Original Research Article

Title: **Aflatoxin M1 Detection in Raw and Ultra-High Temperature Milk Sourced from the Markets of Lahore, Pakistan.**

Running Title: Aflatoxin M1 Detection in Unprocessed and High-Temperature Milk from Lahore

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**Novelty Statement:**

There is a lack of milk quality studies in Lahore markets, and HPLC analysis is rarely applied to assess the raw and UHT milk safety risks to the public. Most of the dairy industries are applying ELISA methods which have low sensitivity in detection as compared to HPLC. In our study, with the HPLC analysis, we have reported the raw milk available in local markets of Lahore is of poor quality and requires strict food safety and control measures. Moreover, some UHT milk samples contained high levels of AFM1, which is indicative of its heat stability.

**Abstract**

Aflatoxin M1 (AFM1) is a carcinogenic compound in milk and can put the population at health risk. This study examines the concentration of AFM1 in unprocessed (raw) and ultra-high temperature (UHT) milk samples from different local markets in Lahore, Pakistan. AFM1 samples were purified by high-performance liquid chromatography (HPLC) and immunoaffinity columns (IAC). AFM1 quantification in unprocessed milk samples surpassed the European Union (50 ng/L) and Food and Drug Administration (500 ng/L) tolerance limits by 76.7 and 10%, respectively. There were 26.67% of UHT milk samples that tested positive for AFM1, 23.3% greater than the European Union (EU) limit (50 ng/L), while none were greater than FDA limits (500 ng/L). Thus, both milk types containing AFM1 pose a serious health risk to end-users, especially children. Raw milk samples retained a mean of 205 ng/L of AFM1, while UHT milk contained 61 ng/L. Our results provide strong evidence of the heat stability of AFM1 and the importance of adhering to food safety and security recommendations.

**Keywords:** Raw milk; UHT milk; Aflatoxin M1; Carcinogenic; High-performance liquid chromatography; Health risk.

**Introduction**

Pakistan is a large producer of milk, ranking as one of the leading countries in the world for milk production therefore, the consumption of milk is increasing steadily ([Farooq 2013-2014](#_ENREF_12)). The consumption of unprocessed milk by more than 90% could be a potential source of aflatoxin AFM1(Muhammad et al. 2010). Aflatoxin M1 (AFM1) is detected in various agricultural and animal products, including ultra-high treated milk resulting from the ingestion of animal feed contaminated with this fungus. Aflatoxins are originally mycotoxins explicitly synthesized by *Aspergillus flavus* and *Aspergillus parasiticus.* Consequently, AFM1 represents a hydroxylated derivative of aflatoxin B1 ([Chifiriuc](#_bookmark4) *[et al](#_bookmark4)*[. 2010](#_bookmark4)). According to Ghazani (2009), AFM1 concentration in milk has linearity with AFB1 quantity in the consumed feed. Moreover, AFM1 is detectable in milk within the time span of 12 to 24hrs of humans or dairy animals consuming fungus-spoiled food and feed, respectively. Several authors have found that there is a strong binding capacity of AFM1 and casein as milk protein (Govaris *et al*. 2001, Kamkar *et al*. 2008, Abdallah *et al*. 2012).

All in all, this prevalence of natural toxins, such as mycotoxins, summons a major threat to food safety in under-developing countries. Several chronic diseases in humans and animals are associated with AFM1. However, children are more susceptible to this toxin due to their comparatively higher milk consumption (Abdallah *et al*. 2012, Al Zuhair and Omar 2012).

Hepatotoxicity, carcinogenicity, nutritional impairment, immunosuppression, and teratogenicity are the crucial alarming situations of AFM1 ingestion (Al Zuhair and Omar 2012, Caloni et al. 2006). As a consequence of its interactions with biosynthetic proteins, genetical material e,g. DNA and nucleoproteins, AFM1 exhibits a high degree of toxicity (Henry and Emmanuel-Ikpeme 201). The carcinogenicity of AFM1 ranges between 2 and 10% and both its genotoxicity and carcinogenicity have been demonstrated in vivo analyses (Amer and Ibrahim 2010). As reported by IARC (2002), based on these studies the international cancer research institute (IARC) has switched M1-toxin to Group-1 which has higher human carcinogenicity potency as compared to Group 2B.

In milk, the levels of AFM1 are not affected by the thermal treatment of food and no reduction in contamination has been observed (Bakirci 2001 Kamkar 2006; [Atasever *et al*. 2010](#_bookmark1)). Furthermore, despite the usual cold and heat techniques for milk preservation and treatment, there is no affirmation that contaminated milk alters AFM1 content and persistency (Atasever *et al*. 2010, [Anfossi *et al*. 2011](#_bookmark0)). The milk AFM1 standards have a variation of 50 to 500 ng/L, in accordance with the European Union and Food and Drug Administration, respectively (EC 2003b; U.S. Food and Drug Administration, FDA 2011). Milk plays an essential role in human nutrition, especially for infants, making commercially available milk and derived products a significant food hygiene risk. Therefore, the best way to limit susceptibility is to ensure that safe food is consumed and that regular monitoring of animal feed for AFM1 is an effective control (Cucci *et al*. 2007, Iqbal *et al*. 2011, Guergues *et al*. 2013). Through this research, the AFM1 contamination level is assessed through the unprocessed, loose, and UHT milk collection from the local markets of Lahore to evaluate the risk of M1- mycotoxin that can be posed to consumers and to stress the need for quality control measures of the milk.

**Materials and methods**

**Chemicals and Regents:**

HPLC-grade acetonitrile, purchased from Sigma-Aldrich (Steinheim, Germany), was used for the analysis. Procurement of AflaM1 TM-HPLC and IAC (Immunoaffinity columns) was done by the VICAM (Watertown, USA). Doubled distilled and Millipore water purification system (698 K) was used for the testing (Bedford, MA, USA). The standardized solution (AFM1, 10 µg/mL with acetonitrile) was sourced through Supelco (Bellefonte, PA, USA). Analytical grading of the used chemicals was ensured.

**HPLC Instrumentation**

In order to determine AFM1, the 2000.08 method was opted from Association of Official Agriculture Chemists (Egmond and Dragacci 2001).Thesamples were processed for AFM1 determination using an HPLC system (Agilent 1100 series, USA). In addition to the autosampler (LAS G1313A), the instrument was manifested with a fluorescence detector (FLD-G1321A). Whereas, in this experiment, the wavelengths of 365 and 435 nm were used for excitation and emission, respectively. A column, such as ZORBAX Eclipse XDB-C18 was used for the HPLC from Agilent company which contained particles 5 µm in diameter and 25 to 75% acetonitrile with water as the mobile phase.

**Sampling**

The samples of raw milk (n=30) were arbitrarily collected from markets in Lahore city. Milk samples were stacked in sterile polythene pouches to avoid contamination. The transportation of the samples was done in an ice cooler. The samples were taken to the quality laboratory of the University (UVAS) in Lahore and stored at -20 ºC until analysis. Similarly, UHT milk samples (n=30) were collected and stored at room temperature (25 ºC) for AFM1 analysis.

**Extraction**

Samples were heated to 37 °C in a water bath, centrifuged at 600 x g for 10 min and filtered through Whatman, filter paper after removing the surface fat layer. Fifty millilitres of milk samples were drawn up into a clean syringe barrel and connected to the immunoaffinity column. The flow rate of the test portion was 2 to 3 mL/min. With a volume of 20 mL of water, the column was rinsed moreover, the toxin eluted with acetonitrile for 60 sec. Eluted toxin drying was done in a water bath till the usage upon experiment (Egmond and Dragacci 2001; [Muhammad *et al*. 2010](#_bookmark1)

**Determination of AFM1 by HPLC.**

The eluted AFM1 was added to the mobile phase to determine milk toxin M1 by chromatography (Egmond and Dragacci 2001). Milk toxin M1 was determined by fluorescence detection. Each sample was processed by HPLC. The fixation of the flow rate at 0.8 mL/min opted for the HPLC. The standard stocks of the M1-toxin were prepared at the concentrations of 5.0, 10.0, 20.0, and 40.0 ng/L in acetonitrile and the calibration curve was determined. The retention time was reported to be 7.87 min. The calibration curve of standardized stock solutions (AFM1) with 5, 10, 20, and 40 ng/L and the coefficient of determination (r2) noted 0.9993.

**Statistical analysis**

The Z-test was used on Microsoft Excel 2013 to statistically calculate the means, standard deviations (SD), and significant variables among the means values of the M1 toxin.

**Results & Discussion:**

In this study, the statistical analysis revealed a significant difference in the calculated values of M1-toxin in unprocessed milk (Zcal ˃Zcrt). Also, the comparison of EU and FDA tolerable standards of AFM1 are given in Table.1.

**Table.1: AFM1 concentrations in raw and UHT milk compared to EU as well as FDA standards.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample | Min (ng/L) | Max (ng/L) | Mean (ng/L) | Std.Dev | >50 ng/L (EU) | >500ng/L (US) |
| Raw Milk | 11 | 729 | 205 | 185.24 | 23 (76.7%) | 3 (10.0%) |
| UHT Milk | 119 | 435 | 61 | 121.85 | 7 (23.3%) | Nil (0%) |

Twenty-five (83.33%) of untreated milk samples were positive for AFM1 whereas, 23 samples (76.7%) were higher than the European standards (50 ng/L) and 3 (10.0%) elevated the FDA tolerable quantity (500 ng/L). The noticeable, smallest count of the toxin was 11 ng/L however, the biggest was 729 ng/L. Meanwhile, the mean value of the M1-toxin observed was 205 ±185.24 ng/L.

According to Sani and Nikpoyaan (2013), 100% of their samples were reported with M1- toxin with an average amount of 16.16 ng/L. However, the highest quantification of the toxin was 64 ng/L which is very low in comparison to the 729 ng/L of the M1 toxin noticed in this study. Nina et al. (2014) estimated AFM1 content in cow milk samples, where the highest AFM1 content, in 6.7% of cow milk samples, was 162.3 ng/L. In a similar study conducted elsewhere in Pakistan elevated AFM1 levels with variations of 46 to 350 ng/L were detected in raw milk samples and 16.3% surpassed the EU limit (Iqbal et al.,2011). Moreover, Kamkar (2006) found the M1-toxin contamination rate of raw milk ranging from 0.00 to 2930 ng/L with 84.32% of positive samples presenting significant contamination. These reports highlight the poor-quality control practiced in developing countries. In contrast, of the UHT milk samples, 8 (26.67%) were positive, while 22 (73.33%) were negative. As shown in Table 1, 7 positive samples (23.3%) exceeded the permissible EU limit (50 ng/L). No evidence of a positive sample exceeding the FDA limit (500 ng/L) was detected. However, the lowest distinguishable concentration of 119 ng/L, whereas, the highest concentration of 435 ng/L, with 61 ±121.85 ng/L as the mean was recorded. This lowest concentration remained higher than the tolerable limit of EU and in comparison, evidently higher than unprocessed milk. It reflects the inconsistency of the AFM1 level in the milk and the probability to control the risk considering the food safety practices.

In some dairies around the world, there are no standardized practices for milk handling, processing, and monitoring system verification, which is an evident factor of M1 toxin retention in processed milk. According to Shundo et al. (2009), AFM1 was found in UHT milk in 95.2% of milk samples in Brazil, with quantification of 2 to 439 ng/L. Similarly, in Turkey, Bekirci (2001) quantified a range of AFM1 from 10 to 630 ng/L in sixty-seven UHT milk samples, with 31% exceeding the EU limit. Tekinsen (2008), also reported in Turkey, that 59% of UHT milk samples contained AFM1, and Cano-Sancho et al. (2010) determined M1 in milk with 94.4% of ultra-heat-treated samples in Catalonia, meanwhile, 9.69 ng/L was the noted mean of the toxin. However, in our study, the average value found in the packed treated milk was 61 ng/L. Although there is a significant difference between the Mean M1 toxin values of unprocessed and ultra-high temperature milk the possibility of getting a high quantity in packed milk is not neglectable, and the lowest quantity of packed milk was 119 ng/L which surpassed the EU standard. Therefore, this studyindicates that this milk toxin can survive at ultra-high temperatures. This endangered hydroxylated derivative of AFB1 must be tested to certify the safety and quality of the milk and the derived products.

**Conclusion**

This study concludes that AFM1 is a common contaminant in raw and UHT milk, making it a public health problem, especially in under-developing countries. Contaminated feed consumed by dairy cows leads to the marked formation of aflatoxin (M1) in their milk. Nowadays provincial food authorities, especially in Lahore, are focusing on the testing of AFM1 in the loose milk and raw material being used in dairy industries however, a stringent approach to reduce M1 toxin levels and preventive measures is to limit the formation of AFB1 in feed, which is the primary source of formation of AFM1 in the milk. In addition, feed should not be produced and stored under adverse conditions to prevent the accumulation of AFM1 in the bodies of dairy animals. Since storage conditions must be controlled, they should also be monitored regularly. As the incidence of aflatoxin-M1 in Ultra high-temperature milk reflects resistance to the toxin, the dairy industry must strictly control the quality of raw milk it receives for commercial processing. Food authorities must take the necessary measures to ensure food safety. It is essential to document the permitted aflatoxin limits and producers to prevent substandard dairy farming.

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**Authors Contribution**

IA, AAS, and MN planned and designed the study. SA and IA conducted the experiments and analysis of the results. TN helped in research work. AYS helped in writing the manuscript. The authors have critically reviewed the manuscript with consent and given approval of the article.

**Conflict of Interest**

 It has been ensured that none of the authors have any conflicts of interest.

 **Data availability**

The data presented would be produced on demand.

**Ethical Approval**

Not applicable to this paper.

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