# Aflatoxin M1 levels in raw milk, pasteurised milk and infant formula

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## Abstract

The incidence of contamination of aflatoxin M1 (AFM1) in milk samples collected from the Jordanian market was investigated by using the competitive enzyme linked immunosorbent assay (ELISA) technique. A total of 175 samples were collected during 2014-2015. All tested samples were contaminated with various levels of AFM1 ranging from 9.71 to 288.68 ng/kg. The concentration of AFM1 in 66% of fresh milk samples was higher than the maximum tolerance limit accepted by the European Union (50 ng/kg) and 23% higher than the maximum tolerance limit accepted by the US (500 ng/kg). Percentages of contaminated raw cow, sheep, goat and camel milk exceeding the European tolerance limit were 60, 85, 75 and 0%, respectively. Of AFM1 contaminated pasteurised cow milk samples, 12% exceeded the European tolerance limit with a range of contamination between 14.60 and 216.78 ng/kg. For infant formula samples, the average concentration of AFM1 was 120.26 ng/kg (range from 16.55 to 288.68 ng/kg), the concentration of AFM1 in 85% of infant formula samples was higher than the maximum tolerance limit accepted by the European Union and the US (25 ng/kg).

## Introduction

Aflatoxins are mycotoxins *i.e.* a group of naturally occurring toxins produced mainly by moulds such as *Aspergillus flavus* and *Aspergillus parasiticus*, and have adverse effects on humans, animals, and crops that result in illnesses and economic losses (Hussain and Anwar, 2008). Aflatoxin M1 (AFM1) is the hydroxylated metabolite of aflatoxin B1 (AFB1) and can be found in milk or milk products obtained from livestock that have ingested contaminated feed (Ardic *et al*., 2009). AFM1 has a potency approximately one order of magnitude lower than that of AFB1 (Prandini *et al*., 2009). Milk and milk products are a good source of many nutrients such as proteins and calcium and are mainly consumed by children. According to the Food and Agriculture Organization of the United Nations, at least 25% of the world's food crops are contaminated with mycotoxins and the production of agricultural commodities is barely sustaining the increasing population of the world. Therefore, the presence of AFM1 in milk is a concern. On the other hand, milk is not only consumed as liquid milk, but also utilised for the preparation of infant formulas, yoghurt, cheese, and milk-based confectioneries including chocolate, and pastry (Gürbay *et al*., 2006). Therefore, it is important to determine AFM1 levels in milk and dairy products in order to protect consumers of various age groups from its potential hazards (Fallah *et al*., 2009). Milk and dairy products are considered as a part of the main nutrient in Jordan. However, the percentage of daily consumption could reach 100% and may change depending on the economic status of people. Therefore, it is important to determine not only AFM1 levels in certain milk samples, but also routine-monitoring surveys should be considered in this regard (Kav *at al*., 2011). Due to the high toxicity and carcinogenic properties of AFM1, its presence in milk is a concern. AFM1 is resistant to thermal inactivation, pasteurisation, autoclaving and other varieties of food processing procedures (Boudra *et al*., 2007; Hussain *et al.,* 2008). Thus, to produce high quality milk, it is essential to keep feeds free from contamination by AFB1 (Sadeghi *et al.,* 2010). The aim of this study is to investigate the presence of AFM1 in various types of milk samples consumed in Jordan by enzyme linked immunosorbant assay (ELISA).

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Key words: Aflatoxin M1; Raw animal milk; Pasteurised milk; Infant formula; ELISA.

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## Materials and Methods

### Sampling

A total of 175 samples composed of raw cow milk (50), raw sheep milk (20), raw goat milk (20), raw camel milk (10), pasteurised cow milk (30), evaporated milk (10), full cream powdered milk (15) and infant formula milk (20) were collected during 2014-2015. All milk samples were thawed gradually at 4°C and then vigorously mixed.

### Sample preparation

For raw milk samples and evaporated milk, 5 mL were incubated for 30 min at 4°C, and centrifuged at 3000 g for 10 min. The milk serum below the fat layer was sampled and directly assayed for AFM1 using a specific ELISA kit (Romer Labs, Singapore). For full-cream milk powder and infant formula, 9.1 g of the powder was dissolved in 100 mL double-distilled water, the solution was warmed up to about 50°C and homogenised using a magnetic stirrer. Then, the sample was prepared as described above for raw milk sample.

### Analysis of aflatoxin M1 in samples by competitive enzyme linked immunosorbent assay

Two types of ELISA kits were used: AgraQuant aflatoxin M1 fast (100/2000 ng/kg) (Romer Labs) and AgraQuant aflatoxin M1 sensitive (25/500 ng/kg) (Romer Labs). These kits were stored at 2-8°C. Before their use, the kits were left for 1 h at room temperature.

The kits were used according to the manufacturer's instruction: AFM1-antibody-coated microtiter plate (supplied with the kit) was pipetted into each well (100 µL/well/standard). Test samples were also pipetted in duplicate (100 µL/well/sample). The plate containing the samples was incubated at room temperature for 60 min using a titer plate shaker at a speed of approximately 100 rpm. Following a washing step with washing solution (supplied with the kit), AFM1 conjugate was added to the wells, and the plate was incubated again at room temperature for 30 min on a microtiter plate shaker at a speed of approximately 100 rpm. The plate was washed with the washing solution in order to remove the unbound conjugate. A 100 µL of substrate solution was added into the wells and the reaction was allowed to proceed in the dark for 40 min at room temperature, at the end of which a blue colour developed. The reaction was stopped by adding 100 µL of stop solution to the wells, and the colour changed from blue to yellow. The absorbance was measured at 450 nm in Multiskan Ascent ELISA Plate Reader (LabSystems, Vantaa, Finland), and the absorption intensity was found to be inversely proportional to AFM1 concentration in the samples. The log-logit AFM1 sheet supplied with the kit was used to generate a standard curve and to calculate the concentration of AFM1 in the samples.

### Calculation of extrapolated values of aflatoxin B1 concentration in animal feeds

Many researchers reported that there was a linear relationship between the amount of AFM1 in milk and AFB1 in feed consumed by the animals like cows, sheep and goats. It has been suggested that only 1.6% of ingested AFB1 is converted to AFM1 by the dairy cattle. The values of AFB1 in dairy cattle feeds are extrapolated from the back calculation of the values of AFM1 obtained from the analysis of cow milk samples. Therefore, the values of AFB1 contamination in dairy animal feeding stuffs were back calculated by the formula given below (Price *et al*., 1985): AFB1 µg/kg=[AFM1 (ng/kg)×100]/1.6*1000.

## Results and Discussion

### Performance of analytical method

The ELISA method was validated to ensure data quality. Validation of ELISA was carried out by determination the recovery and the mean variation coefficient for fresh milk spiked with different concentrations of AFM1 (20, 100, 300 ng/L) and analysis of AFM1 in fresh milk. The recovery of AFM1 in spiked milk samples was found to be 102.1% [coefficient of variation (CV)=3.12], 94% (CV=1.11) and 99% (CV=1.06) for spiking concentration of 20, 100, 300 ng/L, respectively. All experiments were made in five times (Table 1).

### Occurrence of aflatoxin M1 in infant formula and other milk products

All samples from infant formula, full cream powdered milk and evaporated milk were contaminated with AFM1. The average of AFM1 in each group was 120.26, 103.95 and 195.91 ng/kg ranging between 16.55-154.14, 18.0-288.68 and 149.39-264.8 ng/kg, respectively (Table 2). European Community (EC) and Codex Alimentarius prescribe a limit of 50 ng/kg AFM1 in milk and 25 ng/kg for infant milk products. However, US regulation fixed the limit to a maximum of 500 ng/kg for milk and 25 ng/kg for infant milk products. In Austria and Switzerland the maximum level is further reduced to 10 ng/kg for infant food commodities (European Commission, 2006).

There are differences in maximum permissible limit of AFM1 in various countries, and some, including Jordan, have no legal limit for AFM1 in milk and dairy products. In general, the amount of AFM1 in 85% of infant formula samples, 33.3% of full cream powdered milk, and all evaporated milk was higher than the maximum tolerance limit accepted by EC/Codex limits. All tested samples were accepted according to the US regulation recommended limit.

### Concentration of aflatoxin M1 in different milk samples

All samples from cows, sheep, goats, camels and pasteurised cow milk were found to be contaminated with AFM1 (Table 3). The range of contamination levels varied among different categories of milk samples. In total, 66% of fresh animal milk samples exceed EC regulation and 23% was unacceptable according to the US regulation (Table 4). Pasteurised cow milk showed the highest value of contamination (216.78 ng/kg) range of 14.60-216.78 ng/kg with a mean value of 59.45 ng/kg. Pasteurisation plants in Jordan usually collect milk from different farmers at different locations in the country and collected milk is pooled and pasteurised. No testing of milk for contamination with AFM1 is done prior to pasteurisation.

AFM1 contamination in camel milk was relatively lower than that in other animal milk samples. Camels in Jordan freely graze and feed on wild plants for major part of the year; they are fed with stored grains for two to three months only. Other animals are fed with manufactured feedstuffs made of various stored grain products and by products of agricultural industry. The latter type of feedstuff is prone to fungal infection and to subsequent contamination with aflatoxins during storage (Dashti *et al.,* 2009; Kamkar, 2006).

The findings of this study are in close agreement with the results reported by previous investigators, confirming the presence of AFM1 in milk and other dairy products. Hussain *et al*. (2010) studied AFM1 contamination in milk from five dairy species in Pakistan and found that 15.8% of contaminated buffalo milk samples and 20% of contami-

**Table 1. Performance of analytical method for enzyme linked immunosorbent assay of aflatoxin M1.**

| AFM1 spiked (ng/L) | Repetitions | AFM1 (ng/L) | Recovery (%)* | CV (%) |
|---|---|---|---|---|
| 20 | 5 | 20.4 | 102.1 | 3.12 |
| 100 | 5 | 94 | 94 | 1.11 |
| 300 | 5 | 297 | 99 | 1.06 |

AFM1, aflatoxin M1; CV, coefficient of variation. *Determined by the following formula: detected AFM1 (ng/mL) divided by the concentration of AFM1 used for spiking and multiplied by 100.

**Table 2. Occurrence of aflatoxin M1 in baby infant formula and other milk products.**

| Sample | N | Positive samples (%) | AFM1 contamination (ng/kg) | |
|---|---|---|---|---|
| | | | Range | Mean±SD |
| Infant formula | 20 | 100 | 16.55-154.14 | 120.26±33.54 |
| Full cream powdered milk | 15 | 100 | 18.0-288.68 | 103.95±76.56 |
| Evaporated milk | 10 | 100 | 149.39-264.82 | 195.91±34.72 |

AFM1, aflatoxin M1; SD, standard deviation.

**Table 3. Concentration of aflatoxin M1 in different milk samples.**

| Samples | N | Mean±SD (ng/kg) | Range (ng/kg) |
|---|---|---|---|
| Fresh cow milk | 50 | 68.91±23.15 | 9.71-129.79 |
| Fresh sheep milk | 20 | 70.25±14.85 | 23.56-137.18 |
| Fresh goat milk | 20 | 60.25±33.41 | 20.25-125.89 |
| Fresh camel milk | 10 | 37.15±12.10 | 23.57-96.52 |
| Pasteurised cow milk | 30 | 59.45±42.12 | 14.60-216.78 |

SD, standard deviation.

**Table 4. Aflatoxin M1 contamination in different kinds of milk samples, exceeding limits established by the European Community/Codex and United States regulations.**

| Sample category | Positive samples (%) | Exceeding EC regulation (%) | Exceeding US regulation (%) |
|---|---|---|---|
| Fresh animal milk | 100 | 66 | 23 |
| Pasteurised animal milk | 100 | 12 | 0 |
| Baby infant formula | 100 | 85 | 85 |
| Full cream powdered | 100 | 33 | 0 |
| Evaporated milk | 100 | 100 | 0 |

**Table 5. The extrapolated aflatoxin B1 concentration in animal feeds.**

| Sample category | Positive samples | Extrapolated AFB1 | Exceeding EC/Codex regulation |
|---|---|---|---|
| Fresh cow milk | 100% (50) | 0.6-8.1 | 60% (30) |
| Pasteurised cow milk | 100% (30) | 0.9-13.5 | 73% (22) |

AFB1, aflatoxin B1. Values other than percentages are expressed as µg/kg.

nated cow milk samples were above the EU limit, while none of the contaminated milk samples from goat and sheep milk exceeded the EU limit. No contamination of AFM1 in the analysed samples of camel milk samples was detected. AFM1 was determined in dairy cattle milk samples in Khartoum State of Sudan: it was found that 95.45% of collected samples were contaminated with AFM1 with 2.07 ppt (Elzupir and Elhussein, 2010).

Accordingly, to produce high quality milk, it is essential to keep feeds free from contamination by AFB1 (Polychronaki *et al.,* 2007). In Jordan, the pasture is not widely available; feeding dairy animals with feedstuffs is more common, especially at milk industry. Therefore, it is important to inform producers and consumers about the toxicity potential of aflatoxins in order to reduce their potential health risk and economic loss.

### The extrapolated values of aflatoxin B1 concentration in animal feeds

Results of AFM1 concentrations found in cow milk samples tested in the present study indicates the likelihood that feeds provided to dairy cattle, as well as other dairy animals like sheep, goat, camels in Jordan, contain higher concentrations of AFB1 than those prescribed by the European Community (5 mg/kg) (Table 5). It is therefore important to monitor the levels of AFB1 in feedstuffs of dairy animals in Jordan and to devise mechanisms to improve their quality in such a way to reduce AFM1 contamination of milk and milk products (Oliveira and Ferraz, 2007).

## Conclusions

The contamination of animal milk samples with AFM1 was found to be higher than the European Community regulation limits, which indicates that Jordanian fresh milk may pose a serious public health problem to human health. For this reason, specific regulations to control AFB1 in animal feeds and AFM1 in milk products must be performed by Jordanian governmental agencies.

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