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Occurrence of Aflatoxin M_1 in flavored UHT milk

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Summary: In this study a total of 90 flavored Ultra High Temperature (UHT) milk samples (cacao, strawberry and banana flavors, 30 samples for each) were analyzed in order to determine the occurrence and the level of Aflatoxin M_1 (AFM_1) contamination by using Enzyme Linked Immuno-Sorbent Assay (ELISA) method. The results showed that none of the samples exceeded the maximum limit of AFM_1 prescribed by Turkish Food Codex (TFC) and European Union Codex (50 ppt; 50ng/l). However, 75.6% of the samples (68 samples) examined were determined to be contaminated with AFM_1 at various levels ranging between 0.39 and 26.6 ng/l. The results showed that, although the detected AFM_1 levels were lower than the accepted limits, AFM_1 contamination of flavored UHT milk, especially preferred and consumed by children with high susceptibility due to their slower biotransformation capacity for carcinogens, could be a public health concern.

Key words: Aflatoxin M_1, ELISA, flavored UHT milk, public health.

Aromalı UHT sütlerde AFM_1 varlığı

Özet: Bu çalışmada toplam 90 aromalı UHT süt örneğinde (Kakolu, çilekli ve muzlu, her birisinden 30 adet olacak şekilde) AFM_1 varlığı ve seviyesi ELISA metodu kullanılarak araştırıldı. Sonuçlar, incelenen örneklerin hiçbirisinin AFM_1 miktarları açısından Türk Gıda Kodeksi ve Avrupa Birliği Kodeksi sınırlarını (50 ppt; 50 ng/l) aşmadığını gösterdi. Buna rağmen, incelenen örneklerin %75.6'sının (68 örnek) 0.39'dan 26.6 ng/l'ye değişen seviyelerde AFM_1 ile kontamine olduğu belirlendi. Sonuçlar, incelenen örneklerde belirlenen AFM_1 seviyelerinin kabul edilen sınırların altında olmasına karşın, özellikle çocuklar tarafından fazlaca tercih edilen ve tüketilen aromalı UHT sütlerin AFM_1 ile kontamine olmaları durumunda, çocuklarda karsinojenlerin biyotransformasyon mekanizmasının daha yavaş olması nedeniyle, bir halk sağlığı problemi olabileceğini gösterdi.

Anahtar sözcükler: Aflatoksin M_1, aromalı UHT süt, ELISA, halk sağlığı.

Introduction

Mycotoxins are a group of secondary metabolites produced by various fungal species under appropriate conditions such as nutrients available, ambient temperature, water activity and oxygen (24). These toxic compounds can contaminate foodstuffs, crops or human foods. That is because of the widespread nature of fungi in the environment, mycotoxins are considered unavoidable contaminants in foods and feeds (63). Due to their resistance to food processing and high temperatures, mycotoxins can be easily transmitted through processed food representing a serious hazard for the food chain, and for human and animal health (20,42).

Aflatoxins are a group of mycotoxins produced by three Aspergillus *spp*. including *Aspergillus flavus*, *Aspergillus parasiticus* and in a lower extend *Aspergillus nomius*, which contaminate plants and plant products (9). Aflatoxins have both acute and chronic toxic effects on animals and humans, and can cause serious health hazards (16, 54). Their major adverse effects on health are stated as carcinogenity, teratogenity and mutagenity and they are considered as major etiological factors for hepatocellular carcinoma (HCC) (33). Their role in hepatocarcinogenesis often in conjunction with hepatitis B is also well established (61). There are some evidences for their association with Reye's syndrome, kwashiorkor, and acute hepatitis (18,60). In addition, exposure of aflatoxins in early life has been associated with impaired growth, particularly stunting (26).

Among 20 different types of aflatoxins, only the aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are associated with acute liver damage and cirrhosis. AFM_1 is a hydroxylated metabolite of AFB_1, which can be found in milk. Although AFM_1 is classified as hepatotoxic and carcinogenic, the toxicity of AFM_1 is lower than AFB_1 (28). It possesses a high level of genotoxic activity and definitely represents a health risk because of its possible accumulation and linkage between DNA (51). The International Agency for Research on Cancer (IARC) of World Health Organization (WHO) reconsidered carcinogenicity categorization of AFM_1 and changed it from Group 2 to Group 1 (2,23).

The concentration of AFM_1 in milk and dairy products depends on the levels of exposure and the amount of AFB_1 ingested by the animal (11). In addition, contamination of milk and dairy products with AFM_1 showed variations according to geography, country and season. The contamination level of AFM_1 was found to be different in hot and cold seasons (13). About 0.3-6.2% of AFB_1 in animal feed is transformed to aflatoxin M_1 in milk (14). AFM_1 could be detected in milk within 12 h after the first ingestion of AFB_1. Following the withdrawal of contaminated source, AFM_1 concentration in the milk decreases to an undetectable level within 72 h (49). Battacone *et al.* (7) stated that AFM_1 could be detected in sheep's milk within 6 hours after consumption of feed contaminated with AFB_1. AFM_1 level of milk is not affected significantly neither by thermal processes used in dairy industry, i.e. pasteurization and UHT treatments nor during stages in the preparation and storage of various dairy products (44).

Although several methods successfully used in aflatoxin detection, including thin-layer chromatography (TLC), liquid chromatography/electrospray-tandem mass spectrometry, high-performance liquid chromatography (HPLC) (12,30,49) as a rapid and sensitive technique for examining milk and other dairy products, immunoassays, especially ELISA has been used more than 20 years. ELISA is considered as a suitable method for quick and sensitive analysis with high sample throughput, and also it is cost effective, fast, and requires only a small sample volume for analysis (36,50). The European Commission (EC) has established a maximum admissible level as 50 ng/l for AFM_1 in milk (21), as it is accepted in Turkey (3) whereas Food and Drug Administration (1) has established 500 ng/l as accepted level.

Presence of AFM_1 in milk and dairy products is a worldwide concern since these products are frequently consumed in market (22), and probably the main consumers of these products are children which also gives another importance to this issue (34). Flavors (chocolate, strawberry, banana etc) are added to UHT milk mainly for children who do not like the taste and flavor of pure UHT milk. Although flavored milk is criticized as a source of added sugars, children consuming flavored milk meet their calcium requirements without consuming significantly more added sugars compared with children who are not milk drinkers (32,39). Data from the Third School Nutrition Dietary Assessment Study shows that most of the elementary school students (71%) choose flavored milk (15). Research in schools shows that students purchase more milk when milk offerings are enhanced and include flavored milk (45). In another report prepared by Tetrapak (4) it was stated that the consumption of flavored milk sold in ready-to-drink liquid form increased by a compound annual growth rate of 9.6%. According to a report prepared by Pınar Süt (5), a well-known dairy company of Turkey, market share indorsement of flavored milk increased to 10% of packed milk market, which was about 26 million litres of milk in volume, and had 105 million Turkish liras indorsement in the market share in Turkey. As it is mentioned above, there is an increase in the consumption of flavored milk especially by children. Therefore, in this study, it was aimed to find out the occurrence and the levels of AFM_1 in flavored UHT milk samples sold in Turkey.

Materials and Methods

Sampling: A total of 90 flavored UHT milk samples (30 chocolate, 30 banana 30 strawberry) from different brands (250 ml) were randomly purchased from supermarkets in October 2012. The samples were transported to the laboratory and stored at 4°C then tested in order to determine the occurrence and levels of AFM_1.

Test Procedure: The quantitative analysis of AFM_1 in the flavored UHT milk samples was conducted by using a competitive enzyme immunoassay based on antigen-antibody reaction carried with Enzyme-Linked Immuno Sorbent Assay (ELISA) test kit according to the procedure given by the commercial company (6).

Flavored UHT milk samples were not subjected to centrifugation procedure, because the homogenization had already been carried out during the processing. Therefore, fat globules were very stable and it would be very difficult to extract milk plasma from homogenized milk with fat. All the reagents were hold at ambient temperature in order to warm the reagents up. 200 µL from each standard was added into wells. Then 200 µL of flavored UHT milk samples were added into the rest of the wells. Microplate was covered with aluminum foil and incubated for 2 hours at room temperature (19-25°C) in the dark. The liquid was poured off into the wells and the micro well holder was tapped upside down vigorously (three times) against absorbent paper to ensure the liquid was completely removed from the wells. All the wells were filled with washing buffer and emptied as described earlier. The washing procedure was repeated three times. After this washing procedure, 100 µL of aflatoxin HRP conjugate was added to each wells and incubated at room temperature in the dark for 15 min. The liquid was poured off into the wells and the washing sequence was repeated three times by using washing buffer. Following the washing procedure, 100 µL of TMB substrate solution was added to each well and incubated at room temperature (19-25°C) in the dark for 20 min. Then 100 µL of the stop reagent was added to each well. Finally, the measurement of AFM_1 was carried out photometrically at the wavelength of 400 nm in ELISA reader (Mindray MR 96A, China). Then the results were evaluated based on the programme provided by the manufacturer.

Results

In this study, a total of 90 flavored UHT milk samples were analyzed by using competitive ELISA technique. The levels of aflatoxin M_1 in the UHT samples collected during October 2012 are shown in Table 1. AFM_1 contamination was detected in 68 (75.6%) of the flavored UHT milk samples examined. The AFM_1 levels in the flavored UHT milk samples were ranging between 0 and 26.6 ng/l with an overall mean value of 9.20 ng/l, and a mean value for positive samples of 12.17 ng/l. Most of the contaminated samples (17 samples; 18.9%) were between 5-10 ng/l interval. The concentration of AFM_1 in all samples was found to be lower than the level prescribed by TFC and European Union Codex (50 ppt; 50ng/l).

Discussion and Conclusion

The results of previous studies regarding to the occurrence and the levels of AFM_1 in UHT and pasteurized milk samples carried out in various countries are given in Table 2. The occurrence of AFM_1 in UHT and pasteurized milk samples showed variations ranging between 27.5% and 100%, and the levels were reaching up to 4100 ng/kg.

In a study carried out in Iran, Fallah (23) reported that 62.3% and 71.5% of the UHT and pasteurized milk samples were contaminated with AFM_1 with a contamination level of 5.6-515.9 and 5.8-528.5 ng/kg, respectively which was higher than our results . A total of 19 samples (17.4%) exceeded the legal limits for USA and Iran (ISIRI). In another study conducted by Cano-sancho et al. (10) in Spain showed that 94.4% of the UHT milk samples analyzed were contaminated with AFM_1 (5-30 ng/kg), which was in agreement with the results of the study presented here. No samples exceeded the legal limits required by EU. Siddappa et al (53) also investigated AFM_1 levels in UHT and pasteurized milk samples in India. They found that 64.4% of UHT and 42.9% of pasteurized milk samples were contaminated with AFM_1. As it was reported Iran by Fallah (23) the results were higher than our results ranging between 60-700 ng/kg for UHT milk samples and 1800-3800ng/kg for pasteurized milk samples. In a study carried out by Unusan (58) in Turkey showed that 58.1% of UHT milk samples were contaminated with AFM_1 with a mean contamination level of 108.17ng/l.

The contamination level of milk and dairy products with AFM_1 may show variations according to geography,

Table 1. AFM_1 levels in flavored UHT milk samples.
Tablo 1. Aromalı UHT süt örneklerindeki AFM_1 seviyesi.

	Level ng/l							Σ
	0	0-5	5-10	10-15	15-20	20-25	25-30	
N	22 (24.4%)	14 (15.6%)	17 (18.9%)	12 (13.3%)	11 (12.2%)	11 (12.2%)	3 (3.3%)	90 (100%)
Min		0.39	5.16	11.01	15.57	20.52	25.03	
Max		4.98	9.57	14.01	19.03	24.47	26.60	
Mean		2.83	7.27	12.37	17.50	22.43	25.70	
SD		1.47	1.46	1.0	1.24	1.36	0.81	

Table 2. Occurrence and level of AFM_1 in UHT milk samples reported in previous studies.
Tablo 2. Önceki çalışmalarda bildirilen UHT süt örneklerinde Aflatoksin M_1 varlığı ve seviyesi.

Country	Sample	No. of Samples Positive	Range of Samples Positive (ng/kg)	Exceed Legal Limit [a]	References
Iran	UHT Milk	68 (62.3%)	5.6-515.9	19 (17.4%)	(23)
	Pasteurized Milk	83 (71.5%)	5.8-528.5	31 (26.7%)	
Portugal	UHT and Pasteurized Milk	11 (27.5%)	6.9-69.7	3 (7.5%)	(17)
Brazil	UHT Milk	23 (30.7%)	1000-4100	23 (30.7%)	(41)
Spain	UHT Milk	68 (94.4%)	5-30	0	(10)
Brazil	UHT Milk	40 (100%)	-	12 (30%)	(52)
Argentina	Pasteurized Milk	8 (50%)	-	0	(37)
Greece	UHT Milk	14 (82.3%)	-	0	(48)
Japan	Pasteurized Milk	207 (99.5%)	5-30	0	(40)
India	UHT Milk	29 (64.4%)	60-700	29 (64.4%)	(53)
	Pasteurized Milk	3 (2.9 %)	1800-3800	3 (42.9%)	
Turkey	UHT Milk	61 (47.2%)	0-543.64	57 (43.9%)	(58)
Turkey	UHT Milk	67 (67%)	10-630	31 (31%)	(55)

[a] The maximum tolerance limit accepted by some countries is 50 ng/l.
[a] Bazı ülkeler tarafından kabul edilen maksimum tolerans sınırı 50 ng / l'dir.

country and season. The contamination level of AFM_1 is found to be different in hot and cold seasons. This is due to grass, pasture, weed, and rough feeds are found more commonly in spring and summer than in winter. At the end of summer, greens are consumed more than concentrated feed, causing a decreased level of AFM_1 in milk (13). This could be explained as a result of linear relationship between the amount of AFM_1 in milk and AFB_1 level in feed consumed by animal.

Milk is stated as the major source of macronutrients, calcium, magnesium, phosphorus, vitamin D, vitamin A, riboflavin, vitamin B_{12}, zinc, and potassium for children and teenagers. Children who do not consume cow's milk have been shown to have a lower intake of many essential nutrients (39) and poor bone health when compared with children who consume cow's milk (9). However, milk and dairy products are the most potent source of aflatoxin among foods. AFM_1 intake, even at trace concentrations, causes a significant risk to human health, especially to infant and children who are the major consumers of infant formulas and milk (19,47,56). Although, The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) did not establish a tolerable daily intake (TDI) for aflatoxins, strongly recommended that the level of aflatoxin should be kept as low as possible (43). Kuiper-Goodman (35) reported a tolerable daily intake 0.2 ng/kg bw for $AFM_{1.}$

Adding flavorings, such as strawberry, banana, chocolate flavors etc, is a commercial issue for increasing profit and is also another option for meeting the recommended intakes of milk. Data from the Third School Nutrition Dietary Assessment Study showed that most elementary school students (71%) chosen flavored milk (15). Research in schools showed that students purchased more milk when milk offerings were enhanced and included flavored milk (45). Therefore in the case of possible AFM_1 contamination, drinking more milk with flavorings might be a potential hazard for children who are more susceptible comparing to adults. Exposure of children, including infants, to AFM_1 is stated to be troublesome, because they are considered more susceptible to its adverse effects, and their capacity for biotransformation of carcinogens is generally slower than occurring in adults (37). As a result of this, circulation and exposure time to the toxin is extended in children and infants' organs and tissues. Even the levels in the milk are within the regulatory limits, it cannot prevent the chronic effect of aflatoxins, principally the carcinogenic effect, due to sustained exposure to low levels of aflatoxins (25). Repeated and high consumption of flavored milk contaminated with AFM_1 in early childhood period might be a great problem for public health irrespective from accepted limits.

Apart from carcinogenic effects, mycotoxins, in general, may have adverse effects on the immune status of both adults and children (31,57). A reduced level of salivary sIgA has been associated with aflatoxin exposure in Gambian children (57). It was hypothesized that early and repeated exposures to aflatoxins intrauterine and through childhood might predispose to cancer in liver. Moreover, immunosuppression due to consumed aflatoxins is another contributing factor (59,38). Aflatoxins thus, may increase child susceptibility to infections and may cause failure of immunizations (29). Beside these effects, it has also been reported that children exposed to aflatoxins may become stunted and underweight (8). For example, study results showed that aflatoxins have been directly related to underweight status in children in Benin and Togo (26, 27) and to the condition of kwashiorkor (46,62).

From the results of the current study, flavored UHT milk could be regarded as a hazardous source of AFM_1 for children even the toxin is occurred in low levels. Although none of the positive samples examined exceeded the accepted limits of TFC (50ng/l), and EU legal limits (50ng/l), 75.6% of flavored UHT milk samples (68 samples) were contaminated with AFM_1 at various degrees. It is recommended that in order to reduce the incidence of AFM_1 in raw milk and to keep its levels low, AFB_1 contamination of feedstuff should be reduced, and all precautions must be taken for limiting fungal growth in feedstuff. Beside this AFM_1 analysis and controls should be carried out regularly in dairy industry.

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