection (**LOD**) for this method was defined as the concentration at which the signal-to-noise ratio (measured from the injection of standard solutions containing melamine) was 3:1. The limit of quantification (**LOQ**) was defined as the lowest concentration of analytes that could be determined with acceptable precision and accuracy.

Samples

In total, 300 domestic and imported dairy products (UHT milk, pasteurized milk, fruit yogurt, milk powder, powdered infant formula, and soft cheese, 50 samples of each) were purchased from national chain grocery stores in Ankara, Turkey, between June 1 and June 30, 2010. All of the milk, yogurt, and cheese samples were domestic products, but 84% of milk powders (42 samples) and 78% of infant formulas (39 samples) were imported. Samples were obtained via convenience sampling, where every unique item containing milk as a major ingredient was purchased for analysis.

Table 3. Selective methods for the quantification of melamine in milk and dairy products[1]

Method type	Matrix	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%)	Reference
HPLC	Yogurt, milk, cheese, milk powder, infant formula	35–110	105–340	82–122	Current study
HPLC	Infant formula	100	200	97–101	Venkatasami and Sowa, 2010
HPLC	Milk and milk powder	200	1,000	81–84	He et al., 2008
HPLC	Milk		20	84–91	Wei et al., 2009
HPLC	Milk and milk powder	5	20	85–109	Rambla-Alegre et al., 2010
HPLC	Milk	18	60	85–99	Sun et al., 2010b
HPLC	Milk products	21			Wang et al., 2010
HPLC-MS/MS	Infant formula		25	75–125	Smoker and Krynitsky, 2008
HPLC-MS/MS	Infant formula		250	72–110	Turnipseed et al., 2008
HPLC-MS/MS	Milk, infant formula	25	50	99–116	Desmarchelier et al., 2009
HPLC-MS/MS	Milk powder	100	500	85–97	Ibánez et al., 2009
HPLC-MS/MS	Milk		30	101–119	Tran et al., 2010
	Infant formula		50		
HPLC-MS/MS	Infant formula, milk, yogurt		4	92–105	Tittlemier et al., 2009
GC-MS	Dairy products	10		94–102	Xu et al., 2009
GC-MS	Milk	0.3	1	65–106	Li et al., 2009
GC-MS/MS	Milk products	2	5	81–92	Miao et al., 2009

[1]LOD = limit of detection; LOQ = limit of quantification.

RESULTS AND DISCUSSION

Method Performance

Several dairy products, in either liquid or solid form, were used to develop the method. They were fortified with melamine and then tested under the same conditions to reveal matrix effects and possible interferences.

Melamine is a weakly alkaline compound that can hydrolyze in strong acid or alkali solutions. Melamine extraction can be carried out under neutral, acidic, or alkali conditions, but acidic (pH $\leq$ 3) and neutral extraction conditions are most common for food. Neutral extraction can be carried out using acetonitrile:water or methanol:water mixtures (Sun et al., 2010a). In this study, an acetonitrile:water mixture was used.

Sample purification is of major importance in various analytical fields. Dairy products contain many proteins and fats that can interfere with melamine analysis, so suitable and effective purification procedures are nec-

Table 4. Range of melamine levels detected in milk and dairy products sold in some markets

Dairy product	Positive products (no.)	Contamination range (mg/kg) (geometric mean)	Reference
UHT milk	0	—	Current study
Pasteurized milk	0	—	Current study
Powdered infant formula	0	—	Current study
Cheese	1	0.121	Current study
Milk powder	4	0.505–0.86 (0.694)	Current study
Yogurt	22	0.136–0.479 (0.294)	Current study
Liquid milk	1	22.8[1]	Chen and Yan, 2009
Powdered infant milk	3	1.32–23.63 (8.5)	Chen and Yan, 2009
Ice cream, liquid milk and milk powder	61	0.01–6,175	Xu et al., 2009
Powdered infant formula	22	0.1–2,563	Gossner et al., 2009
Liquid milk and yogurt	52	0.6–648	Gossner et al., 2009
Powdered milk products	56	<1–6,196	Gossner et al., 2009
Frozen dairy products	6	4.4–60.8	Gossner et al., 2009
Raw liquid milk	2	2.09–2.19 (2.14)	Sun et al., 2010b
Semi-finished liquid milk	3	1.67–1.89 (1.81)	Sun et al., 2010b
Flavored liquid milk	5	0.08–1.74 (0.36)	Sun et al., 2010b
Infant formula	71	0.0431–0.346 (0.016)	Tittlemier et al., 2009
Cheese, soft	0	—	Tittlemier et al., 2010
Milk	1	0.00742	Tittlemier et al., 2010
Milk, condensed and evaporated	4	0.0175–0.0307 (0.0254)	Tittlemier et al., 2010
Milk powder	2	0.00528–0.0122 (0.00802)	Tittlemier et al., 2010
Yogurt	0	—	Tittlemier et al., 2010
Milk powder	3	0.5–5.5	Schoder, 2010

[1]mg/L for liquid milk.

essary before instrument analysis (Sun et al., 2010a). For this reason, disposable syringe filters were used in this study. This hydrophilic membrane removes water-soluble oligomers and polymers such as proteins, with a binding capacity for proteins of 82 μg per 25-mm filter.

Reversed-phase liquid chromatography is an excellent separation technique for ionizable molecules. Because melamine is a basic analyte ($pK_a = 5.0$) under acidic conditions, the analyte is fully protonated in the mobile phase and the residual silanol groups on the silica support of the column packing are also protonated (Venkatasami and Sowa, 2010). In the present work, a Nucleosil C8 column was used for the separation of melamine in milk and dairy products. The mechanism of separation was ion-pairing with sodium octane sulfonate. Because the particle size of column was small (3 μm), the flow rate was adjusted to 1.0 mL/min to compensate for the higher back pressure. The calibration curves (Figure 1) indicated no interference from milk and dairy products, and the melamine signal was clearly distinguished at 4.836 min (Figure 2). In addition, samples spiked with 0.05 to 5 mg/kg of melamine showed recoveries ranging from 95 to 109%, with relative standard deviation values ranging from 0.8 to 2%, indicating that the method was accurate over the tested concentration range (Table 2).

The LOD, LOQ, and recovery of this method for the determination of melamine in some dairy products were compared with other reported methods (Table 3). A sensitive and validated HPLC method for the determination of melamine residue in milk and dairy products was developed. The proposed method was sensitive, reliable, and accurate, and permitted the detection of melamine residues at levels as low as 105 to 340 μg/kg in different dairy products. The method can be used for the routine determination of melamine residues in different dairy products.

Presence of Melamine in Milk and Dairy Products Sold in Turkey

Melamine was not found in infant formulas and pasteurized or UHT milk samples, whereas 2% of cheese samples (1 sample), 8% of milk powder samples (4 samples), and 44% of yogurt samples (22 samples) contained melamine at 121, 694 ± 146 (range 505–860), and 294 ± 98 (range 136–479) μg/kg levels, respectively, and compared with the melamine levels detected in milk and dairy products sold in some markets (Table 4). These findings were below the limits set by Codex Alimentarius Commission (2010) and EU legislation (European Commission, 2002, 2009; 1 mg/kg for infant formula, 2.5 mg/kg for dairy products). We assumed that the lower amounts detected were the result of contamination during the preparation of dairy products and the higher amounts the result of deliberate addition. The consumption of foods containing these low levels of melamine does not constitute a health risk for consumers.

CONCLUSIONS

A simple, precise, reliable, accurate, and validated reversed-phase HPLC method was developed for the determination of melamine in UHT and pasteurized milk and powdered infant formula, fruit yogurt, soft cheese, and milk powder. The LOD and LOQ of the method were sufficient to detect melamine in milk and dairy products under the safety limits recommended by the Codex Alimentarius Commission. The proposed method can be used for the routine determination of melamine residues in milk and dairy products.

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