Occurrence of Aflatoxin M1 in Pasteurized Doogh Commercialized in Tehran, Iran

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Abstract: Doogh is a popular dairy product in Iran owing to its beneficial influence on human health and nutritional value. The aim of this study was to evaluate aflatoxin M1 (AFM1) contamination in 225 commercial doogh samples composed of traditional (115 samples) and industrial pasteurized doogh (110 samples) obtained from popular markets of Tehran, Iran. The occurrence and concentration range of AFM1 in the samples was investigated by the high-performance liquid chromatography (HPLC) method. Then data were analyzed statistically by applying ANOVA. AFM1 was found in 100% of the examined doogh samples by average concentration of 49.7 ng/l. Aflatoxin M1 was detected in 151 (67.1%) samples, consisted of 83 (72.1%) traditional doogh samples (mean:52.3 ng/l; range: 5.8–211 ng/l) and 68 (61.8%) industrial pasteurized doogh samples (mean: 46.4 ng/l; range:7.8–152 ng/l) in Tehran. Just, 27 of the doogh samples, 15 samples (12.8%) of traditional and 12 samples (10.8%) of industrial in Tehran had higher AFM1 level than the maximum tolerance limit (50 ng/l) accepted by European Union but the contamination level was lower than 500 ng/l in all the samples, which is accepted by Codex Alimentarius and National Standard. The results indicated that the contamination of the samples with AFM1 in such a level could not be a serious public health problem at the moment. This paper represents the data of the first survey on the occurrence of AFM1 in commercial doogh marketed traditional and industrial in Tehran of Iran.

Key words: Immunoaffinity column clean-up, HPLC, Doogh, Aflatoxin M1.

INTRODUCTION

Mycotoxins are secondary metabolites of molds, which are associated with certain disorders in animals and humans. In addition to being acutely toxic, some mycotoxins are now linked with the incidence of certain types of cancer and it is this aspect which has evoked global concern over feed and food safety, especially for milk and milk products (Castegnaro and McGregor, 1998). AFM1 is a hepatocarcinogen found in milk of animals that have consumed feeds contaminated with aflatoxin B1 (AFB1), the main metabolite produced by fungi of the genus Aspergillus in particular A. flavus, A. parasiticus and A. nomius (Galvano et al., 2001). About 0.3–6.2% of AFB1 in animal feed is transformed to AFM1 in milk (Creppy, 2002) which is not destroyed by common heat treatments. Although its potency of toxicity is less than those of its parent compound, it has been suggested as a probable Group 2B human carcinogenic by the International Agency for Research on Cancer (IARC, 1993), while AFB1 was classified as a group 1 carcinogen. The importance of AFM1 as a food safety hazard is reflected in the existence of a maximum permissible level of 0.05µg/kg AFM1 in milk and milk products like doogh established by the European Commission (BS EN ISO 14501, 2007; Van Egmond and Wagstaffe, 1992) and additional 17 national regulations.

The International Agency for Research on Cancer (IARC) classified aflatoxin B1 in group 1, as agents that are certainly carcinogenic to human: evaluation of the risk is well known, so much so that it is considered the most powerful hepatocarcinogenic agent in mammals. Aflatoxin M1, on the other hand, is included in Group 2B, as an agent with possible carcinogenic effects to humans (IARC, 1993). The formation of AFM1, a metabolite of AFB1, occurs in the liver and it is excreted into milk in the mammary gland of dairy cows (Cathey, Huang, Sarr, Clement and Philips, 1994). When doogh was made from milk contaminated with AFM1, AFM1 was carried over into this product.

Tabari (2010) also found out that the AFM1 levels differed also with the milk products. Pasteurization processes, even those using UHT techniques, do not affect drastically AFM1 concentration because of its heat stability (Tabari, 2010; Galvano, Galofaro and Galvano, 1996).

To protect consumers, particularly children, from contaminated milk and dairy products, several countries have established legislation to regulate the levels of AFB1 in feeds and AFM1 in milk. Thus, the EU has set a maximum admissible level of 0.05µg/l in raw milk, heat-treated milk and milk for the manufacture of milk-based products like doogh (EC, 2001), whereas the Food and Drug Administration (FDA) has established an
action level of 0.05µg/l in whole, low-fat and skim milk (US FDA, 1996). The importance of AFM1 as a food safety hazard is reflected in the existence of a maximum permissible level of 0.05µg/l AFM1 in milk products like doogh established by the European Commission (BS EN ISO 14501, 2007) and additional 17 national regulations Union but according to the Codex Alimentarius and National Standard contamination level are lower than 500 ng/l.

The objective of this work was to evaluate the level of aflatoxin M1 in both industrial and traditional doogh in the region. This study reports the data of a first survey on the presence of AFM1 in doogh in Tehran, Iran.

**Materials:**

**Sampling:**
In this study, AFM1 was analysed in 2009 in a total of 225 doogh samples were collected based on the Iranian National Standard Sampling Method INS No.419. A total of 225 samples (115 samples commercial and 110 samples traditional) of doogh were collected. The industrial dooghs were manufactured by dairy plants and the traditional doogh samples were made by local people, which offered their products to sell on the markets of Tehran province.

**Chemicals and Standards:**
Acetonitrile (HPLC grade) of Sigma-Aldrich (Steinheim, Germany) was used for AFM1 analysis. The immunoaffinity columns AflaM1 TM HPLC were obtained from VICAM (Watertown, MA, USA). The water was double distilled with Millipore water purification system (Bedford, MA, USA) and was used for analysis. Standard of AFM1 (10 µg/ml in acetonitrile) was purchased from Supelco (Bellifonte, PA, USA). All the other chemicals used were of Analar grade.

**Methods:**

**Determination of Aflatoxin M1:**
The method used for determination of AFM1 was the AOAC Official Method 2000.08 reported by Dragacci, Grosso and Gilbert (Dragacci, Grosso and Gilbert, 2001).

**Extraction Procedure:**
Doogh samples, warmed at 37 ºC in water bath, were centrifuged at 2000 × g. The fat layer was removed completely and then doogh was passed through filter paper (Whatman No. 4). Then 50 ml of this prepared test portion was taken in a syringebarrel which was attached with immunoaffinity columns (IAC). The test portion was passed at the flow rate of 2–3 ml/min. The column was washed with 20 ml water and then blown to dryness. Acetonitrile (4 ml) was allowed to pass and to be in contact with the column for at least 60 seconds and consequently aflatoxin M1 was eluted. The gentle stream of nitrogen was passed to evaporate the eluate to dryness and it was diluted with the mobile phase at the time of LC determination.

**LC Determination With Fluorescence Detection:**
The HPLC system of Agilent 1100 series (Agilent, USA), equipped with an auto sampler LAS G1313A and a fluorescence detector FLD G1321A with excitation and emission wavelength of 365 nm and 435 nm, respectively, was used for AFM1 determination. The ZORBAX Eclipse XDB-C18 (Octadecyl silane chemically bonded to porous silica) column (Agilent, USA), 150 mm with particle size 5 lm in diameter, was used. Acetonitrile/water (25/75, v/v) was used as mobile phase. The flow rate was set to be 0.8 ml/min. Standard solutions AFM1 with concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 µg/l in acetonitrile were used to obtain the calibration curve. The retention time for aflatoxin AFM1 was 7 min.

**Calculations:**
Calculations were made according to the following equation:

\[ W_m = W_a \times \left( \frac{V_f}{V_i} \right) \times \left( \frac{1}{V_s} \right) \]

where \( W_m \) = amount of AFM1 in the test sample in µg/l; \( W_a \) = amount of AFM1 corresponding to area of AFM1 peak of the test extract (ng); \( V_f \) = the final volume of re-dissolved eluate (l); \( V_i \) = volume of injected eluate (l); \( V_s \) = volume of test portion (doogh) passing through the column (ml).

**Statistical Analysis:**
The AFM1 concentration results were statistically analyzed by applying one-way analysis of variance (ANOVA).
RESULTS AND DISCUSSION

Doogh are good sources of bioavailable calcium and proteins; and presence of contaminants like aflatoxins in doogh could be a serious health hazard for consumers specially children who are more sensitive to adverse effects of aflatoxins than adults.

In this study, AFM1 was analysed in 2010 in a total of 225 in Tehran, Iran. In the earlier effective methods for determination of aflatoxin M1 in doogh, water and methanol were used as extraction solvents. A more recent advancement in quantitative extraction of aflatoxin M1 and subsequent clean-up is the use of immunoaffinity columns. The first published method for AFM1 with immunoaffinity columns (Mortimer, Gilbert and Shepherd, 1987).

Modifications of the immunoaffinity-based methods for AFM1 were subsequently published and studied collaboratively, under the auspices of the International Dairy Federation and AOAC International, by a group mainly of European laboratories that could determine AFM1 in doogh at concentrations of 0.05 µg/l. A collaborative study resulted in approval of AOAC method 2001.

The standard solutions of concentration from 0.05 µg/l to 1 µg/l AFM1 were used to find calibration/standard curve as described by the following regression equation: y = 17.579x-1.520, where y = area and x = amount of AFM1. The results showed the linearity of the standard curve over the range studied. The coefficient of determination (R2) was 0.9998.

To determine LOD, a series of standard solutions in the range of 50–0.01 µg/l AFM1 were injected into the HPLC equipment and observed the signal to noise ratio. The limit of determination (LOD) was determined to be 0.04µg/l. Recovery was studied by spiking the doogh samples with AFM1 standard at the levels of 10, 20 and 50ng/l. The recoveries were found to be 88%, 90% and 96%, respectively.

AFM1 was detected in all the examined doogh samples by average concentration of 49.7 ng/l doogh. The results on an amount of AFM1 are shown in Table 1. Only 27 samples had higher AFM1 level than the admissible level (50 ng/l) for adult established by the Commission of the European Communities (10.8% of commercial dooghs and 12.8% of traditional dooghs were contaminated higher than 50 ng/l).

**Table 1**: Occurrence of Aflatoxin M1 in commercial and traditional doogh in Iran.

<table>
<thead>
<tr>
<th>Sample</th>
<th>NO. of samples</th>
<th>NO. of positive samples (%)</th>
<th>Concentration (ng/l) Of Total samples (mean ±SEM)</th>
<th>Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional doogh</td>
<td>115</td>
<td>82 (72.1)</td>
<td>52.3 ± 6.8</td>
<td>5.8 211</td>
</tr>
<tr>
<td>Commercial doogh</td>
<td>110</td>
<td>68 (61.8)</td>
<td>46.4 ± 10.0</td>
<td>7.8 152</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
<td>151(66.95)</td>
<td>49.7 ± 6.5</td>
<td>5.8 211</td>
</tr>
</tbody>
</table>

In addition, 50 of doogh samples had higher AFM1 level than the acceptable level (25ng/l) for children (17.2% of commercial and 26.5% of traditional dooghs). The range of contamination level varied from 7.8 to 152ng/l and 5.8 to 211ng/l in commercial and traditional doogh samples respectively.

**Table 2**: Levels of aflatoxin M1 in Traditional and Commercial doogh in Iran.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AFM1 contamination of samples (ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10</td>
</tr>
<tr>
<td>Traditional doogh</td>
<td>33 (28.4%)</td>
</tr>
<tr>
<td>Commercial doogh</td>
<td>41 (37.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>74 (32.9%)</td>
</tr>
</tbody>
</table>

The distribution of negative samples.

The results demonstrate that there were not significant differences (P< 0.01) in the levels of AFM1 in commercial and traditional dooghs however Contamination level in traditional dooghs was higher than commercial ones.

As it can be seen, 82.4 % (90 samples) of commercial dooghs had lower AFM1 level than 25ng/l and 37.6 % (41 samples) had lower AFM1 level than 10ng/l (table 2). In addition, 62.4 % of these samples had the higher contamination levels than 25 ng/l and only 17.6% of samples had higher AFM1 level than 50 ng/l. 15 % of AFM1-contaminated traditional doogh samples exceeded the European tolerance limit (50 ng/l).The percentage of samples with the lower level of AFM1 decreased in traditional dooghs. The concentration of AFM1 in 33 samples (28.4%) was lower than 10 ng/l. In addition, 72.7% of doogh samples had lower AFM1 levels than 25 ng/l. AFM1 was found in 100% of examined traditional samples by mean concentration of 52.3 ng/l.

In order to face the problem of aflatoxin M1 in milk and dairy products, it is necessary to focus the attention on the most sensitive steps of feedstuff production for lactating cows. In order to prevent losses of yield and hazards for human and animal health, it is necessary to take care of cultural phases that can represent a critical point for fungal growth and mycotoxin production in an organic, low and a high-input farming system (e.g.
excessive nitrogen fertilization in a high-input farming system; in organic and low-input farming system). To prevent future aflatoxin’s outbreaks it is needed to communicate about the potential risk deriving from unsuitable farming managements that could lead to the development of contaminated feeds and foods. We cannot find any surveys refer to the occurrence of AFM1 in doogh and it is the first survey for evaluate it.

REFERENCES


