Effect of Storage on the Level of Aflatoxin M₁ in Milk and Other Dairy Products Sold at Tripoli Province, Libya

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Abstract

Milk and dairy products are one of the chief sources of nutrition for human beings particularly for infant and children. Aflatoxin M₁ (AFM₁) a hydroxylated metabolite of aflatoxin B₁ found in milk and milk products causes serious health issues for human beings. The objective of this study was to evaluate the effect of storage on the level of aflatoxin M₁ in milk and other dairy products sold at retail stores of Tripoli Province, Libya. Selected samples (Skimmed and cream milk, infant milk formula, butter, cheese, Cheddar, spread and slice) were evaluated by using specialized RIDASCREEN AFM₁ competitive enzyme linked immune sorbent assay (ELISA) technique. Our investigation revealed that, the concentration of AFM₁ increased with the duration of storage. Furthermore, we found that the newly manufactured samples had very low concentration of AFM₁ and within the permitted range. Moreover, AFM₁ concentration in skimmed and cream milk having 6 month shelf life had 5.00 ngkg⁻¹ and 5.03 ngkg⁻¹ respectively. Furthermore, both the expired skimmed and cream milk had AFM₁ concentrations 121.8 ngkg⁻¹ and 108.18 ngkg⁻¹, respectively. In addition to that, we found that the levels of AFM₁ in different dairy products varies with different shelf lives (12 and 1 month), such as cheddar (5.0 and 72.79 ngkg⁻¹), Spread (5.30 and 60.03 ngkg⁻¹), Slice (5.50 and 61.18 ngkg⁻¹). Additionally, infant milk formula with shelf life of 24 months and expired samples had AFM₁ less than 5.00 ngkg⁻¹ and 60.8 ngkg⁻¹, respectively. Based on our investigation, we found that the presence AFM₁ in milk and milk products at high concentration may cause serious illness to consumers’ health and the consequent economic losses.

Keywords: Mycotoxins, Aflatoxin M₁, ELISA, milk and milk products, Shelf lives.

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INTRODUCTION

In recent years, occurrence of food contamination has been increased raising serious issue concerning human health and safety (Negash, 2018; Ibrahim et al., 2018). Milk and other dairy products are the key source of human nutrition especially for children across the globe (Tahoun et al., 2017). However, natural food contaminants present in milk may cause risk to human health. Therefore, it is not astonishing that a significant consideration has been paid over the several years to improve quality of milk (Garbaj et al., 2013). The occurrence of aflatoxin in milk and other dairy product has been well known across the world dairy industries (Iqbal et al., 2015). However, occurrence of aflatoxin from these fungal species Aspergillus flavus, Aspergillus nomius and Aspergillus parasiticus are more prominent. In addition to that, Aspergillus flavus produces only aflatoxin B, while the other species produces both aflatoxins B and G (Abdolgader et al., 2017). Furthermore, aflatoxin B (AFB) produces a hydroxylated metabolite aflatoxin M, (AFM), which is excreted in milk when lactating animals are nurtured with contaminated food. Consumption of feedstuff contaminated with aflatoxin B during lactation in bovine causes production of AFM (monohydroxy derivative of B) which is excreted in the bovine milk (Iha et al. 2013). Among the aflatoxin, aflatoxin B is particularly the most toxic metabolite, when consumed by dairy animals. In addition, biotransformation of metabolites AFM occurs at the hepatic level using microsomal cytochrome p450 (European Commission 2003). Subsequently AFM excreted in animal milk used for human intake (Baskaya et al., 2006; Battaccone et al., 2005). In addition to that, aflatoxins does not have flavor or smell, they are fluorescent under the ultraviolet light and they are resistant to high temperatures more than 320°C without fragmenting (Garmakhany et al., 2011). Moreover, pasteurization of milk diminishes the chance of infection caused by pathogenic bacteria, but thermal treatment does not control other contaminants such as aflatoxin (Omer, 2016). AFM is reasonably stable in raw and processed milk products and cannot be destroyed by heat treatments (Iha et al., 2011). Aflatoxin has been suggested as highly toxic, carcinogenic, teratogenic and mutagenic compounds, and it is well known as a causative agent for human hepatic and extrahepatic carcinogenesis. It has been reported that, high levels of aflatoxin in the bloodstream depress the immune system, which could cause stunting growth of children as well as low immunity levels in patient with HIV and cancer (Villers, 2014). There are various published reports, suggesting mycotoxins are interrelated for the prevalence of cancer in human beings. Which includes such as aflatoxin, zearalenone, fumonisins, sterigmatocystin, ochratoxin and patulin, (Ostry et al., 2009; Stec et al., 2009). International Agency for Research on Cancer (IARC) included aflatoxin B as main causative agent for carcinogenic effect however AFM is included into secondary groups of carcinogenic compounds (Ahmad et al., 2011; Kamkar et al., 2011). The European Community and Codex Alimentarius recommended tolerance range of AFM in liquid milk and dried or processed milk foodstuffs should not exceed more than 50 ng kg⁻¹. However, as per the guideline from US, the permitted range of AFM in milk should not go beyond 500 ng kg⁻¹. In Austria and Switzerland, the maximum levels are further reduced to 10 ng kg⁻¹ for infant food commodities (Abdolgader et al., 2017). However, we found that detection of AFM with respect to shelf life of the milk and other dairy products have not been previously studied.

The objective of this study was to assess the Aflatoxin M in milk and other dairy products having different shelf lives to make sure the safety of milk and dairy products that’s been used in the markets of Libya as milk and dairy products are major source of energy in diet and consumed in great amounts yearly.

MATERIALS AND METHODS

Chemicals and standard solutions

AFM reference standard (Aspergillus flavus, 10μg, A6428) was purchased from Sigma–Aldrich (Germany). Dichloromethane, methanol, PBS (Phosphate Buffered Saline), heptane, and deionized water of HPLC grade were purchased from Sigma-Aldrich (Germany). Dichloromethane, methanol, PBS (Phosphate Buffered Saline), heptane, and deionized water of HPLC grade were purchased from Baker (Deventer, Netherlands).

Sample collection

A total of 21 samples were obtained (3 samples of skimmed milk, 3 samples of cream, 6 samples of Infant milk formula, 3 samples of Cheddar and 3 samples of spread and 3 samples of slice and 3 samples of butter) randomly from...
the local markets of Tripoli, Libya and aflatoxin concentrations were determined by using enzyme linked immunosorbant assay, ELISA (Ridascreen, aflatoxin M1).

Sample preparation

Liquid milk

Degreasing of milk samples were performed by centrifuging at 3500g for 10 minutes and temperature was regulated at (10°C or 50 °F). Later on, the upper cream layer was distanced completely by aspirating through a Pasteur pipette. The skimmed milk was directly used for the experiment (100 µl per well) (Montagna et al., 2008).

Milk powder

10 g of milk powder weighed into a flask and 100 ml deionizer water were added and stirred for 5 min. Rest of sample preparation were performed as of milk preparation.

Cheese and Butter

The samples preparations were performed as mentioned above with slight modifications. A representative sample was chopped into coarse slice and later on thoroughly mixed, without the addition of liquid. 2g thoroughly mixed cheese weighed and placed into a centrifugal glass vial. 40 ml dichloromethane were added and extraction was done by stirring for 15 min. In addition to that, samples were filtered and suspension evaporated at 60 °C under nitrogen stream. After that, oily residues were re-dissolved with 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml heptane. Later on, samples were mixed and centrifuged for 15 minutes at 2700 g at 15 °C. The upper layer of heptane was removed completely. Poured off an aliquot of the lower methanolic-aqueous phase carefully using a Pasteur pipette 100 µl of this aliquot diluted with 400 µl buffer 1 (1:5 dilution) and 100 µl per well are used in the test (Tihrumala-Devi, et al., 2002).

ELISA Analysis

To analyze the prepared samples, Ridascreen AFM1 competitive enzyme immunoassay techniques were used for the estimation of AFM1 in milk and other dairy products. Moreover, for estimation of AFM1 required number of microtiter wells was inserted into the micro well holder for all standards and samples to be run in duplicate. Standards and samples position was recorded. 100µl AFM1 standard solutions or prepared sample were added to separate duplicate wells and analyzed as per the instruction mentioned over the ELISA kit. Analysis of AFM1 was performed using ELISA plate reader (Thermo Labsystems Multiskan Spektrum, 1500) at 450 nm against blank (Montagna et al., 2008).

Statistical Analysis

All the experiments were carried out in triplicates. Results were expressed as mean ± SD of three independent experiments (n = 3)

RESULTS AND DISCUSSION

In this study we tested the presence of AFM1 in milk and dairy products by using specific ELISA (RIDASCREEN®) AFM1 30/15 technique used for AFM1. Skimmed and creamed milk samples were investigated for AFM1 with different duration of expiry dates (6 months, 1 months and expired) (Table 1). Skimmed and creamed milk sample whose expiry dates was 6 months did not show high level of AFM1 (5 and 5.03 ngkg-1, respectively). Although, high values of AFM1, recorded, but under threshold level (50 ngkg-1) for both skimmed (33.9 ngkg-1) and creamed (29.1 ngkg-1) milk samples having expiry of 1 month. However, both expired samples of skimmed (121.8 ngkg-1) and creamed (108.18 ngkg-1) exhibited high amount of AFM1 that were more than the permissible limit. Both skimmed and creamed milk samples that were already expired exhibited maximum concentration of AFM1 that is much higher than the permissible international value (50 ngkg-1) for AFM1 (European Codex 2000). Moreover, expired skimmed milk samples showed

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shelf life (months)*</th>
<th>Aflatoxin M1 (ngkg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed*</td>
<td>6</td>
<td>5.00±0.05</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>40.7±2.24</td>
</tr>
<tr>
<td></td>
<td>Expired</td>
<td>121.5±5.52</td>
</tr>
<tr>
<td>Cream milk*</td>
<td>6</td>
<td>5.03±0.12</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>28.1±1.17</td>
</tr>
<tr>
<td></td>
<td>Expired</td>
<td>108.2±4.47</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD

*Number of replicates for each sample; n=3
more concentration of AFM$_1$ as compared to the expired creamed milk samples. However, AFM$_1$ has been found to be unaffected to thermal energy, pasteurization, autoclaving and other varieties of food processing procedures (Boudra et al., 2007; Hussain et al., 2008). Value of 0.05 µg kg$^{-1}$ of milk is currently the legal limit in those European countries that have the most stringent regulations for AFM$_1$. This value was proposed by the Codex Committee on food additives and contaminants in 2000 (European Codex 2000). Therefore, it is important to stringent screening and routine-monitoring of AFM$_1$ levels in milk samples. However, it has been reported that due to high toxicity and carcinogenic properties of AFM$_1$, its presence in milk has been put consumer health on alarm.

Newly born and infants are highly dependent on infant milk formulas constitute an important or often sole source of food for them during the first months of life. However, these milk products are often given to the infants up to the age of 2 years. Moreover, AFM$_1$ levels in infant milk formula were presented in table 2. Which showed that, out of 6 samples of infant milk powder with different duration of expiry, only expired sample showed high concentration of AFM$_1$, i.e.; >60.18 ng kg$^{-1}$. However, there were no significant differences (5 - 5.09 ng kg$^{-1}$) observed in concentrations of AFM$_1$ in other 5 samples with 24, 12, 6, 3 and 1 month(s) to expire. Milk powder has been a frequently marketed product in retail stores during the recent years. It is used in chocolate, ice-cream, yoghurt, cake and baby food. Although infant milk powder has long duration of expiry dates, the samples with 24, 12, 6, 3 and 1 month gave less amounts of AFM$_1$. The samples from expired milk powder showed high concentration of AFM$_1$. From this we interpreted that there was no risk to give milk powder to babies until it was expired but it would be very harmful to the babies if unknowing expired milk given to them, as AFM$_1$ work as an immunosuppressant and babies have weak immune system and are therefore more prone to the diseases (Akhtar et al., 2017).

The widespread presences of aflatoxin M1 in cheese may be considered to be potential hazards for human health and that is why it is also important to identify the amount of AFM$_1$ in various cheese (cheddar, spread and slice) available in the retail markets. Different cheese such as cheddar, spread and slice were also

### Table 2. Level of aflatoxin M1 in infant milk powder with different duration of expiry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shelf life (months)*</th>
<th>Aflatoxin M1 (ng kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>&lt;5.00±0.13</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&lt;5.80±0.15</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&lt;5.00±0.11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;5.03±0.18</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&lt;5.90±0.17</td>
</tr>
<tr>
<td></td>
<td>Expired</td>
<td>60.8±3.9</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD; *Number of replicates for each sample; $n=3$

### Table 3. Level of aflatoxin M1 in infant milk powder with different duration of expiry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shelf life (months)*</th>
<th>Aflatoxin M1 (ngkg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar</td>
<td>12</td>
<td>5.00±0.13</td>
</tr>
<tr>
<td>Cheddar</td>
<td>1</td>
<td>72.79±4.2</td>
</tr>
<tr>
<td>Spread</td>
<td>12</td>
<td>5.30±0.70</td>
</tr>
<tr>
<td>Spread</td>
<td>1</td>
<td>60.03±2.9</td>
</tr>
<tr>
<td>Slice</td>
<td>12</td>
<td>5.50±0.22</td>
</tr>
<tr>
<td>Slice</td>
<td>1</td>
<td>61.18±4.5</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD
* Number of replicates for each sample; $n=3$

### Table 4. Level of aflatoxin M1 in butter with different duration of expiry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shelf life (months)*</th>
<th>Aflatoxin M1 (ng kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td>12</td>
<td>5.00±0.19</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>18.0±0.88</td>
</tr>
<tr>
<td></td>
<td>Expired</td>
<td>88.2±2.84</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD
* Number of replicates for each sample; $n=3$
investigated and values presented in table 3. Samples from cheddar, spread and slice having 12 month of expiry showed similar values of AFM1 i.e.; (5.0, 5.3 and 5.5 ngkg\(^{-1}\), respectively). However, cheese samples from cheddar, spread and slice having 1 month shelf lives showed higher amount of AFM1, 72.79, 60.03 and 61.18 ngkg\(^{-1}\) respectively. The values were higher than the permissible limits (Ishikawa et al., 2016).

In addition to that, level of AFM\(_1\) in butter sample showed gradual increase in residual concentration as shown in table 4. Maximum amount (88.18 ngkg\(^{-1}\)) of AFM\(_1\) was detected in expired sample of butter. Previous studies showed that AFM\(_1\) concentration in butter was lesser than in the milk from which it was made. This study was also supported by our results for the presence of AFM\(_1\) in butter. Only expired sample of butter contains toxic level of AFM\(_1\).

**CONCLUSION**

Aflatoxins are among the most toxic contaminant produced by *Aspergillus* species. Based upon our result, we found that the concentration of AFM\(_1\) was increasing with the increase in duration of storage of sample. Freshly prepared samples had insignificant amount of AFM\(_1\) and was within the limit of European Regulations. However, incidence of AFM\(_1\) in milk and other dairy products is a serious health issue and must be monitored as well as prophylactic measures should be taken to prevent factors responsible for AFM\(_1\) toxin production. We found that, it is very unsafe to use any dairy product and milk that is near to expiry or expired. We are regularly using milk in our daily life. In addition to that, this is high time to screen milk and other dairy products for the presence of toxic AFM\(_1\) in Libya and set permissible levels for such products.

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**CONFLICT OF INTEREST**

The authors do not have any conflict of interest.

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