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A survey on the occurrence of aflatoxin M_1 in raw milk produced in Adana province of Turkey

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ABSTRACT

During 2012, a total of 176 samples of raw milk obtained from dairy plants of Adana province of Turkey were analysed for the presence of aflatoxin M_1 (AFM_1). Aflatoxin M_1 analysis was carried out by centrifugation, liquid–liquid extraction, immunoaffinity column clean-up and high performance liquid chromatography with fluorescence detection (HPLC-FD). The limits of detection (LOD) and quantification (LOQ) of the analytical method were 0.021 μg kg^{-1} and 0.025 μg kg^{-1}. Accuracy of the method obtained from bias ranged from 2.94 to 8.70. Aflatoxin M_1 was detected in 53 out of 176 samples analysed (30.1%). The ranges for positive samples were 0.042–0.552, 0.033–1.01, 0.047–0.150 and 0.025–0.102 μg kg^{-1} in autumn, winter, spring and summer seasons, respectively. Thirty samples of raw milk (17%) were above the legal limits of Turkey and EU regulations.

1. Introduction

Aflatoxin M_1 is the hydroxylated metabolite of AFB_1 and can be found especially in milk and in other dairy products when lactating animals are fed with contaminated feedstuffs (Prandini et al., 2009). The rate of carring over AFB_1 from feed to milk in dairy animals is influenced by various nutritional and physiological factors, including feeding regimens, rate of ingestion, rate of digestion, health of the animal, hepatic biotransformation capacity, and actual milk production (Duarte et al., 2013). The forming of AFM_1, occurs in liver and it is secreted into milk. There is a linear relationship between the amount of AFM_1 in milk and AFB_1 in feed consumed by the animals (Kamkar, 2005). If contaminated foodstuffs are used, AFM_1 will appear in the milk 2–3 days following ingestion. In the same way two-three days are the time necessary to reduce to zero the AFM_1 level in milk, when a diet without aflatoxins is fed (Prandini et al., 2009).

AFM_1 level in milk and dairy products may vary according to geographic location, development level of the country and climatic conditions. High temperatures and extreme weather events such as droughts and floods may influence milk production and its quality as a result of changes in the availability and quality of food and water provided to animals (Picinin et al., 2013).

Aflatoxin M1 has been classified as possibly carcinogen (group 2B), based on inadequate evidence for carcinogenicity in humans and sufficient evidence in humans (IARC, 1993). Milk and dairy products are a good source of many nutrients such as proteins and calcium. In addition, certain milk lipids such as butyric acid are known to present anticarcinogenic feauters (Tsakiris et al., 2013). Therefore, humans are potentially exposed to these metabolites and it is generally assumed that neither storage nor processing provides a reduction of AFM_1 content (Unusan, 2006). Presence of AFM_1 in commercially available milk and dairy products and the high intake of these products by human population, may have negative health implications for consumers, particularly for infants and children. For this reason, many countries regulated AFM_1 level in milk and dairy products and established measurement to control AFM_1 contamination (Kabak & Ozbey, 2012; Marnissi, Belkhou, Morgavi, Bennani, & Boudra, 2012). For example, The European Commission Regulation 165/2010 and Turkish Regulation set a maximum permissible limit of 0.05 μg kg^{-1} for AFM_1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products (European Commission, 2010; Turkish Food Codex, 2011a). This limit has been established following the ALARA (As Low As Reasonable Achievable) principle.

Contamination of raw milk with AFM_1 has been shown in several surveys conducted in Portugal (Martins, Guerra, & Bernardo, 2005; Martins & Martins, 2000), Spain (Velasco, Delso, & Escudero, 2003), Iran (Kamkar, 2005; Mohammadian, Khezri,

Abbreviations: AFM_1, aflatoxin M_1; HPLC-FD, high performance liquid chromatography-fluorescence detector; IAC, immunoaffinity column; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; EU, European Union; TFC, Turkish Food Codex.

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Ghasemipour, Mafakheri, & Langroudi, 2010; Rahimi, Bonyadian, Rafei, & Kazemeini, 2010), Brazil (Shundo & Sabino, 2006), France (Boudra, Barnouin, Dragacci, & Morgavi, 2007), Pakistan (Hussain & Anwar, 2008; Hussain, Anwar, Asi, Munawar, & Kashif, 2010; Iqbal & Asi, 2013), Indonesia (Nuryono et al., 2009), Syria (Ghanem & Orfi, 2009), Egypt (Motawee, Bauer, & McMahon, 2009), Serbia (Horvatovic, Juric, & Glamocic, 2009), Argentina (Alonso et al., 2010), Croatia (Bilandzic, Varenina, & Solomun, 2010), Lebanon (Assem, Mohamad, & Oula, 2011), Morocco (Marnissi et al., 2012), India (Siddappa, Nanjegowda, & Viswanath, 2012) and Turkey (Ertas, Gonulalan, Yildirim, & Karadal, 2011; Var & Kabak, 2009).

The aim of this study was to determine the presence of AFM_1 in raw milk collected from different seasons in Adana province of Turkey and estimate mean daily intake of AFM_1 through milk consumption for Turkish consumers. A total of 176 raw milk samples were collected from various dairy plants in 2012 and analysed for AFM_1 by immunoaffinity column (IAC) cleanup and high performance liquid chromatography coupled to a fluorescence detector (FD). Results were compared with the EU and Turkish legislation concerning AFM_1 in raw milk the available literature.

2. Materials and methods

2.1. Samples

Between the months of January 2012 and December 2012, 176 raw milk samples were collected based on the Turkish Food Codex milk sampling method for the official control of mycotoxins in foodstuffs (TFC, 2011b). Samples of raw milk (1000 ml) were taken from the plants' raw milk tanks with raw milk jar samplers and transported at 4–8 °C in an icebox.

2.2. Chemicals and reagents

Acetonitrile and methanol (both of HPLC grade) were supplied by Sigma–Aldrich (St. Louis, MO, USA). The IACs Afla M_1 HPLC (Cat. No. #G1007) were purchased from VICAM (United States of America). Ultrapure water, for the HPLC mobile phase and all analytical steps was produced in a Synergy 185 water purification system (Millipore, Molsheim, France).

2.3. Standard solutions

The AFM_1 standard was supplied by R-Biopharm Rhone (Glasgow, Scotland) (Afla standard solution, Cat No. P42). The bottle consists of 1 μg AFM_1 in one ml of methanol. Stock solution standard was diluted with freshly in HPLC mobile phase consisting of water-acetonitrile-methanol (6.8/2.4/0.8, v/v/v) to obtain concentration of 5 ng ml^{-1}. From this intermediate solution, a series of working standards from 0.05, 0.25, 0.5, 1 and 2 ng ml^{-1} were prepared.

2.4. Extraction and IAC cleanup

The contamination and levels of AFM_1 in raw milk samples were determined using Turkish Official Method TS EN ISO 14501 (Turkısh Standard, 2002). This method involves centrifugation, acetonitrile-water extraction, IAC cleanup and liquid chromatography coupled with fluorescence detector.

An aliquot of 40 ml of milk was warmed at 37 °C and centrifuged (Hermle Z330K, Gosheim, Germany) at 4000× *g* for 15 min. After centrifugation, the upper cream layer was completely removed and the remaining milk was filtered through Whatman No. 4 filter paper. A 50 ml of filtered skimmed milk was passed through an IAC, placed in a vacuum manifold (Agilent Technologies, Santa Clara, CA, USA). The column was washed 10 ml ultrapure water and AFM_1 was eluted from the column with 4 ml acetonitrile. The extract was evaporated until the volume remaining approximately 500 μl acetonitrile at 45 °C under N_2 stream and then diluted to 5 ml with ultrapure water. It was collected in HPLC vials (Supelco, Bellefonte, PA, USA), shaken on the vortex mixer and analysed by HPLC-FD (TS, 2002).

2.5. HPLC-FD analysis

HPLC analysis was performed with Agilent 1100 series HPLC system consisted of a G1310A isocratic pump, a G1379A degasser, a G1313A autosampler, a G1316A column oven and a fluorescence detector model G1321A (Agilent Technologies, Palo Alto, California). Chemstation 3 D software was used to control the system and the process signals. Chromatographic separations were performed on a silica 5 μm ACE 5 C18, 100 Å, 25 × 4.6 mm column supplied by Advanced Chromatography Technologies (Aderden, Scotland). The column temperature was maintained at 25 °C. The injection volume into HPLC system for both standard and sample was 100 μl.

The mobile phase consisted of the mixed solution of water-acetonitrile-methanol (6.8/2.4/0.8, v/v/v). The fluorescence detector was set to an excitation and emission wavelengths of 360 and 430 nm, respectively. The run time for one cycle was 11 min, and the retention time of AFM_1 under these conditions was approximately 8.7 min.

2.6. Analytical quality parameters

The following performance characteristics were evaluated to ensure the method quality: linearity, sensitivity, recovery and accuracy (precision and trueness). The linearity was assessed by constructing five-point calibration curves over the concentration range of 0.05–2 ng ml^{-1}. Linear regression lines were plotted using the peak area versus the analyte concentration. The linearity was determined by linear regression analysis and expressed as coefficient of determination (R^2).

The sensitivity of the method was expressed by the limits of detection (LOD) and quantification (LOQ). The LODs and LOQs were calculated according to EURACHEM Guide based on data of recovery experiment (EURACHEM, 1998). Blank samples were spiked with 0.02 μg kg^{-1} for each analyte and measured in 10 independent replicates. The LOD and LOQ were calculated using the following relations:

$$\mathrm{LOD} = X + 3s,$$

$$\mathrm{LOQ} = X + 10s,$$

where "*X*" is the mean concentration of fortified sample blank values, and "*s*" is the sample standard deviation.

For recovery experiment, non-infected raw samples were spiked with AFM_1 at two concentration levels of 0.07 and 0.2 μg kg^{-1}. Spiking was carried out in eight replicates. The spiked materials were then analysed according to method protocol previously described and analytes were quantified. The observed signal was plotted against the actual concentration. The measured concentration was determined using the obtained calibration curves and the recovery value was calculated by the following equation:

$$\%\ \text{recovery} = 100 \times \text{measured concentration for spiked sample/spiked (added) concentration}$$

Table 1
Performance of analytical method in raw milk samples for AFM_1.

Spiking level (μg kg^{-1})	Mean recovery (%)	Intra-day repeatability[a] RSD (%)	Inter-day repeatability[b] RSD (%)	Bias[c] (%)	LOD[d] (μg kg^{-1})	LOQ[e] (μg kg^{-1})
0.07	95.71	5.45	5.66	2.941	0.021	0.025
0.2	91	6.05	6.06	8.696		

[a] Intra-day repeatability was estimated by analysis of eight replicate samples at two concentration levels on the same day.
[b] Inter-day repeatability was estimated by analysis of eight replicate samples at two concentration levels on the two different days.
[c] Bias was estimated by analysis of eight replicate samples at two concentration levels on the two different days.
[d] LOD, limit of detection of the chromatographic method.
[e] LOQ, limit of quantification of the chromatographic method.

The accuracy refers to a combination of precision and trueness. The precision of the method in terms of repeatability was evaluated by analysis of eight replicate samples at two concentration levels on the two different days. The precision was calculated as the relative standard deviation (RSD) of replicate results. The trueness, in terms of bias (a measurement of systematic error) was calculated according to the following equation:

$$\text{Bias}\ (\%) = [(X_i - X_t)/X_t] \times 100,$$

where "X_i" is the expected value and "X_t" is measured value.

3. Results and discussion

3.1. Method validation

Based on linear regression analysis, AFM_1 showed good linearity over a concentration range of 0.05–2 μg kg^{-1}, with coefficient of determination greater than 0.99.

The LOD and LOQ, the results of recovery, intra-day and inter-day precision of the analytical method are summarised in Table 1. The LOD and LOQ are 0.021, 0.025 μg kg^{-1}, respectively, which allowed a toxin determination well below the regulated limits.

The recovery values, ranging between 85.5 and 110%, are in good agreement with the Commission Regulation (EC) No 401/2006 (European Commission, 2006) performance criteria for quantitative methods of mycotoxin analysis. The intra-day precision of method was calculated as RSD for first and second person by analysis of eight replicate samples at two concentration levels (0.07 and 0.2 μg kg^{-1}) on the same day. The RSD values under intra-day repeatability conditions were ranging from 5.45 to 6.05%. The inter-day precision of method was calculated as RSD by analysis of eight replicate samples on the two different days by different persons. The RSD values under inter-day repeatability conditions were ranging from 5.66 to 6.06%. In light of these values, the repeatabilities (always lower than 10%) indicate the good precision of the method at two concentration levels. The bias values ranged between 2.94 and 8.70%.

Additionally inter-laboratory proficiency testing with the internationally recognised Food Analysis Performance Assessment Scheme (FAPAS®), confirmatory testing of results was done with certified reference material whose assigned value was 0.19 μg/l. A satisfactory range of determinations was said to be 0.191–0.491.

3.2. Occurrence of AFM_1 in raw milk

The occurrence and distribution of AFM_1 in raw milk samples are presented in Table 2. AFM_1 was detected in 53 out of the 176 raw milk samples (30.1%) at concentrations between 0.025 and 1.101 μg kg^{-1}, with a mean level of 0.153 μg kg^{-1}. HPLC-FD chromatogram of naturally contaminated raw milk sample with AFM_1 (0.033 μg kg^{-1}) is shown in Fig. 1. Maximum concentrations of AFM_1 from September until November (autumn season) were in the range from 0.042 to 0.552 μg kg^{-1}, from December until February (winter season) were in the range from 0.033 to 1.101 μg kg^{-1}, from March until May (spring season) were in the range from 0.047 to 0.150 μg kg^{-1}, from June until August (summer season) were in the range from 0.025 to 0.102 μg kg^{-1}, respectively. The mean concentration of AFM_1 in autumn season was 0.082 μg kg^{-1}, in winter season 0.275 μg kg^{-1}, in spring season 0.099 μg kg^{-1} and in summer season 0.055 μg kg^{-1}, respectively. The highest concentration (1.101 μg kg^{-1}) exceeding maximum permitted value was determined in winter season. Results showed that AFM_1 levels determined in autumn and winter season were significantly higher in accordance with concentration of AFM_1 in spring and summer season. In similar studies, the higher levels of AFM_1 in milk occurred in the winter months and these researchers have shown a seasonal differences in AFM_1 contamination (Bilandzic et al., 2010; Kamkar, 2005; Marnissi et al., 2012; Picinin et al., 2013; Tajkarimi et al., 2008). These differences are presumably due to seasonal variations in the type and quality of feed given to cows. Although, in autumn and winter, stored feeds, concentrates and silages are used more often; in spring and summer there is fresh animal feed available such as grass, weeds and raw feed. The most important factors on the amount of AFB_1 are temperature and moisture, since some moulds like *Aspergillus flavus* and *Aspergillus parasiticus* can easily grow in feeds having moisture between 13 and 18%, and environmental moisture between 50 and 60% (Unusan, 2006).

In this study, there was a high incidence by 30% of analysed raw milk samples exceed the maximum limit for AFM_1 (0.05 μg kg^{-1})

Table 2
Distribution of AFM_1 concentration by season in 2012.

Season	Number of Samples	Positive *n* (%)		Frequency distribution, n (%)				Contamination (μg kg^{-1})	
			<LOQ[a]	LOQ–0.050 μg kg^{-1}	0.05–0.25 μg kg^{-1}	0.25–0.50 μg kg^{-1}	>0.50 μg kg^{-1}	Range[b]	Average[c]
Autumn	63	20 (31.8)	43 (68.2)	13 (20.6)	6 (9.5)	–	1 (0.2)	0.042–0.552	0.082
Winter	47	19 (40.4)	28 (59.6)	7 (14.9)	7 (14.9)	1 (0.2)	4 (8.5)	0.033–1.101	0.275
Spring	33	11 (33.3)	22 (66.7)	1 (0.3)	10 (30.3)	–	–	0.047–0.150	0.099
Summer	33	3 (9.1)	30 (91.1)	2 (0.3)	1 (0.3)	–	–	0.025–0.102	0.055

[a] Limit of quantification (0.025 μg kg^{-1})
[b] Min–max.
[c] Mean of positive samples.

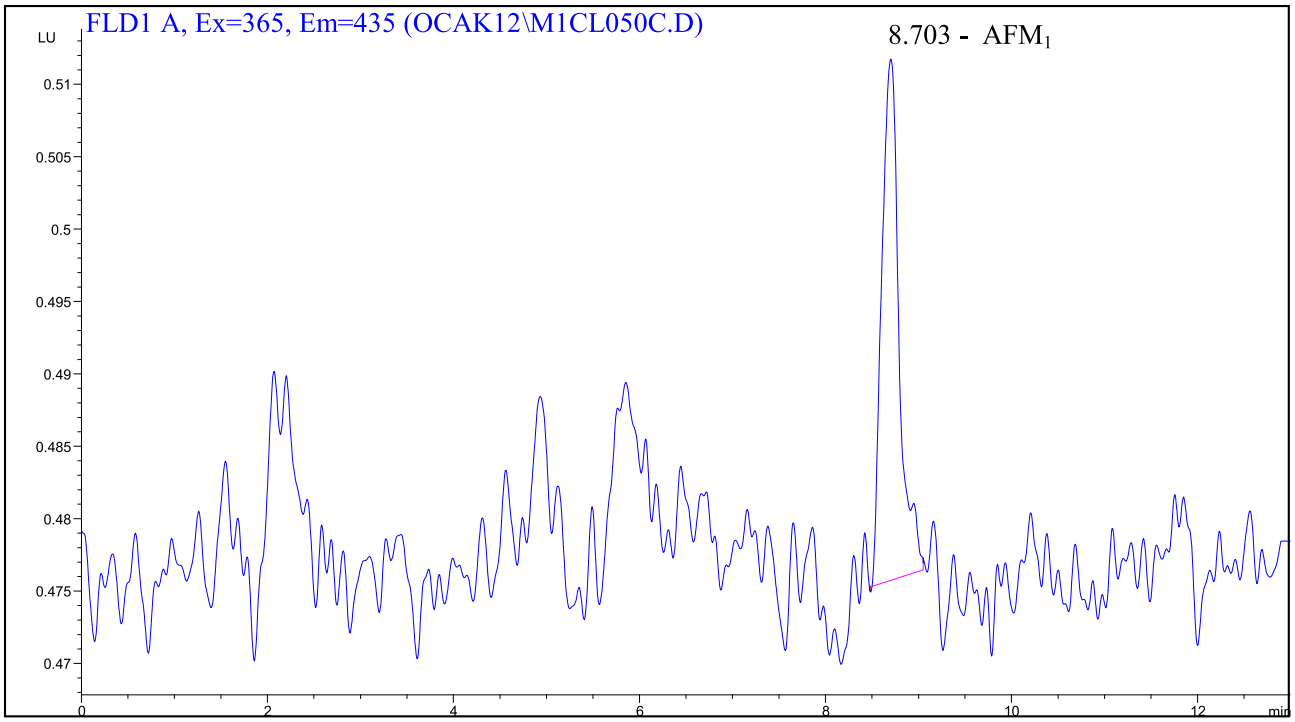

Fig. 1. HPLC-FD chromatogram of naturally contaminated raw milk with 0.033 μg AFM_1 kg^{-1}.

set by Turkish and EU regulations. During the last decade, because the natural occurrence of AFM_1 in milk and dairy products is a global problem; there are many surveys have been carried out to assess the occurrence and levels of AFM_1. The results of these studies are summarised in Table 3. In Pakistan, India and Syria, the incidence rate of AFM_1 contamination above maximum levels was 41.1, 48.9, 58.3, 99.4%, respectively (Ghanem & Orfi, 2009; Hussain & Anwar, 2008; Iqbal & Asi, 2013; Siddappa, Nanjegowda, & Viswanath, 2012).

Contamination of feeds is influenced by local weather conditions during pre-harvest and harvest stage, but is exacerbated by inadequate storage conditions (Duarte et al., 2013). AFB_1 contamination is prevalent in warm and humid climates and reported in temperate countries following severe drought (EFSA, 2004; Marnissi et al., 2012).

The European Commission publishes weekly overviews of alert and information notifications on its website. During the year 2012, there were only 3 notifications in the presence of AFM_1 in milk and milk products in Italy, of all them were in raw milk. While 2 notifications were originated from Hungary (0.073 and 0.072 μg kg^{-1}), one notification was originated from Slovenia (0.074 μg kg^{-1}). (http://webgate.ec.europa.eu/rasff-window/portal).

Table 3
Occurence and levels of AFM_1 in raw milk in various countries.

Country	No. of samples	Positive *n* (%)	Method	Mean (μg kg^{-1})	Exceeding % EU ML (μg kg^{-1})	Reference
Portugal	31	25 (80.6)	HPLC-FD	n.r.	0	Martins & Martins, 2000
Spain	92	5 (5.4)	ELISA	n.r.	0	Velasco et al., 2003
Portugal	598	394 (65.8)	HPLC-FD	n.r.	8.2	Martins et al., 2005
Iran	11	85 (76.6)	TLC	0.061	40	Kamkar, 2005
Brazil	22	13 (59.1)	TLC	0.013	9	Shundo & Sabino, 2006
France	264	9 (3.4)	HPLC-FD	0.014	0	Boudra et al., 2007
Pakistan	168	168 (100%)	Fluorometer,	0.371	99.4	Hussain & Anwar, 2008
Indonesia	113	65 (57.5%)	ELISA	0.009	0	Nuryono et al., 2009
Syria	74	70 (95%)	ELISA	0.14	58.3	Ghanem & Orfi, 2009
Egypt	50	26 (52%)	ELISA	n.r.	34	Motawee et al., 2009
Serbia	23	23 (100%)	TLC	n.r.	30	Horvatovic et al., 2009
Pakistan	40	15 (37.5%)	HPLC-FD	0.014	20	Hussain et al., 2010
Iran	240	226 (94.2%)	ELISA	0.013	4.2	Mohammadian et al., 2010
Argentina	94	60 (64%)	LC-MS/MS	0.028	11	Alonso et al., 2010
Iran	75	59 (78.7%)	ELISA	0.06	36	Rahimi et al., 2010
Croatia	61	n.r.	ELISA	0.01	1.64	Bilandzic et al., 2010
Turkey	50	43 (86%)	ELISA	0.009	0	Ertas et al., 2011
Lebanon	38	28 (73.7%)	ELISA	n.r.	45	Assem et al., 2011
Morocco	48	13 (27%)	HPLC-FD	n.r.	8	Marnissi et al., 2012
India	45	45 (100%)	HPLC-FD	n.r.	48.9	Siddappa et al., 2012
Pakistan	107	76 (71%)	HPLC-FD	0.15	41.1	Iqbal & Asi, 2013

HPLC-FD: High performance liquid chromatography-fluorescence detection; TLC: Thin-layer chromatography; ELISA: Enzyme-linked immunosorbent assay; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; AFM_1: Aflatoxin M_1; EU: European Union; ML: Maximum level; n.r.: Not reported; n.d.: Not detected.

3.3. Estimated daily intake of AFM_1

The average contamination of AFM_1 is 0.046 μg kg^{-1} in according to present survey. While there is a lack of data concerning milk consumption by different age groups in Turkey, it has been assumed an average consumption of milk of about 26 kg per year (i.e. 71 g of milk per day) (IDF, 2010). In light of these, the estimated daily intake of AFM_1 for Turkish consumers was 0.054 ng kg^{-1} b.w. day^{-1}, assuming an adult body weight (b.w.) of 60 kg. It has been reported that a tolerable daily intake of (TDI) of 0.2 ng kg^{-1} b.w. day^{-1} for AFM_1 based on a risk level of 10^{-5} (Kuiper-Goodman, 1990). The value of estimated AFM_1 intake through milk by this exposure assessment is 3.7 times lower than the TDI proposed by Kuiper-Goodman (1990). In the present study, the contribution to daily intake of AFM_1 through milk could be considered to be rather higher in comparison with daily intake value of 0.008 ng kg^{-1} in another study conducted in Turkey (Kabak & Ozbey, 2012). However, the intakes of AFM_1 through milk consumption were estimated to be 0.002 ng kg^{-1} b.w. day^{-1} in Africa, 0.11 ng kg^{-1} b.w. day^{-1} in Europe (JECFA, 2001), 0.122 ng kg^{-1} b.w. day^{-1} in Argentina (Signorini et al., 2012), 0.054 ng kg^{-1} b.w. day^{-1} in Morocco (Zinedine et al., 2007) and 0.036–0.043 ng kg^{-1} b.w. day^{-1} in Spain (Cano-Sancho, Marin, Ramos, Peris-Vicente, & Sanchis, 2010).

4. Conclusions

According to results obtained in Adana province of Turkey, incidence and contamination levels of AFM_1, seem to be a serious problem for the public health, since all the age groups including infants and children consume these products worldwide. For this reason, milk and dairy products have to be inspected and controlled continuously for AFM_1 contamination. The different climate conditions showed influence on AFM_1 contamination of raw milk samples. The higher contamination levels were found in spring and summer season. The most effective way of controlling AFM_1 in the food supply is to reduce contamination with AFB_1 of raw material and supplementary feedstuffs for dairy cattle. Reduction can be achieved by good manufacturing practice and good storage practices. It is clear that detoxification method can be suitable for animal feeds if preventive measures fail to reduce fungal growth and AFB_1 formation. Feeds that have higher concentrations of AFB_1 may be acceptable for feeding to dairy animals if they are blended with feed that has lower concentrations, provided that the resultant AFM_1 concentration in milk does not exceed levels considered to be safe.

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