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A study on the aflatoxin M1 rate and seasonal variation in pasteurized cow milk from northwestern Iran

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Abstract Present study aims to assess aflatoxin M1 (AFM1) contamination in 100 samples of pasteurized milk which were conventionally gathered during spring, summer, autumn, and winter from supermarkets located in Maragheh city of northwestern Iran. Samples were evaluated for AFM1 with a high-performance liquid chromatography (HPLC) method and with fluorimetric detection. The results showed that approximately 44% (11.25) of samples in winter, 32% (8.25) of samples in spring, 24% (6.25) of samples in summer, and 20% (5.25) of samples in autumn had AFM1 concentrations that exceeded the limit (0.05 μg/l) set by the European, Codex Alimentarius Commission and Iran standards. According to the statistical analysis of the data, there was no significant variation between the mean content of AFM1 during different seasons ($P = 0.076$). The results of our study suggest a high level of contamination of AFM1 in pasteurized milk in all seasons which may be due to the fact that milk supply for dairy factories is provided from dairy farms that are low in livestock feed quality. In Iran, pasteurized milk is consumed more than other milk products by all age groups. The total daily aflatoxin intake from contaminated milk and possibly other food products will be a significant risk to public health.

Keywords Aflatoxins · Milk · Dairy products · HPLC-FD · Seasonal variation

Introduction

Milk and milk products are generally considered of particular importance in feeding all human age groups, in all parts of the world and in all social, cultural, and economic classes. Therefore, the absence of infectious

Highlights • Analysis of aflatoxin M1 in samples of cow pasteurized milk from was carried out.0
•Cow milk samples exceeded the maximum limit fixed by the European Union standard.
•The pasteurized milk samples in all seasons indicated a high hazard for AFM1 contamination.

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and poisonous agents in milk is very important for society (Pereira 2014). Mycotoxins are chemical hazards produced by molds which can be transferred from contaminated feed to the milk. Aflatoxins are considered the most important mycotoxins in milk and they are the only mycotoxins for which maximum limits have been established in dairy products (Regulation (EC) 1881/2006) (Asselt et al. 2017). This aflatoxin is a threat to human health and can cause serious complications such as hepatotoxicity, teratogenicity, and immunotoxicity (Kumar et al. 2017). Aflatoxins are produced by certain species of Aspergillus species such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomosis*. *Aspergillus flavus* only produces aflatoxin B, while two other species produce B and G aflatoxins. Aflatoxin M1 (AFM1) and M2 (AFM2) are respectively hydroxylated metabolites of aflatoxins B1 (AFB1) and B2 (AFB2). The molds grow on different kinds of animal feeds such as maize, sorghum, barley, wheat, rice, and other legumes especially in humid climatic conditions (Akande et al. 2006). They produce AFB, which is consumed by livestock and then metabolized to AFM1 and subsequently appears in the milk (Davis et al. 1966; Tajkarimi et al. 2007; Tajkarimi et al. 2008; Akande et al. 2006).

AFM1 is resistant to usual milk processing methods such as pasteurization and sterilization or other industrial processes, and if this toxin exists inside raw milk, it cannot be inactive in final products (D. L. Park 2002; Van Egmond et al. 1977; Bullerman and Bianchini 2007). AFM1 is a highly toxic compound, immunosuppressive, mutagenic, and carcinogenic. Furthermore, human and animal food contamination is also attributed to AFM1. It has been classified as the human carcinogen in group 1. In this regard, epidemiological studies on malignant liver cancer in humans show that AFM1 has a synergistic activity with the hepatitis B virus (De Roma et al. 2017; Tajkarimi et al. 2008). If the level of AFM1 in raw milk is less than 0.3 μg/l, it will be acceptable. In Iran, the AFM1 limit is modeled on the European Union standard and is 0.05 μg/l (Bahrami et al. 2016; Ghazani 2009).

The sources of livestock feedstuffs, financial aspects on the farm, farm controlling and management, ecological factors, and especially weather parameters including temperature and humidity are the factors affecting rates of AFM1 contamination in dairy products and especially in milk (raw, pasteurized, and sterilized) (D. Park et al. 1999; Tajkarimi et al. 2008; Kabak et al. 2006).

The cities of northwestern Iran, especially the cities of East Azerbaijan province, are the main areas of livestock farming, milk production, and milk factories in Iran. Livestock products are distributed from here throughout Iran or are exported overseas (Ghazani 2009). One of the most important livestock farming areas in this zone is represented by the southern cities of the province and in particular Maragheh and its surrounding villages, which are located on the slopes of Sahand Mountain. Table 1 shows the climate properties of Maragheh which may favor the contamination of livestock feed via the *Aspergillus* fungi.

In this investigation, we report the data of monitoring the AFM1 contamination in pasteurized cow milk from this area throughout diverse seasons in 2017. To detect AFM1 in milk samples, analyses were carried out via high-performance liquid chromatography (HPLC) equipped with a fluorimetric detector.

Materials and methods

Sampling

During March 2017 until February 2018 (in 12 months and 4 seasons), pasteurized milk samples (1000 ml each) were conventionally gathered from supermarkets located in Maragheh of northwestern Iran (totally 100 samples: 25 samples in Spring, 25 samples in Summer, 25 samples in autumn, 25 samples in winter). All samples were chilled on ice (4 °C) and sent to the laboratory where they were stored at − 20 °C until HPLC analysis.

Table 1 Climate properties (min–max) of the Maragheh City of Iran and percentage of pasteurized milk samples had AFM1 concentrations that exceeded standard limit (0.05 μg/l)

Season	Precipitation (mm)	Mean relative humidity (%)	Mean temperature (°C)	% of milk samples higher than 0.05 μg/l
Winter	0.0–0.3	40–76	− 2–7	44.0
Spring	0.0–2.1	25–96	− 2–27	32.0
Summer	0.0–0.7	11–55	19–35	24.0
Autumn	0.0–0.0	19–60	5–32	20.0

Source of climate properties: https://www.worldweatheronline.com/maragheh-weather-history/east-azarbaijan/ir.aspx

Chemical substances and reagents

The solvents acetonitrile and methanol (liquid chromatography grade) were purchased from Merck (Darmstadt, Germany). Purified water (deionized) was obtained by a Milli-Q water system (Milli-Q Millipore 18.2 MΩ/cm resistivity). Other chemicals included standard solution of AFM1 (10 μg/ml, in acetonitrile; R-Biopharm Rhone Ltd., Scotland) and AFM1 immunoaffinity column (VICAM, Watertown, MA, USA). The AFM1 working standard solution was separately made via diluting of an intermediary standard solution in HPLC mobile phase.

High-performance liquid chromatography technique for analysis of AFM1

Milk samples (100 ml) were warmed up to 37 °C in a bain-marie and then were centrifuged at 4000 rpm for 15 min. Upon centrifugation, the upper fatty layer was discarded and the residual aqueous segment was passed over filter paper Whatman # 4 which was purchased from Fisher Scientific (Fair Lawn, NJ). The filtrated quota was then loaded onto the immunoaffinity column (Polar-RP) at a rate of 2 ml/min. The column was rinsed with distilled water, and AFM1 was eluted via 2 ml methanol and acetonitrile (60:40, *v*/*v*) at a flow rate of approximately 2 drops/s. The extract was vaporized to desiccation underneath a gentle stream of nitrogen at 42 °C. The remainder was dissolved in 1 ml of mobile phase and filtered through a 0.45-μm nozzle filter previous to HPLC system analysis. HPLC system (Agilent 1100 chromatograph; Agilent Corporation, Santa Clara, USA) equipped with a vacuum degasser, quaternary pump, and a fluorescence detector was applied for quantification of AFM1. The chromatographic parting was performed on a Discovery® C18 HPLC column (250 mm × 4.6 mm × 5 μm) guarded with a Discovery® C18 Supelguard column (2 mm × 4.6 mm × 5 μm) both from Supelco (Bellefonte, USA). The mobile phase was methanol/acetonitrile/water (20:20:60) in isocratic type at a flow rate of 1.0 ml/min with the column oven set at 25 °C. The injection volume was 100 μl. FLD was controlled at wavelengths of 360 and 440 nm for excitation and emission, respectively (De Roma et al. 2017; Bahrami et al. 2016).

Statistical analysis

A Kolmogorov-Smirnov test was carried out to check normality of AFM1 level data. The distribution of data was not normal according to the test; therefore, the mean of AFM1level across different seasons was compared by the Kruskal-Wallis nonparametric test. Data analysis was performed using IBM-SPSS (Version 21) and values of $P < 0.05$ were considered statistically significant. The graph (Fig. 1) was plotted by GraphPad Prism software (Version 7.01). The confidence interval (CI) of the estimates of proportions was calculated by the modified Wald method using GraphPad software.

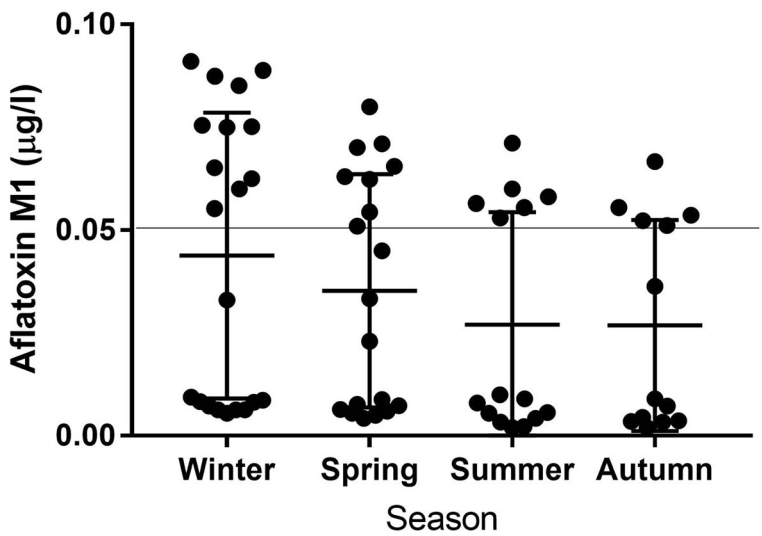

Fig. 1 The scatter plot of the distribution of AFM1 level (μg/l) in samples from different seasons. The error bars represent the standard deviation, and the horizontal line shows the standard limit for AFM1 (0.05 μg/l)

Results and discussion

In the HPLC test, determination of AFM1 concentration in the pasteurized milk samples was performed via an external standard calibration curve. The attained recovery rates of the HPLC process were according to the suggested recovery of mycotoxins in foodstuffs. The LOD and LOQ of the HPLC technique were found to be 0.0001 and 0.0005 μg/l, respectively (LOD, limit of detection—the smallest quantity or concentration of analyte in the test sample that can be reliably discriminated from zero; LOQ, limit of quantitation—the lowest concentration of analyte that can be determined with an acceptable repeatability and trueness). The calibration curve of AFM1 was linear, and the value of r^2 was upper than 0.999 (EC 2006, 2014).

Effects of seasonal variation on the AFM1 concentration of pasteurized milk

The level of AFM1 in pasteurized milk in different seasons is indicated in Table 2. According to the EU, Codex Alimentarius Commission and Iran standard (maximum allowed rate for AFM1 is 0.05 μg/l), approximately 44% (11.25) of samples in winter, 32% (8.25) of samples in spring, 24% (6.25) of samples in summer, and 20% (5.25) of samples in autumn had AFM1 concentrations that exceeded this limit (see Fig. 1 and Table 2).

Although aflatoxin levels were observed from highest to lowest in winter, spring, summer, and autumn, respectively, the statistical analysis of the data indicated no significant alteration between the mean content of AFM1 during different seasons ($P = 0.076$). This indicates that the amount of AFM1 contamination in all seasons is high and the confidence interval overlaps in all seasons, as shown in Table 2. In a similar study, Tajkarimi et al. (2007) surveyed seasonal incidence of AFM1 contamination in milk in five cities (Gorgan, Hamedan, Rasht, Shiraz, and Tehran) in Iran. Seasonal evaluation of the results did not show significant seasonal differences in contamination level (Tajkarimi et al. 2007).

Nevertheless, various investigations show a significant alteration among different seasons and it is probably due to different feeding supply strategies. In spring and summer, fresh forages are available; however, in autumn and winter, the feeding is mainly relied on the silo and processed concentrates. These feeding supplies are stored for a while before consumption, where molds can have the opportunity to grow and produce mycotoxins. Storing under humid, hot, and air full of dust situations can lead to accumulation of mycotoxins in feeds. According to the results of some similar studies, the incidence of AFM1 contamination was higher in the cold season, and this fact can be caused based on lactating livestock feeding mainly by deposited silage and grains in winter (Tajkarimi et al. 2008; De Roma et al. 2017; Bahrami et al. 2016).

In this study, the reason for the high levels of contamination in all seasons of the year may be due to different natural, economic, and political conditions. In recent decades, Iran has encountered climate alterations such as an increase of the minimum temperature and the decreased annual precipitation (Alizadeh-Choobari and Najafi 2018). In such a situation, the need for stored

Table 2 Aflatoxin M1 in pasteurized milk determined by HPLC by season

Season	Sample tested, *n*	Positive samples *n* (%), CI 95%[a]	Positive samples		Exceed regulation, *n* (%)[c], CI 95%[a]
			Mean±SEM[b] (μg/l)	Min–max (μg/l)	
Winter	25	21 (84.0), 65–94	0.04 ± 0.03	0.005–0.09	11 (44.0), 27–63
Spring	25	19 (76.0), 56–89	0.03 ± 0.03	0.004–0.08	8 (32.0), 17–52
Summer	25	15 (60.0), 39–79	0.03 ± 0.03	0.002–0.07	6 (24.0), 11–44
Autumn	25	13 (52.0), 33–70	0.03 ± 0.03	0.002–0.07	5 (20.0), 8–40

[a] Computed by modified Wald method using GraphPad software

[b] There was no statistically significant difference between seasons ($P = 0.076$)

[c] The standard limit for aflatoxin M1 is 0.05 μg/l

feeding is not restricted to a special season anymore, because fresh forage may not be available even in spring. Iran has also been under severe economic sanctions from last decade which have reduced the exchange value of Iranian currency to a significant level (Reza and Robert 2018). Most of the ingredients of feeds are imported from other countries; therefore, farmers are not able to provide high-quality feeds for their domestic animals and the low-quality cereals and silage and even the bread waste (mostly moldy) are fed to livestock. After all, the policies for controlling the level of aflatoxins such as adequate drying, insect activity elimination, and separation of contaminated grains are not followed in Iran. In rural areas, farmers grow and store the feeds by themselves and most feeds are stored in small and traditional granaries and there is a low investment in contamination control (Alizadeh-Choobari and Najafi 2018). In Iran, the collected milk is not tested for mycotoxins; therefore, it is not possible to recognize the contamination source and enforce the controlling policies at the farm level.

Although differences in temperature and humidity are important and effective in different seasons especially in winter, it seems that the quality of the raw material used for the livestock's feed formulation is more important. Another cause can be the unsuitable and bad conditions for stocking livestock's feed in the farm. In other words, high quality and ensuring food safety of milk manufactured goods can verify the low existence of aflatoxins in milk.

According to results acquired in different cities of Iran, occurrence and contamination rates of AFM1 in different types of milk appear to be a severe problem (Bahrami et al. 2016; Tajkarimi et al. 2007; Tajkarimi et al. 2008; Ghazani 2009; Fallah 2010a, 2010b; Fallah et al. 2011). In Iran, pasteurized milk is consumed more than other milk products by all age groups (Ghazani 2009). The total daily aflatoxin intake from milk will be a significant risk factor for public health.

Conclusions

This study presents the results on the AFM1 incidence determination in pasteurized milk gathered in Maragheh, Iran, in different seasons during March 2017 until February 2018. This study disclosed that the pasteurized milk samples in all seasons indicated a high hazard for AFM1 contamination. It is needed that the ranchers and dairy farmers be trained and notified thru health and management organizations such as the Ministry of Health and the Veterinary Organization on potential health concerns of aflatoxins. It is also important for local authorities to set specific testing standards and enforcement for raw milk collection and before marketing the pasteurized milk.

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References

Akande, K., Abubakar, M., Adegbola, T., & Bogoro, S. (2006). Nutritional and health implications of mycotoxins in animal feeds: a review. *Pakistan Journal of Nutrition, 5*(5), 398–403.

Alizadeh-Choobari, O., & Najafi, M. (2018). Extreme weather events in Iran under a changing climate. *Climate Dynamics, 50*(1–2), 249–260.

Asselt, E., der Fels-Klerx, H., Marvin, H., Bokhorst-van de Veen, H., & Groot, M. N. (2017). Overview of food safety hazards in the European dairy supply chain. *Comprehensive Reviews in Food Science and Food Safety, 16*(1), 59–75.

Bahrami, R., Shahbazi, Y., & Nikousefat, Z. (2016). Aflatoxin M1 in milk and traditional dairy products from west part of Iran: occurrence and seasonal variation with an emphasis on risk assessment of human exposure. *Food Control, 62*, 250–256.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology, 119*(1–2), 140–146.

Davis, N. D., Diener, U., & Eldridge, D. (1966). Production of aflatoxins B1 and G1 by Aspergillus flavus in a semisynthetic medium. *Applied Microbiology, 14*(3), 378–380.

De Roma, A., Rossini, C., Ritieni, A., Gallo, P., & Esposito, M. (2017). A survey on the aflatoxin M1 occurrence and seasonal variation in buffalo and cow milk from Southern Italy. *Food Control, 81*, 30–33.

EC (2006). Commission Regulation (EC) No 1881/2006 of December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). Official Journal of the European Union L 364/5.

EC (2014). Commission Regulation (EC) No 519/2014 of 16 May 2014 amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis Text with EEA relevance. Official Journal of the European Union L 147/29.

Fallah, A. A. (2010a). Aflatoxin M1 contamination in dairy products marketed in Iran during winter and summer. *Food Control, 21*(11), 1478–1481.

Fallah, A. A. (2010b). Assessment of aflatoxin M1 contamination in pasteurized and UHT milk marketed in central part of Iran. *Food and Chemical Toxicology, 48*(3), 988–991.

Fallah, A. A., Rahnama, M., Jafari, T., & Saei-Dehkordi, S. S. (2011). Seasonal variation of aflatoxin M1 contamination in industrial and traditional Iranian dairy products. *Food Control, 22*(10), 1653–1656.

Ghazani, M. H. M. (2009). Aflatoxin M1 contamination in pasteurized milk in Tabriz (northwest of Iran). *Food and Chemical Toxicology, 47*(7), 1624–1625.

Kabak, B., Dobson, A. D., & Var, I. l. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical Reviews in Food Science and Nutrition, 46*(8), 593–619.

Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., & Kang, S. G. (2017). Aflatoxins: a global concern for food safety, human health and their management. *Frontiers in Microbiology, 7*(2170). https://doi.org/10.3389/fmicb.2016.02170.

Park, D., Joint, F., Njapau, H., & Boutrif, E. (1999). Minimising risks posed by mycotoxins utilising the HACCP concept. http://agris.fao.org/agris-search/search.do?recordID=XF2000390599. Accessed 15 April 2018.

Park, D. L. (2002). Effect of processing on aflatoxin. *Advances in Experimental Medicine and Biology, 504*, 173–179.

Pereira, P. C. (2014). Milk nutritional composition and its role in human health. *Nutrition, 30*(6), 619–627.

Reza, G. M., & Robert, W. (2018). A computable general equilibrium model of international sanctions in Iran. *The World Economy, 41*(1), 287–307. https://doi.org/10.1111/twec.12528.

Tajkarimi, M., Aliabadi-Sh, F., Nejad, A. S., Poursoltani, H., Motallebi, A., & Mahdavi, H. (2008). Aflatoxin M1 contamination in winter and summer milk in 14 states in Iran. *Food Control, 19*(11), 1033–1036.

Tajkarimi, M., Aliabadi, F. S., Nejad, M. S., Pursoltani, H., Motallebi, A., & Mahdavi, H. (2007). Seasonal study of aflatoxin M1 contamination in milk in five regions in Iran. *International Journal of Food Microbiology, 116*(3), 346–349.

Van Egmond, H., Paulsch, W., Veringa, H., Schuller, P., Sizoo, E., Wilhelmina, E., et al. (1977). The effect of processing on the aflatoxin M1 content of milk and milk products. Archives de l'Institut Pasteur de Tunis. http://agris.fao.org/agris-search/search.do?recordID=TN19790418127. Accessed 1 March 2018.