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Determination of Melamine in Infant Milk Formula, Milk Powder and Basaa Fish Samples by HPLC/DAD

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Abstract

Thirty two samples were collected of milk powder for baby and human consumption and Basaa fish 8 infant milk formula, 8 growing up milk formula purchased from pharmacy in Saudi Arabia, 6 Sweetened Full Cream Milk Powder purchased from China and 10 fish samples (Basaa fish) Ui Tnam purchased from popular markets in Saudi Arabia. The samples were analyzed and determination for Melamine (MEL) by HPLC/DAD. The incidence of MEL in powder milk and in fish, was about 100 and 80 % respectively. High concentrations were found in growing up milk formula. Melamine was detected in 28 out of 32 samples with varying concentrations. Of the 28 melamine positive samples, samples had a melamine concentration higher than 258 mg/kg (the median). The lowest and the highest concentrations were found to be 7.5 and 258 mg/kg, respectively in all samples. This application note describes an efficient and simple method for preparing milk powder and fish samples coupled to an HPLC/DAD method for rapid analysis of melamine in these samples. The purpose of the survey was to determine levels of melamine and to monitor the effectiveness of the controls in place to limit consumer exposure to melamine.

Keywords: Exposure; Melamine; Infant milk formula; Powder milk; HPLC; Bassa fish

Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is used in the production of plastics, in finishers for paper, in fertilizer, as a flame retardant, and in the manufacture of wrinkle-free textiles Garber [1]. The use of melamine as a nonprotein nitrogen source for cattle was studied by Newton and Utley [2]. Although the melamine was digested, most of the nitrogen was eliminated in the urine and not assimilated by microbes in the rumen. Since the spring of 2008, an increase of urinary tract stones in infants and young children were noticed by pediatricians in Gansu, Hebei, Beijing, and other cities/provinces. This was followed by an epidemic of urinary tract stones in infants and young children in more than 10 provinces in the mainland of China. In September 2008, the cause of this unusual epidemic was identified, i.e. the illegal use of "protein essence" (melamine, an industrially synthesized chemical) in raw milk (the main ingredient of infant formula) to falsely increase the protein content of raw milk after dilution with water. This was a typical case of raw milk adulteration. When melamine concentration was high enough in the infant formula, because of its very low solubility, it formed crystals or stones in the urinary tract of infants and young children [3]. Melamine is classified by the World Health Organization as not posing a health risk [4]. The use of melamine-formaldehyde resins in the production of molded plastics and as coatings in contact with food is approved by the U.S. Food and Drug Administration (FDA) as long as the yield of chloroform-soluble extractives does not exceed 0.5 mg/in^2 of food contact surface [5].

Since melamine is a raw material in the production of some plastic products used for serving food, low-level migration of melamine into the food has been reported. Thus, melamine was detected using liquid chromatography in beverages at levels of 0.54, 0.72, 1.42 and 2.2 mg/kg in coffee, orange juice, fermented milk and lemon juice respectively, with a limit of detection of 0.05 mg/L [6]. These levels originated from migration of melamine from the cup, made of melamine-formaldehyde resin, into the beverage under experimental hot and acidic conditions (95°C for 30 min) [7].

Much higher levels of melamine have been detected in food, feed or raw materials following fraudulent practices: data reported in 2007 by the U.S. FDA [8] showed melamine in wheat gluten and rice protein concentrate imported from China at levels in the range of 2 to 80 mg/kg, with a limit of detection of 0.05-0.1 mg/kg. Analyses of actual feed product showed melamine in fish feed samples ranging from 53 to 400 mg/kg, respectively [8]. The U.S. Food and Drug Administration (FDA) derived a TDI for melamine of 0.63 mg/kg b.w. [8]. For adults, melamine is only dangerous in very high concentrations. In this regard, the US Food and Drug Administration (FDA) have set a tolerable daily intake (TDI) of 0.5 mg/ kg body weight [8]. Since the spring of 2008, an increasing number of kidney stones in infants were noticed by pediatricians in Gansu, Hebei, Beijing, and other cities/provinces in China [9].

The aim of the work is to perform an easy, fast, accurate and reliable analytical methodology to quantify the level of melamine in milk and fish. Also study on the occurrence of melamine in milk powder, infant milk formula and Bassa fish from Saudi Arabia and China.

Materials and Methods

Sampling

A total of 32 samples, Sweetened Full Cream Milk Powder (6 samples) for a whole family (in convenient pack) from China in Zahnghou, Fujan province, Xiamen and Hangzhou, Zhejiang Province were collected from markets While, infant milk formula (8 samples), growing up milk formula (8 samples) and Bassa fish (Ui Tnam) (10

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samples) were randomly collected from Saudi Arabia, Qassim region (Byraida,Onyza, Al-Ras, Al-Badya and Al-Bokhria) .

Reagents and chemicals

Melamine standard (95% purity) was purchased from Sigma (St. Louis, MO, USA). Water was obtained by distillation through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), reagent-grade formic acid (content 98–100%) and HPLC-grade potassium dihydrogen phosphate (93%) were purchased from Merck Germany).

Stock solution: 10.00 mg of melamine (MEL) was added to a diluted to mark with a solution of 2 % ammonium hydroxide in water (v/v) 100 mL volumetric flask and adjusted concentration to produce a stock solution 100 µg/mL. The solution was sonicated for 20-30 minutes until crystals of melamine were no longer visible.

Intermediate solutions: A 10.0 µg/mL standard was prepared by adding a 1.0 mL aliquot of the MEL stock to a 10.0 mL volumetric flask and diluting to the mark with water. A 1.0 µg/mL standard was prepared by adding a 1.0 mL aliquot of the MEL stock to a 100.0 mL volumetric flask and diluting to the mark with water.

Extraction of melamine from:

Milk powder: Five gram of baby infant or powder milk samples was transferred to 50 ml volumetric flask, then 20 ml methanol grade HPLC was added and mixed well by vortex. The mixture was sonicated for 10 min. and then centrifuged at 4500 rpm 10 min. The extracts were evaporated to dryness by a gentle nitrogen stream. Afterwards, an aliquot of the supernatant was diluted with HPLC-grade methanol – water (8:2) according to FDA [10].

Fish: The extraction of melamine in fish was done according to the procedures of Wendy et al. [11]. Homogenized bassa fish tissue (5.0 g) was weighed into a 50 mL polypropylene centrifuge tube. To this was added 24 mL of a 50:50 (v:v) solution of acetonitrile: water and 1 mL of 1.0 N hydrochloric acid. The sample was capped, shaken vigorously for 30 seconds and then vortex mixed for 1 minute. The sample was centrifuged at 4000 rpm for 5 minutes at 5°C. Breaking through the solid fat layer at the top of the sample with the tip of a pipette, 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 15mL tube, and the sample was shaken for two minutes. The sample was centrifuged at 4000 rpm for 5 minutes 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 minute. The polypropylene tube was again centrifuged at 4000 rpm for 5 minutes 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 seconds. 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 minute. The column was eluted into a glass culture tube using 5 mL of 5 % ammonium hydroxide in methanol. The elute was evaporated to dryness at 55°C under flowing nitrogen at 15 psi for 20 minutes.

Equipment and chromatographic conditions

High-performance liquid chromatography (Agilent 1100 series) equipped with a photodiode array DAD (G 1315 B) analysis was carried out with a liquid chromatograph equipped with solvent delivery systems (Agilent Technologies, Inc.200 Regency Forest Drive, Suite 330 Cary, NC 27511 USA) system containing a G1322A Vacuum Degasser, a G1312A Binary and a reverse-phase analytical column packed with C18 material (Agilent ZORBA, X DB- 5 µm, 150 mm × 4.6 mm). The mobile phase consisted of methanol: water (80:20). Separation was performed at ambient temperature at a flow rate of 1.0 ml min^{-1}; the injection volume was 50 µl for both standard solutions and sample extracts by auto sampler (G1329A). The Dad detector was operated at a 220 nm.

Results and Discussion

The occurrence of melamine in infant milk formula, growing up milk formula and Bassa fish collected from different regions of Qassim, Saudi Arabia and Sweetened Full Cream Milk Powder from China is listed in Table 1 and Figure 1. Melamine was detected in 28 out of 32 samples with varying concentrations. Of the 28 melamine positive samples, concentration was 258 mg/kg (the mean value). The lowest and the highest concentrations were found to be 7.5 and 258 mg/kg, respectively. Figure (1a, 1b and 1c) showed the results obtained, depicts the chromatogram for melamine extraction from samples and HPLC analysis. As can be seen, possible interferences from substances present in samples were not observed. In addition, a detection and identification process based on retention times and a Diode-Array Detector (DAD) was carried out.

The percentage of positive samples with detectable levels of melamine was 100% in infant milk formula, growing up milk formula

No of samples	Mean of melamine concentration (mg/kg ± SD)			
	Infant milk formula Mean ± SD	Growing up milk formula Mean ± SD	Basaa fish Mean ± SD	Full Cream Milk Powder Mean ± SD
1	9.49 ± 0.35 [a]	251.23 ± 0.78	7.5 ± 0.28	35.8 ± 0.44
2	11.0 ± 1.0	61.4 ± 0.78	ND	39.7 ± 0.35
3	13.0 ± 1.15	28.5 ± 0.86	ND	33.5 ± 0.28
4	258 ± 1.15	5.97 ± 0.04	12.26 ± 0.14	35.4 ± 0.30
5	252.0 ± 1.45	7.75 ± 0.75	14.8 ± 0.44	29.1 ± 0.58
6	254.0 ± 0.57	31.66 ± 0.88	13.5 ± 0.28	31.6 ± 0.88
7	185.25 ± 0.25	200.0 ± 0.57	16.6 ± 0.30	-
8	192.7 ± 1.45	182.33 ± 1.45	Nd	-
9	-	-	Nd	-
10	-	-	9.6 ± 0.30	-

ND: not detected
[a] Mean ± S.D. for triplicate determinations

Table 1: Melamine concentrations in Infant milk formula, growing up milk formula, full cream milk powder and Basaa fish.

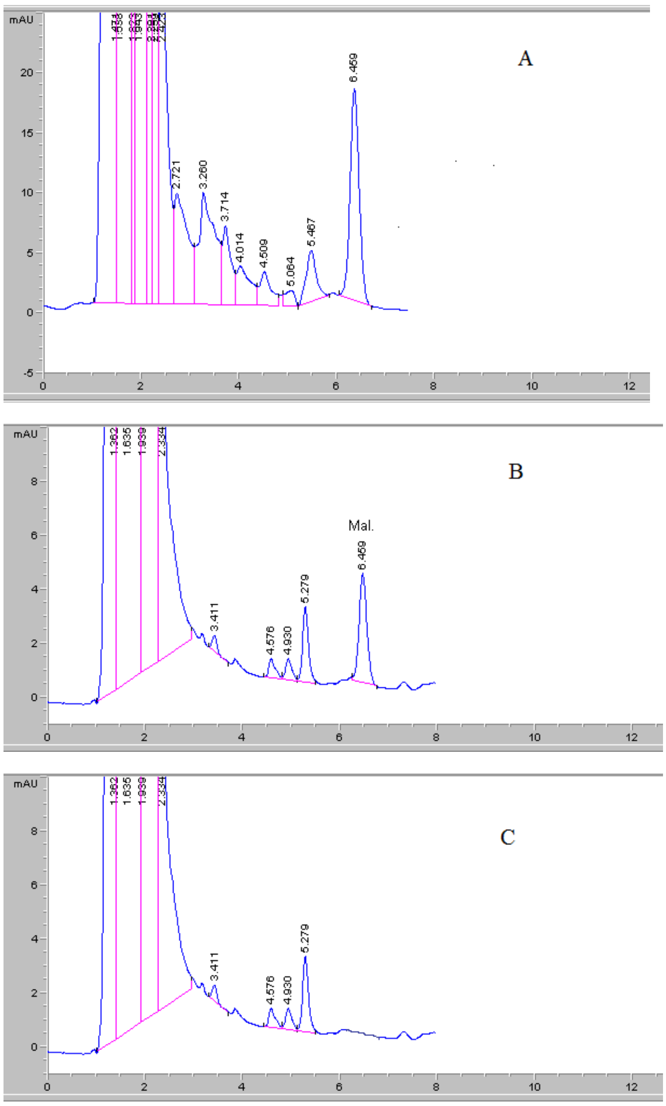

Figure 1: HPLC Chromatograms of melamine separation (A) standard, (B) positive sample and (C) negative sample.

from Saudi Arabia and full cream milk powder from China, while in Bassa fish 60%. The percentage of melamine contamination was high different between the raw material fish and milk powder may be in China, where adulteration had occurred, water had been added into raw milk to increase the volume. As a result of this dilution, the milk had a lower protein concentration and the companies using the milk for further production (e.g. of powdered infant formula) would produce the products which had a low protein content but high melamine. Recently, an increased incidence of kidney stones and renal failure in infants was reported in China which was believed to be associated with the ingestion of infant milk-based formula contaminated with melamine [12]. It seemed that again nitrogen-rich melamine was added to raw milk to boost its apparent protein content.

Human could be also exposure to melamine and its analogues from a number of other sources. Firstly, one of the sources would be from breakdown of the pesticide cyromazine, which is approved for use in many countries [13]. Secondly, migration from melamine resin containers to food has been reported [7]. Thirdly, melamine could be introduced into human diets as it may carry over from the illegal use of melamine in animal feed or feed ingredients although no quantitative data exist. In this regard, some data have shown that the carry-over occurs from feed to products of animal origin including milk, eggs, meat, and fish. Lastly, contamination of melamine into the food chain as a baseline level is possible as it is present in the environment and the widespread use of melamine-containing materials [14].

The results presented in Table 2 shows that melamine was detected at levels greater than 1 mg/kg compared with the control limit set in China in powdered infant formula (1 mg/kg) and in other foods (2.5 mg/kg). The US Food and Drug Administration (FDA) sets 2.5 mg/kg as the concern level, while Hong Kong, China and New Zealand also set a safe limit in food products of 2.5 mg/kg, though Hong Kong has lowered the level for children under 3 and pregnant or lactating women to 1 mg/kg. Compared with 1 mg/kg, the melamine contents of all samples in Table 1 exceeded the limit level notably. The baseline level refers to the amount of melamine from the unintentional use, while the adulteration level is that of melamine in food that result from the intentional addition of melamine or the unapproved use or misuse of melamine and any substances that can degrade to form melamine [14]. Maximum permitted concentration for melamine in food has been set at 2.5 mg/kg by the European Commission [15,16]. Hong Kong established tolerance at 1 mg/kg in infant foods and Taiwanese authorities stated that melamine should not be detected in any food using the most sensitive instrumentation [15].

The UV absorption of melamine is reported in wide range between 210 and 240 nm, such as 214 nm [17], 215 nm [18], 220 nm [19], 236 nm [20], 240 nm [21], etc. The difference of detection wavelength maybe related to the polarity and cut-off wavelengths of the solvents used in the mobile phases. We compared the absorption of melamine in the mobile phase solution within the range of 205-240 nm, and found that the absorption decreased markedly with the increased wavelength. Considering the cut-off wavelength at 220 nm was thus selected for detection of melamine.

The present results showed that the Infant milk formula samples had an unacceptable level of melamine. Furthermore, the high level of melamine was found in the infant milk formula followed by milk formula from the collected samples in Saudi Arabia.

Melamine was detected using liquid chromatography in beverages at levels of 0.54, 0.72, 1.42 and 2.2 mg/kg in coffee, orange juice, fermented milk and lemon juice respectively, with a limit of detection of 0.05 mg/L [6]. These levels originated from migration of melamine from the cup, made of melamine-formaldehyde resin, into the beverage under experimental hot and acidic conditions (95°C for 30 min) [7]. Much higher levels of melamine have been detected in food, feed or raw materials following fraudulent practices: data reported in 2007 by the U.S. FDA [8] showed melamine in wheat gluten and rice protein concentrate imported from China at levels in the range of 2 to 80 mg/kg, with a limit of detection of 0.05-0.1 mg/kg. Analyses of actual feed

Samples	No of samples	% of positive samples	Mean (mg/kg)	Minimum (mg/kg)	Maximum (mg/kg)
Infant milk formula	8	100	146.93	9.49	258
Growing up milk formula	8	100	96.105	7.75	251
Basaa fish	10	60	12.73	9.6	16.6
Full Cream Milk Powder from China	6	100	34.18	29.1	39.7

Table 2: Detection of Melamine in Infant milk Formula , Growing up milk formula , Full Cream Milk Powder and Bassa fish Samples (*n*=3).

product showed melamine in fish feed samples ranging from 53 to 400 mg/kg, respectively [8]. The U.S. Food and Drug Administration (FDA) derived a TDI for melamine of 0.63 mg/kg b.w. [8]. In recent study by Li et al. [22] found an increasing risk of nephrolithiasis with increasing levels of daily melamine intake, starting at a lower intake than 0.2 mg/kg of body weight. This result holds true after adjustment for exposure time, preterm, urinary, malformation, and parents with a history of urinary stones. The TDI for melamine of 0.2 mg/kg of body weight as recommended by the WHO seems to be too high for young children. It should at least be lower than 0.1 mg/kg. In today's world, it is crucial to understand and deal with the global implications of food borne diseases if problems like the melamine epidemic are to be prevented.

Conclusion

The present results showed that the Infant milk formula samples had an unacceptable level of melamine. Furthermore, the high level of melamine was found in the infant milk formula followed by milk formula from the collected samples in Saudi Arabia. This application note describes an efficient and simple method for preparing milk powder and fish samples coupled to an HPLC/DAD method for rapid analysis of melamine in these samples. As can be seen, possible interferences from substances present in samples were not observed. In addition, a detection and identification process based on retention times and a diode-array detector (DAD) was carried out.

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