



# Contamination profile of aflatoxin M1 residues in milk supply chain of Sindh, Pakistan



Sana Jawaid, Farah N. Talpur<sup>\*,1</sup>, Shafi M. Nizamani, Hassan Imran Afridi

National Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro 76080, Pakistan

## ARTICLE INFO

### Article history:

Received 2 July 2015

Received in revised form 18 October 2015

Accepted 18 October 2015

Available online 21 October 2015

### Keywords:

AFM1

Branded and non-branded milk

ELISA

Milk chain

Buffaloes

## ABSTRACT

Aflatoxin M1 (AFM1) is a potent carcinogen, teratogen and mutagen found in the milk when lactating animals consume feed contaminated with aflatoxin B<sub>1</sub> (AFB<sub>1</sub>). In the present study, the contamination of AFM1 was evaluated in the milk supply chain of the province of Sindh, Pakistan. For the broader profiling of targeted toxin, enzyme-linked immunosorbent assay (ELISA) was used for the determination of AFM1 in both branded and non-branded milk samples. The results showed that 96.43% of samples (81 out of 84) were contaminated with AFM1 in the range of 0.01–0.76 µg/L. The average contamination level was 0.38 µg/L. The determined values of AFM1 in the collected milk samples were above the standard limit of the European Commission while 70% of the samples exceeded levels established by United States regulations. According to these results, the estimated daily intake of AFM1 for adults was determined as 3.1 ng/kg of body weight per day.

© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Mycotoxins are secondary metabolites produced by fungal activity. Owing to the humidity and temperature conditions, mold and toxins can dominate growth in tropical and sub-tropical regions as compared to other dry regions of the world. [27]. Due to this fact, such toxins are found in various agricultural-based products like maize, rice, wheat, soybean, barley and corn. Apart from this, the fungal growth remains active during the entire process of production, which includes harvesting, collecting, transporting and processing [1,19]. Mycotoxins are part of a large family, comprising major groups including aflatoxins, ochratoxins, fumonisins, patulin, trichothecenes and zearalenon [17]. *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins (AFs), whose sub-types AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub> are categorized as acute and chronic toxins for animals and humans [6]. Considering its adverse effects, animals are severely exposed to AFB<sub>1</sub> as compare to humans.

When animals consume feed contaminated with AFB<sub>1</sub>, it is bio-transformed to AFM1 by the hepatic microsomal mixed-function oxidase system and gets absorbed in the milk of mammals [11,23]. The residues of AFM1 are stable enough to survive in raw and processed milk, hence they are known as milk toxins [24]. The amount

of AFM1 in milk varies from day to day milking of individual animals and can reach up to 6.2% in cows with high milk yields [3]. Humans become exposed to aflatoxins through the intake of contaminated dairy milk and its products. In 1993, the International Agency for Research on Cancer (IARC) identified AFB<sub>1</sub> as the most acute and highly toxic compound and a category A carcinogen [14]. The carcinogenicity of AFM1 is nearly 2–10% higher than the original form AFB<sub>1</sub>. Moreover, AFM1 together with aflatoxins B<sub>2</sub> and G<sub>1</sub> can cause DNA damage, gene mutations, chromosomal anomalies, immunosuppression and cell transformation in humans [16,34]. They have been re-classified as Group 1 carcinogens after additional studies [15].

AFM1 may be degraded during the processes of pasteurization or ultra-high temperature (UHT) treatments. Thus, it becomes important to provide effective control of raw milk and processed milk in accordance with the maximum residue levels (MRLs) set by the food regulation authorities. The maximum European Commission (EC) [8] limit of residue is 50 ng/kg for AFM1 concentration in dairy milk and 25 ng/kg for milk-based baby foods [8]. MRL levels in other countries like Syria, the US, China, and Brazil are higher (e.g., 200 ng/L in Syria [9]) while they are 500 ng/L in the US [10], China [32] and Brazil [29]. From the global perspective of toxicity profiling, the accurate detection of aflatoxins in milk is a mandatory task for regulatory and health purposes.

Pakistan is the fourth largest milk-producing country in the world. Its annual production of milk is 33 billion liters. Its milk supply chain can be divided into formal and informal sectors, as

\* Corresponding author at: National Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro 76080, Pakistan. Fax: +92 22 9213431.

E-mail address: [farahhtalpur@hotmail.com](mailto:farahhtalpur@hotmail.com) (F.N. Talpur).

<sup>1</sup> Links: [www.ceacsu.edu.pk](http://www.ceacsu.edu.pk).

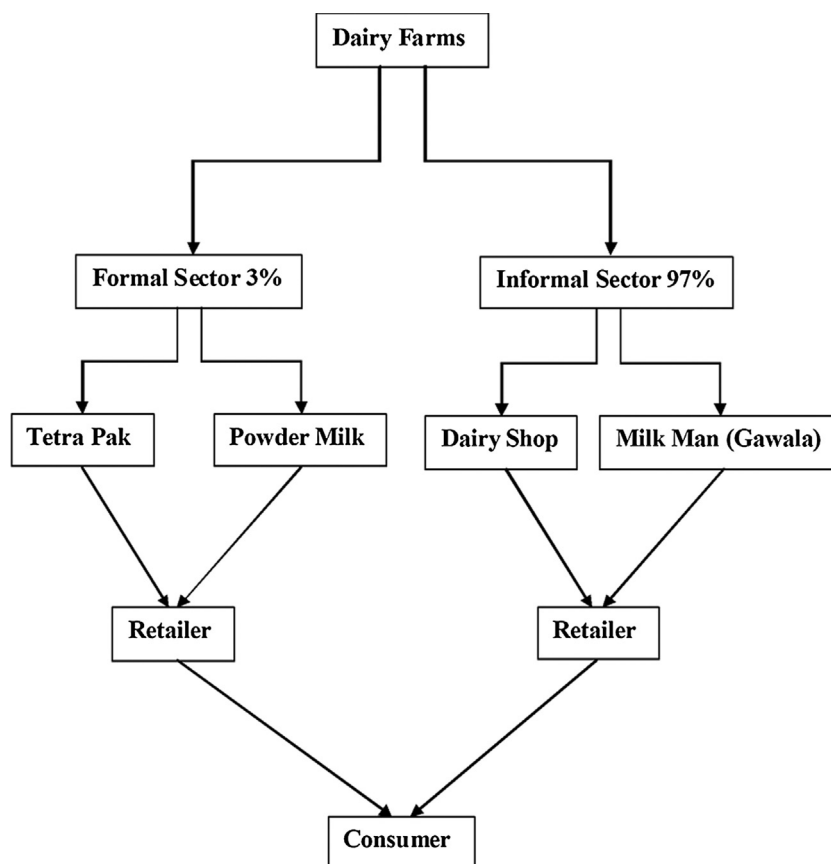


Fig. 1. Milk supply chain of formal and informal dairy sectors in Pakistan.

presented in Fig. 1. The vast majority of milk production (97%) falls in the category of the informal sector, which is sold as raw milk in urban and rural markets while only 3% of the milk falls in the formal sector, which is processed in dairy industries in the form of packaging [21].

The possible presence of toxins in milk may create health hazards for consumers. Therefore, the presence of such toxins has led us to monitor the level of contamination in the market milk. To estimate the health hazard risk for consumers, we have undertaken this study to evaluate the AFM1 contamination in processed and non-processed dairy milk samples collected from Sindh, Pakistan. Various analytical methods were used for the detection of AFM1 in milk, such as: high-performance liquid chromatography (HPLC), capillary electrophoresis, and ultra-performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS). However, in the present study, we used an ELISA method for the detection of AFM1 in milk samples to enable a faster, reliable and yet inexpensive determination of these toxic compounds.

## 2. Material and methods

### 2.1. Sample collection

Eighty-four milk samples were purchased randomly from the local area/market within Hyderabad region of the province of Sindh, Pakistan. The collected milk samples were classified into branded (tetra pack, powder milk) and non-branded (dairy farm, milk man (gawala), dairy shop) categories. The samples were properly labeled and transported to the laboratory in an insulated container and were stored at 4 °C before performing analyses.

### 2.2. Sample preparation

Milk samples were prepared for AFM1 measurement using an ELISA method. Liquid milk (20 mL) was centrifuged at 4000 rpm for 5 min. The upper fat layer was then discarded. The lower layer was diluted with 35% methanol in a ratio of 1:9 (20  $\mu$ L of milk + 180  $\mu$ L of 35% methanol). From the diluted sample, 50  $\mu$ L was taken per well for the analytical assay. For the powdered milk samples, 1 g of solid milk was dissolved in 9 mL of double-distilled water and further processed as described above.

### 2.3. Reagent preparation

Wash solution (supplied with kit) was prepared by mixing with distilled water (1:19) during washing processes.

### 2.4. ELISA analysis method

The quantitative ELISA kit MaxSignal Aflatoxin M1 (Bio scientific corporation, Austin, Texas, USA) was brought to normal room temperature before analysis. AFB1 standard solutions were used for the construction of calibration curves at concentrations of 0, 0.05, 0.1, 0.2, 0.4 and 0.8 ng/mL. Wells in the microtiter plate were coated with antibodies specific to AFM1. All test samples and AFM1 standards (50  $\mu$ L) were pipetted into each well in duplicate followed by addition (100  $\mu$ L each) of aflatoxin antibody. The plate was then incubated at room temperature for 30 min using a titer plate shaker at a speed of 100 rpm. Before the addition of 150  $\mu$ L of aflatoxin horseradish peroxidase (HRP) conjugate, the plate was washed 3 times with 250  $\mu$ L of wash solution by using Fluido 2 Microplate Washer (Biochrom Anthos, Cambridge, UK) and incubated again for 30 min at room temperature. The bound enzyme activity was

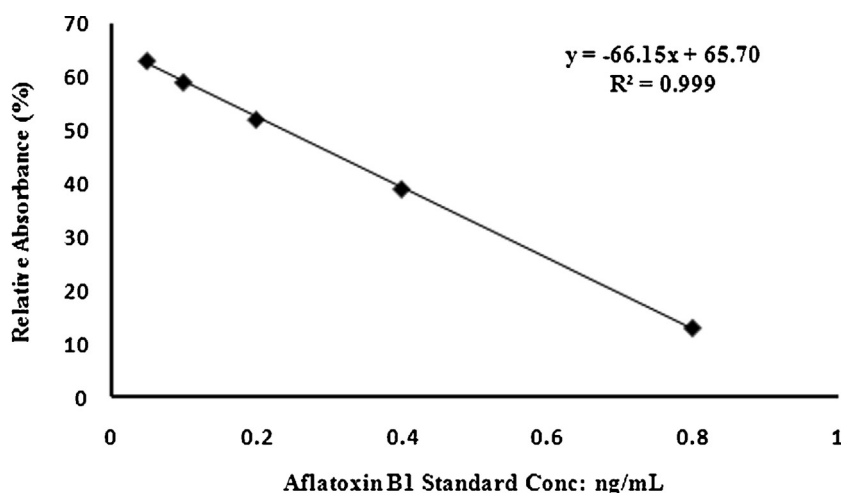


Fig. 2. Calibration curve of AFB1 by competitive ELISA.

determined by adding a chromogenic tetramethylbenzidine (TMB) substrate that converted the colorless chromogen into a blue color during incubation at room temperature for 15 min. After addition of stop buffer (100  $\mu$ L), the absorbance was measured at 450 nm in an Anthos 2010 Microplate Reader; the absorbance intensity was inversely proportional to AFM1 concentration in the samples.

### 3. Results and discussion

#### 3.1. Occurrence of AFM1

The concentration of aflatoxins in milk samples was determined from the standard calibration graph plotted in the range of 0 to 0.8  $\mu$ g/L using ELISA. Linear regression analysis was further used for the quantification of aflatoxin present within the milk samples. The standard calibration graph is presented in Fig. 2, and showed excellent linearity with  $R^2$  value of 0.999.

AFM1 was detected in 81 samples out of 84 (96.43%), with contamination range between 0.01–0.76  $\mu$ g/L (0.38  $\mu$ g/L average concentration) (Table 1). According to the EC, the MRL for AFM1 in milk is 0.05  $\mu$ g/L while in the US, the MRL for AFM1 is 0.5  $\mu$ g/L for milk and dairy products [8,10].

Out of a total of 84 milk samples, three were free from AFM1 contamination while 79 samples (94%) exceeded the established limit of the EC. Only two samples fell within the prescribed limit of the EC in our experiment. According to US regulations, 25 milk samples (29.76%) were above the acceptable limit whereas the remaining

59 (70.24%) contaminated samples were below the approved limit. Though US regulations prescribe 10-fold higher limits of AFM1 as compared to the EC, even then 29.76% of milk samples in the current study exceeded the maximum tolerance limit of US regulations. Hence, there is an utmost need to introduce safety measures for AFM1 in branded and non-branded milk samples in Sindh, Pakistan.

AFM1 concentrations ranged from 0.18 to 0.76, 0.19 to 0.70, 0.20 to 0.57, 0.01 to 0.53 and 0.30 to 0.53  $\mu$ g/L in dairy farm, dairy shop, milk man (gawala), UHT milk and powder milk samples, respectively. When the results were compared in terms of their categories, AFM1 contamination was relatively higher in non-branded milk samples as compared to the branded ones. In Pakistan, sellers mix buffalo milk with cow milk and sell it in open market. More than 60% of milk is produced by buffaloes and nearly 39% of milk is contributed by cows. Owing to high milk production from buffaloes, a large number of farmers prefer to invest money in purchasing more buffaloes than cows [26]. The buffaloes are kept in dairy farms, where farmers feed them manufactured food made of various stored grain products and by-products of the agricultural industry. Hence, the milk from these mammals is greatly exposed to such toxins. In contrast, the UHT plants have their own dairy farms and also buy milk from various resources such as small household farms (normally pasture-raised buffaloes) and other varieties of farms (pasture and grain feed). Hence, the possible cause of the low level of AFM1 in such samples is due to the mixing of a variety of milks obtained from different sources.

Table 1  
Aflatoxin M1 levels in branded and non-branded milk samples.

Concentration ( $\mu$ g/L)	Non-branded			Branded	
	Dairy farm (n=20)	Dairy shop (n=20)	Milk man (gawala) (n=20)	UHT milk (n=18)	Powder milk (n=6)
BDL <sup>a</sup>	–	–	–	3 (16.67%) <sup>b</sup>	–
0.00–0.05	–	–	–	2 (11.11%)	–
0.06–0.10	–	–	–	3 (16.67%)	–
0.11–0.20	1 (5%)	1 (5%)	1 (5%)	6 (33.33%)	–
0.21–0.30	5 (25%)	2 (10%)	3 (15%)	–	2 (33.33%)
0.31–0.40	4 (20%)	6 (30%)	6 (30%)	–	2 (33.33%)
0.41–0.50	2 (10%)	2 (10%)	5 (25%)	2 (11.11%)	1 (16.67%)
0.51–0.60	4 (20%)	5 (25%)	5 (25%)	2 (11.11%)	1 (16.67%)
0.61–0.70	2 (10%)	4 (20%)	–	–	–
0.71–0.80	2 (10%)	–	–	–	–

MRL European commission = 50 ng/kg = 0.05  $\mu$ g/L.

MRL USDA–500 ng/L = 0.5  $\mu$ g/L.

<sup>a</sup> BDL–Below detection limit.

<sup>b</sup> Number of +ve samples (percentage).

**Table 2**

Review of the incidence and range of AFM1 in raw milk, pasteurized and UHT milk in different countries reported in previous studies.

Country	Matrix	Number of samples	Average measured value ( $\mu\text{g/L}$ )	Positive samples	Percent	Reference
Brazil	Different type of milk	125	0.03	119	95.2	[29]
China	Raw milk	72	0.07	60	83.33	[32]
Iran	Raw milk	111	0.06	85	76.6	[20]
India	Infant milk products and liquid milk	87	0.30	76	87.0	[28]
Morocco	Pasteurized milk	44	0.02	39	88.8	[34]
Syrian	Raw milk	126	0.15	101	80.0	[11]
Sudan	Cattle milk	44	2.07	42	95.4	[7]
Serbia	Different type of milk	176	0.20	132	75.0	[23]
Turkey	UHT milk	40	0.03	8	20.0	[19]
Pakistan	Fresh milk	168	0.37	162	96.4	[13]
Pakistan	Milk and milk products	221	0.09	168	76.0	[16]
Pakistan	Branded and non branded milk	84	0.38	81	96.43	Current study

Moreover, contradictory data regarding reduction of AFM1 concentration with various heat treatments is also available in the literature. The reduction in AFM1 reported in these studies varies from 12% to 40%, while boiling, sterilization and pasteurization causes 14.50, 12.21 and 7.62% reduction of AFM1, respectively [2,4,5]. From the aforementioned reports, it was concluded that the degradation of AFM1 relied on the time and temperature of the treatment system. However, some reports have shown that aflatoxins are stable during heat treatments, such as pasteurization and sterilization [12,30,31,33]. Data comparing the AFM1 residues found in milk samples from several countries is presented in Table 2. As is evident from the data, Brazil and the Sudan have similar levels of AFM1 contamination as were found in this study [7,29].

A higher incidence of AFM1 (80%) in pasteurized milk and dairy products was also reported in India [28], Morocco [34], Syria [11] and China [32], whereas lower levels of AFM1 (20%) in UHT milk were reported from Turkey. Although there are no comparative data available for AFM1 contamination in branded and non-branded milk, the concentration of AFM1 determined in this study was supported by previous studies carried out in Pakistan [13]. Comparatively lower AFM1 levels were reported for Morocco, Brazil, China, Iran [20] and Turkey, while a higher contamination (2.07  $\mu\text{g/L}$ ) level was found in the Sudan. The same average extent of AFM1 contamination was also detected in Indian milk samples [28].

### 3.2. Estimated daily intake in adults

AFM1 estimated daily intake (ng/kg per body weight per day) was calculated based on the mean concentrations of the AFM1 detected in the present study and average milk intake (per day and mean body weight for adults). There is no information about the human exposure to AFM1 from milk consumption in Pakistan. However, the Joint Expert Committee on Food Additives [18] established the intake of AFM1 for Latin America as 0.058 ng/kg body weight per day, assuming a body weight of 60 kg and considering an estimate from data on the concentration of AFM1 in milk reported in the respective countries. According to our study, the intake of AFM1 through milk (branded and non-branded milk) consumption was 3.1 ng/kg body weight per day. This value is approximately 53 times higher than the intake calculated for Latin Americans. The wide variations in AFM1 levels among reported studies could be associated with forage and feed quality, dairy animal diet, and geographic and seasonal variations. In addition to this, differences in genetic variation and farm management practices can alter AFM1 levels. Contamination of feeding sources with AFB1 varies with location, because it is highly influenced by weather conditions during harvest and feed storage practices [25]. Identification and understanding factors for determining the presence of toxicants in

milk is important and may provide a strong basis for controlling the transfer of chemicals to humans through milk consumption.

## 4. Conclusion

Milk is an essential source of nutrition and thus its consumption is increasing parallel to the continuous increase of the human population. This study provides a complete profiling of AFM1 toxin present in branded and non-branded milk samples collected from the Sindh region of Pakistan. The study identifies non-branded milk samples as highly exposed to such toxins compared to branded samples. The presence of such toxins in milk is a serious problem considering the extraordinary usage of milk and milk associated products in Pakistan. Henceforth, this report provides a strong basis for food and health regulation authorities of Pakistan to take firm steps for constant monitoring and regulating of such toxins in milk. Strict permissible limits should be implemented to avoid fungal contamination. Awareness programs and education for the dairy farmers and milk processors may also be helpful in this regard.

## References

- [1] S. Alborzi, B. Pourabbas, M. Rashidi, B. Astaneh, Aflatoxin M1 contamination in pasteurized milk in Shiraz (south of Iran), *Food Control* 17 (7) (2006) 582–584.
- [2] I. Bakirci, A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey, *Food Control* 12 (2001) 45–51.
- [3] N. Bilandzic, D. Bozic, M. Dokic, M. Sedak, B.S. Kolanovic, I. Varenina, S. Tankovic, Z. Cvetnic, Seasonal effect on aflatoxin M1 contamination in raw and UHT milk from Croatia, *Food Control* 40 (0) (2014) 260–264.
- [4] P.L. Choudhary, R.S. Sharma, V.N. Borkartria, Effect of chilling and heating on aflatoxin M1 content of contaminated Indian cow's milk, *Egypt. J. Dairy Sci.* 26 (1998) 223–229.
- [5] O. Deveci, Changes in the concentration of aflatoxin M1 during manufacture and storage of white pickled cheese, *Food Control* 9 (2007) 1103–1107.
- [6] M. Edite Bezerra da Rocha, F.da.C.O. Freire, F. Erlan Feitosa Maia, M. Izabel Florindo Guedes, D. Rondina, Mycotoxins and their effects on human and animal health, *Food Control* 36 (1) (2014) 159–165.
- [7] A.O. Elzupir, A.M. Elhussien, Determination of aflatoxin M1 in dairy cattle milk in Khartoum State, Sudan, *Food Control* 21 (6) (2010) 945–946.
- [8] European Commission regulation (EC) no. 1881 (2006) of 19 December 2006, setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L364, 5–24.
- [9] FAO, Worldwide regulations for mycotoxins in food and feed in 2003, Food and Agriculture Organization of the United Nations, 2003.
- [10] FDA, Sec. 527.400 whole milk, low fat milk, skim milk e Aflatoxin M1 (CPG 7106.10) FDA/ORA Compliance Policy Guides, Food and Drug Administration, 2005.
- [11] I. Ghanem, M. Orfi, Aflatoxin M1 in raw, pasteurized and powdered milk available in the Syrian market, *Food Control* 20 (6) (2009) 603–605.
- [12] A. Govaris, V.K. Roussi, N.A. Botsoglou, Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese, *Food Addit. Contam.* 18 (5) (2001) 437–443.
- [13] I. Hussain, J. Anwar, A study on contamination of aflatoxin M1 in raw milk in the Punjab province of Pakistan, *Food Control* 19 (4) (2008) 393–395.
- [14] IARC (1993). IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, some naturally occurring substances: heterocyclic aromatic amines and mycotoxins. Lyon, 56.
- [15] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 83, Suppl. 7), International Agency for Research on Cancer, Lyon France, 2002.

- [16] S.Z. Iqbal, M.R. Asi, S. Jinap, Variation of aflatoxin M1 contamination in milk and milk products collected during winter and summer seasons, *Food Control* 34 (2) (2013) 714–718.
- [17] S.Z. Iqbal, S. Nisar, M.R. Asi, S. Jinap, Natural incidence of aflatoxins, ochratoxin A and zearalenone in chicken meat and eggs, *Food Control* 43 (0) (2014) 98–103.
- [18] JECFA, Fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives, Safety Evaluation of Certain Mycotoxins in Food; WHO Food Additives Series 47, FAO Food and Nutrition, WHO, Geneva, 2001, pp. 74.
- [19] B. Kabak, F. Ozbey, Aflatoxin M1 in UHT milk consumed in Turkey and first assessment of its bioaccessibility using an in vitro digestion model, *Food Control* 28 (2) (2012) 338–344.
- [20] A. Kamkar, A study on the occurrence of aflatoxin M1 in raw milk produced in Sarab city of Iran, *Food Control* 16 (7) (2005) 593–599.
- [21] A. Kamran, S. Rizvi, Reason and trends for using packaged milk in Pakistan: study of urban Pakistani consumers, in: J. Xu, M. Yasinzai, B. Lev (Eds.), *Proceedings of the Sixth International Conference on Management Science and Engineering Management*, 185, Springer, London, 2013, pp. 909–924.
- [23] J. Kos, J. Lević, O. Đuragić, B. Kokić, I. Miladinović, Occurrence and estimation of aflatoxin M1 exposure in milk in Serbia, *Food Control* 38 (0) (2014) 41–46.
- [24] H. Mohammadi, A review of aflatoxin M1, milk, and milk products, *Aflatoxins Biochem. Mol. Biol.* (2011) 397–414.
- [25] Muhammad Rafique Asi, Shahzad Zafar Iqbal, Agustín Ariño, Altaf Hussain, Effect of seasonal variations and lactation times on aflatoxin M1 contamination in milk of different species from Punjab, Pakistan, *Food Control* 25 (2012) 34–38.
- [26] T.N. Pasha, Comparison between bovine and buffalo milk yield in Pakistan, *Ital. J. Anim. Sci.* 6 (2) (2007) 58–66.
- [27] I.R. Pizzutti, A. de Kok, J. Scholten, L.W. Righi, C.D. Cardoso, G. Necchi Rohers, R.C. da Silva, Development, optimization and validation of a multimethod for the determination of 36 mycotoxins in wines by liquid chromatography-tandem mass spectrometry, *Talanta* 129 (0) (2014) 352–363.
- [28] S. Rastogi, P.D. Dwivedi, S.K. Khanna, M. Das, Detection of aflatoxin M1 contamination in milk and infant milk products from Indian markets by ELISA, *Food Control* 15 (4) (2004) 287–290.
- [29] L. Shundo, S.A. Navas, L.C.A. Lamardo, V. Ruvieri, M. Sabino, Estimate of aflatoxin M1 exposure in milk and occurrence in Brazil, *Food Control* 20 (7) (2009) 655–657.
- [30] H.P. Van Egmond, W.E. Paulsch, H.A. Veringa, P.E. Schuller, The effect of processing on the aflatoxin M1 content of milk and milk products, *Arch. Inst. Pasteur Tunis.* 54 (1977) 381–390.
- [31] D.W. Wiseman, R.S. Applebaum, R.E. Brackett, E.H. Marth, Distribution and resistance to pasteurisation of aflatoxin M1 in naturally contaminated whole milk, cream and skim milk, *J. Food Prot.* 46 (6) (1983) 530–532.
- [32] J.L. Xiong, Y.M. Wang, M.R. Ma, J.X. Liu, Seasonal variation of aflatoxin M1 in raw milk from the Yangtze River Delta region of China, *Food Control* 34 (2) (2013) 703–706.
- [33] A.E. Yousef, E.H. Marth, Stability and degradation of aflatoxin M1, in: H.P. Van Egmond (Ed.), *Mycotoxins in Dairy Products*, Elsevier, London, 1989, pp. 127–161.
- [34] A. Zinedine, L. González-Osnaya, J.M. Soriano, J.C. Moltó, L. Idrissi, J. Mañes, Presence of aflatoxin M1 in pasteurized milk from Morocco, *Int. J. Food Microbiol.* 114 (1) (2007) 25–29.