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Reduction of Aflatoxin M_1 during Production of Kashk, a Traditional Iranian Dairy Product

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Abstract

Background: Aflatoxin M_1 (AFM_1) is a toxic compound that could be found in milk and dairy products. AFM_1 is the principle hydroxylated derivative of aflatoxin B_1 (AFB_1), formed in liver and excreted into the milk in the mammary glands of both human and lactating animals that have been fed with AFB_1 contaminated diet. To the best of our knowledge, there is no report on stability of AFM_1 during production of Iranian traditional kashk (ITK). This study designed to assess the effect of ITK processing on AFM_1 content of cow's milk artificially contaminated with AFM_1.
Methods: ITK making consisted of production of yogurt and strained yogurt, boiling the strained yogurt to make a curd and drying the resultant curd, according to common native procedure in Iran. AFM_1 content of initial yogurt, strained yogurt and dried kashk was determined using high performance liquid chromatography (HPLC). Statistical analyses were performed by Student's t-test and ANOVA using the SPSS 16.0 software package program.
Results: The mean concentration of AFM_1 in final ITK was determined as 0.118 µg/kg which was significantly (p<0.05) lower than that of initial milk, yogurt and strained yogurt samples. Also, AFM_1 content of yogurt, strained yogurt and final ITK product was 46.12%, 6.94% and 48.24%, respectively lower than that of initial milk (p<0.05).
Conclusion: This study showed that ITK processing can effectively degrade AFM_1 presented in initial raw milk and could be useful for minimizing AFM_1 content of highly contaminated raw milk in dairy industries.

Introduction

Aflatoxins consist of four teratogenic, mutagenic and carcinogenic toxins including B_1, B_2, G_1, and G_2, which are biosynthesized by toxicogenic strains of *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* in various food commodities (Eslami et al., 2015; Fallah, 2010a; Fallah, 2010b; Iha et al., 2013; Mason et al., 2015).

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A. flavus exclusively produces B aflatoxins, while the two others produce B and G types. Aflatoxin M_1 (AFM_1) is formed in liver and excreted into milk in the mammary glands of both human and lactating animals that have been fed with aflatoxin B_1 (AFB_1) contaminated diet (Creppy, 2002; FAO, 2001; Kamkar et al., 2008; Khodadadi et al., 2014). The rate of alteration of AFB_1 to AFM_1 are various from 0.5 to 5% for lactating animals (Neal et al., 1998). AFM_1 has been classified as group 1 of human carcinogens (IARC, 2002). Among consumers, infants and young children use high amount of dairy products and therefore, effect of AFM_1 in these groups make great concern for public health. The accepted maximum level of AFM_1 content in milk and dairy products has been reported 0.05 µg/kg (EC, 2006b). There is some published data indicated high content of AFM_1 in milk and dairy products of Iran (Fallah, 2010a; Fallah, 2010b; Fallah et al., 2011; Ghazani, 2009; Heshmati, 2010; Heshmati and Milani, 2010; Iha et al., 2013). AFM_1 can be detected in dairy products if the initial milk is contaminated to this toxin (Bakirci, 2001). Unfortunately, a little reduction of AFM_1 occurs during normal heat processing and current food preservation methods (El Khoury et al., 2011; Fallah, 2010a; Iha et al., 2013).

South East Asia including, Iran, Iraq, Syria, and Turkey are important area for production and consumption of traditional fermented milk (Chandan, 2006). Due to nutritional benefits of yogurt, its consumption rate has been increased, recently (McKinley, 2005). Dried yogurt is normally obtained from dehydration of natural yogurt to keep the product stable and extend its shelf life. There are many kinds of dried yogurt and its derivatives worldwide. "Kashk" is local name of a popular and traditional dried yogurt in Iran. It is obtained from natural/plain yogurt when concentrated, boiled, shaped into flat rolls and dried. This product is very nutritious; it contains 95.6% total solid, 54.4%, protein 7.9% fat, and 29.5% lactose content (Tamime and Robinson, 2007).

Many researchers have attempted to study the effect of food processing on the residue of AFM_1 in milk and dairy products. To the best of our knowledge, there is lack of data about the variation of AFM_1 during processing of dried yogurt and its derivatives such as Iranian traditional kashk (ITK). Thus, the main aim of this study was to evaluate the stability of AFM_1 during the production and processing of ITK using high performance liquid chromatography (HPLC) assay.

Materials and methods

AFM_1 standard preparation

AFM_1 standard was purchased from Sigma Chemical Co., St Louis, MO. Working solution of AFM_1 was achieved by suitable dilution in acetonitrile. To obtain final concentration of 0.1 to 10 ng/ml, different portions of the standard solutions were evaporated, and then diluted with mobile phase.

Sample preparation and extraction

The samples were shaken for about 2 min to obtain homogeneous mixtures. Briefly, 10 g kashk and yogurt samples, 10 g celite (Sigma–Aldrich, 5876), and 80 ml dichloromethane (HPLC grade, Merck) were blended using Ultra Turrax (Junke and Kunkel, GmbH, Germany) at 24000 rpm for 3 min in order to form slurry. The slurry was filtered with Whatman no. 1 filter paper. Collected filtrate was evaporated to dryness under vacuum at 35–40 ˚C using laboratory type evaporator (Heidolph Rotary Evaporator VV–2000, Germany). The obtained residue was dissolved in a mixture (30:50:20 v/v/v) of methanol (HPLC grade, Merck), water and hexane (extra pure, Merck). The aqueous phase was separated using a separator funnel. An aliquot (35 ml) of the filtrate from each sample was passed through an immunoaffinity column (Aflaprep M, R-Biopharm Rhone Ltd., Glasgow, Scotland, UK) containing monoclonal antibodies specific to AFM_1 (Montaseri et al., 2014).

Immunoaffinity column purification and isolation

Each test solution obtained from former step was passed through an immunoaffinity column, at a slow steady volume having flow rate of 1 ml/min. Then, column was secured on a vacuum manifold conditioned with 5 ml of phosphate-buffered saline. After double washing of the column with 10 ml of ultrapure water, AFM_1 was removed from the column with acetonitrile. The eluate was then evaporated under nitrogen flow (Dubnof Bath BSD/D). The residue was dissolved again in mobile phase, collected in HPLC vials (Supleco, Bellefonte, PA, USA), and finally injected to HPLC system (Montaseri et al., 2014).

HPLC analysis

Setup of HPLC was carried out according to the published procedure by Montaseri et al. (2014). Briefly, Column (Reverse phase ODS_2–5 µm, 250 m×4.6 m C18 Column TSK–GEL® TosoHas), Guard Column (Guard Column NovaPak® C18 Waters), and Mobile phase (acetonitrile: methanol: H_2O; 20:20:60) were used. The flow rate of 1 ml/min, and injection volume of 20 µl were adjusted. Fluorescence detector was waters 2475 fluorescence detector, excitation 360 nm, emission 440 nm, and Gain and emission units full scale (EUFS) were 10 and 1000, respectively. Retention time was considered 3.5 min. HPLC System was Waters Breeze 1525 HPLC Pump, Waters 1525 Binary HPLC Pump, Waters Column

Heater, Waters Bus SAT/IN, Waters Bus Lace, Waters Breeze Software.

Validation of HPLC analytical method

The HPLC assay was validated based on the selectivity, linearity, sensitivity, accuracy and precision. The selectivity of the assay was determined by evaluation of blank and spiked samples at different levels of 0.1–10 ng/mg. The linearity was analyzed by constructing 9-point calibration curve at content of 0.1 to 10 ng/mg. Each concentration was injected four times and then evaluated by linear regression analysis and stated as correlation coefficient (R^2). The precision of the assay was determined by limits of detection (LOD) and quantification (LOQ). The LOD, as the lowest content of AFM_1 which can be detected above the baseline, was determined by three fold analysis of spiked non-contaminated samples. LOQ, as the lowest content of analyzed sample, can be achieved with acceptable relative standard deviation (RSD) in within and between run procedure. To determine the accuracy (recovery), blank samples were spiked with suitable content of AFM_1 working standards to produce amounts of 0.02, 0.05 and 0.2 µg/kg. The recovery values were analyzed by the evaluation of three spiked samples with HPLC after extraction and immunoaffinity column clean-up mentioned previously (EC, 2006a; Montaseri et al., 2014).

Cow's milk artificially contaminated with AFM_1

Skimmed cow' s milk (dry matter, 9%) artificially contaminated with AFM_1 at level of 0.25 µg/l according to Montaseri et al. (2014) and heated to 42 ˚C. The AFM_1 content of the initial milk was under the limit of detection (0.01 ng/ml). A commercial starter culture (V1, direct vat set type, Chr. Hansen, Horlom, Denmark) with a mix of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (1:1) was used for yogurt making. According to manufacturer' s recommendation the lyophilized starter culture was activated in sterilized skimmed milk and 4 ml of activated culture added to 1 l AFM_1 contaminated milk. After aseptically distributing in sterile plastic bottles, the incubation step was started until the pH of the samples reaches to 3.90±0.02. At the end of the fermentation stage, each sample was quickly cooled in an ice bath and stored at 4 ˚C. Strained yogurt was produced from plain yogurt, for this purpose, yogurt was placed in cheesecloth and hung overnight at 4 ˚C.

Production of dried kashk from strained yogurt

According to ITK making method, the obtained strained yogurt was heated in a water bath at 95 ˚C for 4 h. After cooling, the resultant curd was divided into small rounded pieces of kashk and dried to reach a moisture content of about 10%. AFM_1 content of initial yogurt, strained yogurt and dried kashk was determined. All the experiments were carried out in triplicate.

Statistical analysis

Statistical analyses of the obtained data were performed by Student's t-test and ANOVA using the SPSS, Inc, Chicago, IL (version 16.0) software package program. Probability (p) values of less than 0.05 were considered as significant.

Results

In this study, selectivity of the method was confirmed using immunoaffinity column for clean up as well as a selective fluorescence detector. As shown in Fig. 1, no interfering peak was observed at the retention time of AFM_1 (3.5 min). LODs determined as the lowest concentration of AFM_1 that can be completely detected above the baseline signal and were 0.003 and 0.006 ng/g for yogurt and kashk samples, respectively. Also, LOQs were 0.01 and 0.019 ng/g for yogurt and kashk samples, respectively. Method validation data for AFM_1 determination in yogurt and kashk are indicated in Table 1.

As seen in Table 2, the mean concentration of AFM_1 in final ITK was determined as 0.118 µg/kg which was significantly (p<0.05) lower than that of initial milk, yogurt and strained yogurt samples. Also, AFM_1 content of yogurt, strained yogurt and final ITK product was 46.12%, 6.94% and 48.24%, respectively which were lower than that of initial milk (p<0.05).

Discussion

In this study, we found that there was a significant reduction in AFM_1 content during ITK production stages. Previously, Govaris et al. (2002) investigated the change of AFM_1 in yogurt samples that contaminated artificially with 0.05 and 0.1 µg/kg during 4 week storage at different pH levels of 4 and 4.6. They showed that stability of AFM_1 in test samples were pH dependent; in pH 4, the AFM_1 level was decreased significantly unlike pH 4.6; and at the end of storage time, AFM_1 showed significant decrease at both concentration levels. In contrast, Iha et al. (2013) reported that yogurt storage up to 28 days did not change the level of AFM_1. It has been shown that factors such as low pH and fermentation of organic acids or other fermentation by-products may change the AFM_1 content in yogurt (Govaris et al., 2002). During yogurt production, decrease in pH level resulting from fermentation, changes the proteins structures e.g. casein. This phenomenon consequently resulted in the association of

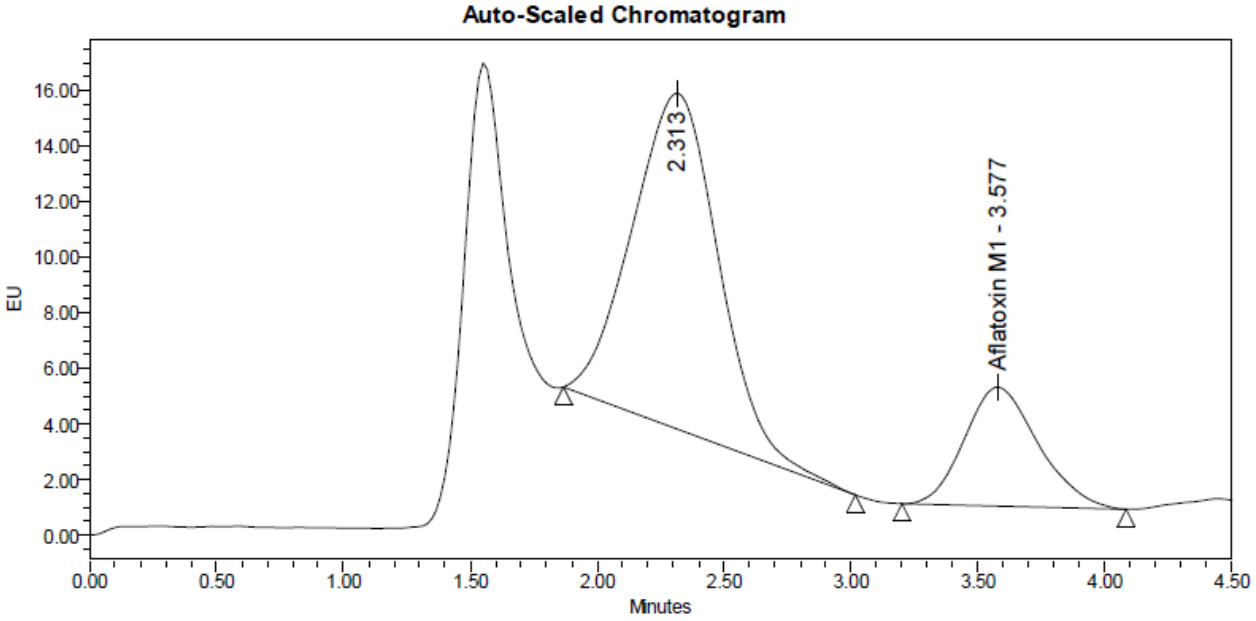

Fig. 1: Chromatogram for sample of milk artificially contaminated with 0.1 (µg/l) AFM_1

Table 1: Method validation data for AFM_1 determination in yogurt and kashk

Matrix	Spiking level (µg/kg)	AFM_1 found (ng/kg)	SD (µg/kg)	Recovery (%)	RSD (%)
yogurt	0.05	48	0.005	96	6.5
	0.2	185	0.004	92.5	6.3
	0.5	470	0.005	94	6.7
kashk	0.05	38	0.008	76	9.1
	0.2	150	0.010	75	8.7
	0.5	365	0.011	73	8.9

Table 2: Changes in AFM_1 concentration during production of Iranian traditional kashk

Sample	Concentration of AFM_1 µg/Kg (mean±SD)	AFM_1 variation (%)	
		In comparison to initial spiked milk	In comparison to previous step
artificially contaminated milk	0.245±0.005		
yogurt	0.132±0.017	-46.12	
strained yogurt	0.228±0.006	-6.94	+72.72
dried kashk	0.118±0.006	-48.24	-51.84

AFM_1 or other toxins with casein (Brackett and Marth, 1982). As illustrated in Table 2, the concentration of AFM_1 in strained yogurt was significantly higher than yogurt sample. In agreement to this result, Govaris et al. (2002) showed that the concentration of AFM_1 in strained yogurt was higher than its correspondence plain yogurt. This could be explained by the effect of increasing the concentration of total solids of yogurt when removing its water content.

The mean concentration of AFM_1 in ITK (0.118 µg/kg) obtained in the present work was significantly lower than milk, yogurt and strained yogurt samples. Previous studies showed that the concentration of AFM_1 was increased after physical water removing from dairy product such as cheese (Kamkar et al., 2008) and strained yogurt (Govaris et al., 2001). On the other hand, heat treatment of milk and dairy product had no remarkable effect on the concentration of AFM_1 indicated by Bakirci (2001) and Govaris et al. (2002). Contrary to mentioned finding, the obtained result of present investigation showed that the concentration of AFM_1 was significantly decreased after transforming the milk in comparison to ITK. It may be explained by the synergistic effect of the combination of heat treatment and low pH during ITK making process

that lead to strongly degradation of AFM_1. Montaseri et al. (2014) stated that processing and storage of probiotic yogurt may decrease the AFM_1 content of initial milk. However, probability of AFM_1 reduction during production and processing of probiotic ITK is not still reported in scientific databases.

Conclusion

AFM_1 is a hazardous component that can seriously threaten the public health by dairy consumption. It is necessary to search methods capable of removing or inactivation AFM_1 in dairy products. The results of this study showed that ITK processing can effectively degrade AFM_1 presented in initial raw milk and could be useful for minimizing AFM_1 concentration of highly contaminated raw milk in dairy industries. More studies are needed to assess the effects of the other aspects of ITK processing on AFM_1 stability.

Conflicts of interest

All the authors of this article state that they had no conflicts of interest.

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