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Original article

Determination of Aflatoxin M1 in Raw Milk: A Review of an Island Sample with the HPLC Method

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Summary. This study has been conducted to determine the frequency of aflatoxin M1 (AFM1) contamination in raw milk produced across six zones of Cyprus between 2018-2020 in accordance with seasonality and production regions. A total of 1,026 samples of raw milk were collected from the different production regions of the 6 provinces between September 2018 and August 2020. AFM1 analyses were conducted using the HPLC method. On average, AFM1 contamination of the total of 1,026 raw milk samples was determined to be 26.4±17.96 ng/L. It was found that only 11.4% of the raw milk samples exceeded the legal limit of the European Union (50 ng/L). AFM1 contamination incidence in raw milk samples is much higher in autumn and winter (average 31.77±19.21 ng/L and 26.96±20.77 ng/L, respectively) compared to spring and summer (average 19.00±18.53 ng/L and 7.51±11.31 ng/L, respectively). For this reason, it is important to monitor AFM1 contamination in raw milk in autumn and winter. This comprehensive study, which is known as the first of its kind in Cyprus, will promote the management and future risk analysis of AFM1 contamination in raw milk across the region.

Key words: Raw milk, aflatoxin M1, HPLC, food safety

Introduction

Aflatoxin M1, which can be found in milk and dairy products, can have adverse effects on the health of individuals who consume dairy products in large amounts (1, 2). These are compounds that have adverse effects on human and animal health, mainly due to their tumorigenesis, liver damage, immune system suppression, mutagenic, teratogenic, and carcinogenic effects (3).

Aflatoxins, which are produced by the mycotoxigenic moulds of the *Aspergillus* genus, are the most studied mycotoxins group in different foods and feed products. Aflatoxins are toxic fungal metabolites may be found in foods (4). They are mainly produced by *A. flavus, A. parasiticus and A. nominus* and they cause contamination of plants, plant products and other foods (5, 6). Among aflatoxins, Aflatoxin B1 (AFB1) is a particularly toxic, mutagenic and teratogenic compound that causes DNA damage, gene mutation, chromosomal abnormalities and cell transformation and it is defined as a class 1 human carcinogen (4, 7).

Mycotoxins, which are produced by certain mould species and have toxic, carcinogenic, mutagenic, teratogenic, and oestrogenic effects on humans and animals, can be present in dairy products due to two reasons (8, 9). The first one is the contamination of the feed consumed by dairy animals followed by the metabolization of the toxins in the feeds and the transfer of the metabolites to the milk which resultantly causes contamination, while the second is the formation of mycotoxins because of the direct exposure of the dairy product to mould contamination (8, 10).

When the animals are fed with feeds contaminated with AFB1 and AFB2, the toxin is metabolised in the digestive tract causing the formation of AFM1 and AFM2 in the milk, which becomes the monohydroxy derivative in the liver of the animal. AFM1 and AFM2 are excreted from the body of the animal via milk, urine and faeces. The level of AFM1 in the milk varies depending on the level of AFB1 the dairy animal has ingested through the feeds. AFM1 can be detected in raw milk from 6 to 24 hours after the ingestion of AFB1 by feeds, where it reaches its highest level within 12-48 hours and decreases 72-96 hours after the AFB1 intake is ceased (11, 12). However, this amount may change depending on the animal`s breed, lactation period and milk levels. In addition, since the AFM1 contaminated raw milk can contaminate all of the milk in the process tanks, even when it is added in very small amounts, the incidence of AFM1 in heat treated milk is higher compared to raw milk (13).

Aflatoxin production of moulds is affected by environmental changes such as temperature, pH, water capacity, moisture, and oxygen. Aflatoxins are only synthesized between 12-42 °C and the optimal temperature for synthesis is 25-35 °C (14). Depending on different conditions, the aflatoxin production duration may vary between 24 hours and 4-10 days. According to previous studies, it has been reported that the minimum water activity in foods should be 0.85 for aflatoxin production. Aflatoxins, which are extremely durable in normal temperatures, require temperatures above 300°C for complete decomposition. Therefore, "Low-temperature long-time" pasteurisation (30 minutes at 63-65 °C), "High-temperature short-time" pasteurisation (15-20 seconds at 72-75 °C), "Very-high temperature" pasteurisation (15 seconds at 85°C and 90°C) and ultra-high temperature (UHT) sterilisation (2-5 seconds at 135°C-150°C) methods do not decrease the aflatoxin amounts in milk (15).

Aflatoxins are easily synthesised in various grains, feed and feed raw materials and foods including peanuts, corn and cotton seeds that contain 15% or higher moisture (16). The presence of CO_2 and O_2 affect the reproduction of aflatoxins and formation of mould. The 20% CO_2 level in the air significantly suppresses the production of aflatoxin and mould growth. A 10% decrease in the oxygen concentration in the air suppresses the production of aflatoxin (17). Although moulds can grow within a wide range of pH (2.1-11.2), the optimum level of aflatoxin occurs at pH 6.0 (15, 18).

To determine AFM1 levels, immunochemical methods are used in general, including HPLC, ELISA (enzyme immunoassay), RIA (radioimmunoassay) and TLC (thin-layer chromatography) (19). The HPLC method is the most widely used and preferred due to its high precision and cost effectiveness (20, 21). The reasons for its wide use include its precision, ease of adaptability to quantitative determination, and suitability for the separation of non-volatile compounds or compounds that are easily degradable in heat. Most importantly, it is adaptable to many materials which are of primary concern for many fields of industry and the community (22, 23).

AFM1 levels in milk and dairy products are not affected by processes such as UHT, pasteurisation, fermentation, cold storage, freezing, concentration or dehydration (24). Since aflatoxins are not inactivated by processes such as UHT and pasteurisation, AFM1 can not only be found in milk, but also in dairy products such as yoghurt, cheese, butter, cream, ice-cream, and dairy-based desserts (16). As AFM1 does not disappear during production processes or heat treatments, it is very important to provide the utmost effective control of these metabolites in raw milk and dairy products according to the maximum residue levels determined by the European Union. According to the Codex Alimentarius standards, EU (European Union) regulations and the Turkish Food Codex, the maximum level of AFM1 in milk should be 50 ng/L (15, 18, 25, 26). The presence of AFM1 in milk and dairy products is very important for adults and especially children, who constitute the group that consumes these products the most. Compared to adults, babies and children are more susceptible to the negative effects of mycotoxins (8).

AFM1 levels in raw milk and dairy products vary depending on the country and geographical location (27). AFM1, which can be found in raw milk in high levels, poses a great threat for the public health. Our study aims to determine the AFM1 levels in samples of raw milk collected from dairy farms in Northern Cyprus and to evaluate the results with regard to public health, mainly due to the fact that no previous studies have been conducted on the aflatoxin levels in raw milk in our country.

Materials and Methods

Study Area and Choice

All milk production facilities and farms under the umbrella of the Dairy Industry Foundation (SUTEK) in Cyprus were included in the study. The milk samples were collected from dairy farms in all provinces – Nicosia (Zone 1), Kyrenia (Zone 2), Famagusta (Zone 3), Trikomo (Zone 4), Morphou (Zone 5) and Lefka (Zone 6) - in all four seasons between September 2018 and August 2020. The samples were analysed at the laboratories of the Dairy Industry Foundation (SUTEK) located in Nicosia.

Sampling

Of the total of 1,026 raw milk samples used in the study, 477 samples were collected from Zone 1, 83 samples from Zone 2, 357 samples from Zone 3, 63 samples from Zone 4, 38 samples from Zone 5 and 8 samples from Zone 6. The reason that the number of samples collected from Zone 4, Zone 5 and Zone 6 provinces were so low is because of minimal milk production in these regions. All samples were collected on site. Milk samples (1 L) were collected from each producer in separate sterile sample containers and transported under cold chain to the laboratory for analysis.

Measuring Methods

In the study, the HPLC (high performance liquid chromatography) method was used to detect the presence of AFM1 in raw milk samples.

AFM1 analysis by HPLC

Preparation of the AFM1 Standards. Calibration standards were prepared with the necessary dilutions from AFM1 standard solution, and the calibration curve was drawn by making the injections. The dilutions were arranged by making the necessary calculations according to the main stock concentrations of the obtained AFM1 standard (Biopharm Rhone Ltd. Aflatoxin M1 Standard is used).

Sample Preparation

Calculation of Aflatoxin M1 in HPLC. Approximately 100-150ml of each of the milk samples obtained from each producer were transferred to a 250ml beaker and heated on a heater up to 35-37°C. Afterwards, they were filtered through Whatman No: 4 filter paper. If the milk contained too much fat, it was centrifuged at 4000 rpm for 10 minutes and the thin fat layer collected at the top was removed and then it was filtered through Whatman No: 4 filter paper. 50ml of milk was taken from the filtrate and prepared to be suitable to flow through IAC immuno-affinity column chromatography. The IAC was brought to room temperature before use. All of the 50ml filtrate flowed through the IAC column at room temperature. The speed that the filtrate flowed through the IAC was approximately 3ml/min. The column was washed by passing 20ml of water through the IAC as the filtrate was about to finish. At the end of the wash, the column was dried by passing air through the IAC using a syringe.

Later, the toxins were obtained in a clean tube after a 12.5ml Methanol-Acetonitrile (20:30) mixture was passed through the IAC. Then, 1.25ml water was passed through the IAC and added to the tube so the total volume increased to 2.5ml. The mixture containing the toxins was transferred to a 2ml amber coloured vial and 100ul was injected into the HPLC. The sample was analysed using the calibration curve and the AFM1 value was determined. These results were divided by 2, representing the dilution factor, and the AFM1 level in the sample was then calculated.

Statistical Analysis

Results were analysed with SPSS statistics programme (IBM® SPSS © Statistics Version 18.0). The results are expressed as average, standard deviation, minimum and maximum AFM1 concentration. The

Mann-Whitney U test was used in order to determine whether there were any differences between the examined milk samples according to years.

Results

A total of 1,026 raw milk samples were collected between September 2018-August 2020. AFM1 levels in the raw milk samples were measured in all seasons (autumn, winter, spring and summer) during the years. The AFM1 levels in milk in the different seasons are presented in Table 1.

The recorded average AFM1 level was highest in autumn (31.77±19.21 ng/L), and lowest in summer (7.51±11.31 ng/L). The recorded average AFM1 levels in four different seasons in descending order were as follows: autumn (31.77±19.21 ng/L), winter (26.96±20.77 ng/L), spring (19.00±18.53 ng/L), and summer (7.51±11.31 ng/L). Seasonally, the percentage of milk samples above the legal limit allowed by the EU in autumn, winter, spring and summer were recorded as 16%, 16.2%, 4.5% and 1.6%, respectively (Table 1).

It was observed that the AFM1 levels increased in September, at the beginning of autumn, and reached the maximum level in November (35.88±18.22 ng/L) as the weather started to cool in Cyprus, which has a Mediterranean climate, and it reached the lowest level in August (5.84±8.45 ng/L). It was also identified that the AFM1 levels periodically increased towards the winter months and started to decrease as the weather began to get warmer. It was detected that there was a significant difference between the AFM1 levels in raw milk in the autumn and winter months compared to the levels in the spring and summer months ($p<0.05$) (Table 1).

Figure 1 shows the seasonality of the AFM1 levels and monthly shifts. AFM1 levels were found to be highest in autumn and lowest in summer. Compared to

Table 1. Average AFM1 levels in raw milk between 2018 – 2020 according to seasons

Months	N	Mean (ng/L)	Min – Max (ng/L)	Above EU limit (%)
Autumn				
September	38	33.68±43.56	0-271	13.2
October	114	29.49±23.24	0-91	13.8
November	167	35.88±18.22	0-107	21.2
Total	319	31.77±19.21	0-104	16.0
Winter				
December	338	32.29±22.99	0-157	22.5
January	166	34.95±19.02	0-83	19.9
February	175	14.77±18.73	0-80	6.3
Total	492	26.96±20.77	0-112	16.2
Spring				
March	97	19.49±20.52	0-96	8.2
April	19	21.78±15.32	0-58	5.3
May	25	14.52±10.55	0-38	0.0
Total	138	19.00±18.53	0-96	4.5
Summer				
June	21	11.61±16.87	0-75	4.8
July	27	7.00±8.00	0-18	0.0
August	32	5.84±8.45	0-27	0.0
Total	77	7.51±11.31	0-75	1.6

There was a significant difference between the AFM1 levels in raw milk in the autumn and winter months compared to the levels in the spring and summer months (respectively p values; autumn-spring p= 0.01, autumn-summer p= 0.00, winter-autumn p= 0.00, winter-summer p= 0.00).

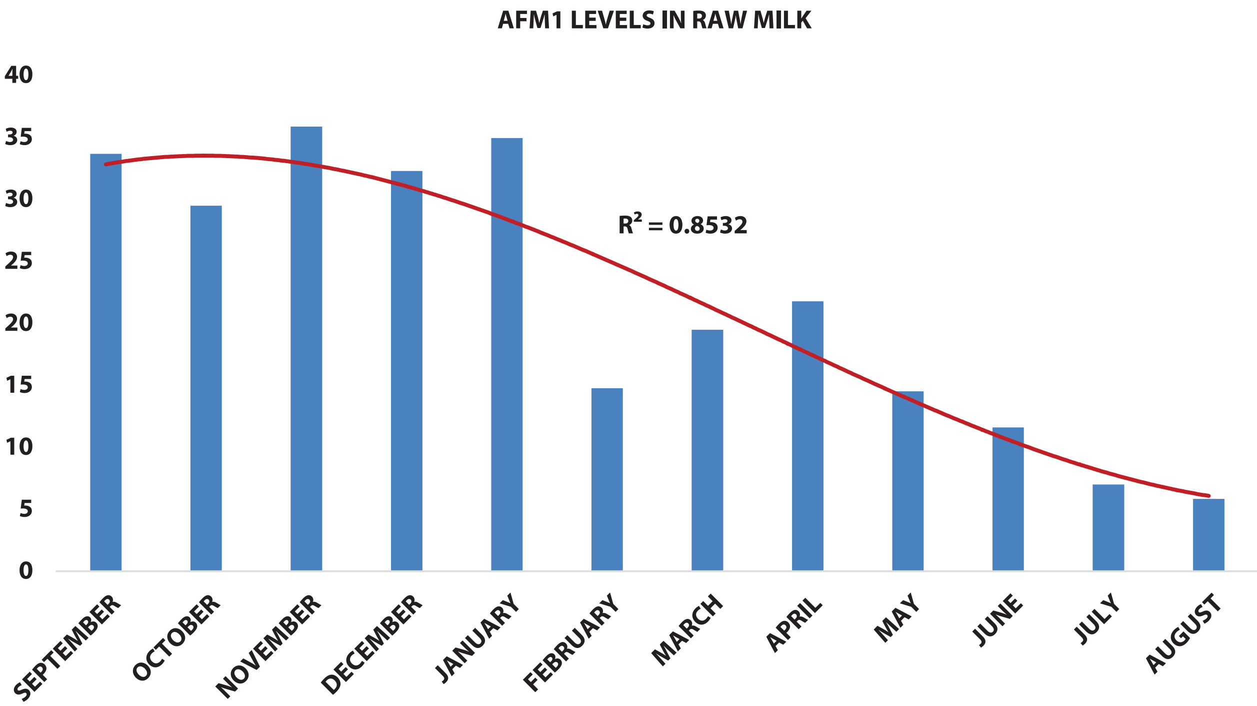

Figure 1. The three-year monthly average frequency of aflatoxin M1 (AFM1) levels in raw milk between 2018 and 2020. The horizontal axis depicts the months. The vertical axis depicts the AFM1 levels (ng/L). The columns represent the relevant average frequency in the specified month. The curve depicts the trendline across the months (R^2 = 0.8532).

the spring and summer months, the incidence of AFM1 levels being above the limits (>50 ng/L) was higher in the autumn and winter months, as the weather cools and rainfall increases. Of the analysed milk samples, it was detected that 16.0% in autumn, 16.2% in winter, 4.5% in spring and 1.6% in summer were above the EU legal limit (50 ng/L). The AFM1 levels of milk samples taken in different seasons were significantly different (Table 1).

The farmers' association was contacted with regard to dairy corporation (i.e., SUTEK). The association acts as a regulatory body to enable clean and accessible milk throughout the population. All of the areas that supplied milk – comprising six zones - were studied. The distribution of all Zone 1 (13.5%), Zone 6 (12.5%), Zone 5 (11.7%), Zone 2 (10.1%), Zone 3 (9.9%) and Zone 4 (7.9%) is as follows (Table 2).

An average of 26.4±17.96 ng/L AFM1 was detected for the total of 1,026 raw milk samples analysed over 2 years within the scope of this study. Detected AFM1 levels of the analysed milk samples according to years are displayed in Table 3. Of the raw milk samples examined by years, it was detected that they contained 30.91±19.04 ng/L, 28.41±18.61 ng/L and 19.95±16.23 ng/L AFM1 for 2018, 2019, and 2020, respectively.

Table 2. Stated according to distribution of AFM1 rates based on provinces that exceeds the EU legal limit

Zone	N	Above EU limit (%)
Zone 1	477	13.5
Zone 6	8	12.5
Zone 5	38	11.7
Zone 2	83	10.1
Zone 3	357	9.9
Zone 4	63	7.9

Discussion

AFM1 toxins, which can be found in milk and dairy products that are among the essential food sources for humans, pose a potential risk for human health. For this reason, it is of utmost importance that the presence and level of AFM1 in milk and dairy products are identified.

Table 3. Average Aflatoxin M1 levels according to years

Year	N	Mean (ng/L)	Min-Max (ng/L)	Above EU limit (%)
2018	311	30.91±19.04	0-85	17.6
2019	397	28.41±18.61	0-82	12.2
2020	318	19.95±16.23	0-96	4.4
Total	1026	26.4±17.96	0-96	11.4

In this study, we analysed 1,026 raw milk samples taken over a period of two years using the HPLC method and detected an average of 26.4±17.96 ng/L AFM1 level where 11.4% of the samples exceeded the legal limits. In a similar study carried out in Malaysia in 2016, it was detected that 35.8% of the 53 milk and dairy product samples contained AFM1 and 7.5% exceeded the legal limits (28). In another study conducted in Italy in 2016, it was detected that 12.3% of 416 raw milk samples contained AFM1 levels above 0.05 µg/L (29). In a field work carried out in Elazig in 2018, it was detected that 27% of 60 raw milk samples did not contain AFM1, 60% contained AFM1 within the legal limits and 13% contained AFM1 above the legal limits (30). In a research conducted in Kars in 2019, the AFM1 levels of 50 raw milk samples were found to be below the limits in 44% of the samples and 56% of the samples contained different levels of AFM1 (31). In Kenya in a project conducted in 2018, a total of 291 milk samples were collected (raw, pasteurised, UHT milk and yoghurt) and AFM1 levels were analysed using ELISA kits. As a result of the study, it was found that 50% of the samples exceeded 50 ng/kg (32). According to the results of a study that included 221 raw milk samples collected from four different dairy farms during autumn 2011 in Varamin, a region in Tehran province of Iran, it was detected that 26.7% of the analysed samples were contaminated with AFM1, which confirmed that preventive measures are necessary to reduce contamination (33). In an investigation conducted in Turkey in 2018, it was reported that the AFM1 concentration detected in 135 milk samples was 8.6 ± 4.57 ng/L and none of the samples exceeded the AFM1 limits set out by the Turkish Food Codex Regulation (34). AFM1 levels vary between countries and according to the geographical location. According to these studies, it is evident that there is a seasonal variation in the presence of AFM1. It is stated that, compared to the summer months, the moisture levels in feeds increase in winter months due to the increased rainfall, the animals are generally given mixed feeds and the feeds given to animals are subject to poor storage conditions in winter, which means that the incidence of AFM1 is higher compared to the warmer seasons (32, 35, 36).

This study also found that AFM1 levels vary according to the seasons. The average recorded AFM1 level was the highest in autumn and lowest in summer. In order from high to low, the AFM1 levels detected in the four different seasons of the year were autumn, winter, spring, and summer, respectively. The reason why the AFM1 levels are higher in the autumn and winter months compared to summer is due to the increased rainfall, suitable heat and higher moisture levels in autumn and winter, which are conducive to toxin production in addition to the fact that the animals are generally given mixed feeds (37). In a research conducted in China in 2016 with 5,650 raw milk samples, it was reported that the incidence of AFM1 contamination was much higher in winter months (10.2%) compared to spring, summer and autumn months (3.0%, 2.1%, 4.4%, respectively) (38). In a study conducted in Pakistan between 2013-2014 with 520 raw milk samples, the detected seasonal prevalence of AFM1 from high to low was identified to be winter, spring, autumn and summer, respectively. They stated that 53% of the milk samples exceeded the legal limits and that the community was at high risk of being exposed to health problems related to AFM1 (27). In another study carried out in 2019 in Ecuador, 209 raw milk samples were collected in both the dry (June and August) and rainy (April and November) seasons. The study findings indicated that the AFM1 levels in 59.3% of the milk samples exceeded the EU legal limits, whereas only 1.9% exceeded the legal limit in Ecuador. As a result of the study, significant differences were found between provinces with respect to months, season, and climate (39). In another report in 2017, which researched the seasonal differences between AFM1 levels, it was reported that among the 360 raw milk samples collected from the Polog and Pelagonia regions, the highest sample incidence that exceeded the maximum residual level (MRL) was observed in the winter months in both regions (0.135 $\mu g.kg^{-1}$; 1.003 $\mu g.kg^{-1}$ respectively). Considering the seasonal variation, it was reported that AFM1 incidence and levels in raw milk samples obtained in the winter months were significantly higher than the samples obtained in summer and there was a statistically significant difference between the samples obtained in spring and summer (40).

In this study, when we compared the AFM1 levels by years, while the AFM1 levels were higher in 2018, a decrease was observed in 2020 due to inspections and trainings. The reason for this is that the dairy institution conducts regional public trainings every year and inspects the milk and dairy product producers. A significant difference was detected between years. It can be stated that the reason that the AFM1 levels were higher in 2018 and then decreased in 2020 is due to the state inspections and trainings given to producers. In an analysis in Italy, which researched the presence of AFM1 in a total of 31,702 milk samples from six dairy processing facilities between April 2013 and December 2018, it was reported that the AFM1 levels detected in 2017 and 2018 were approximately the same as the AFM1 levels detected between December 2014 and December 2015. In addition, the results indicated that AFM1 contamination between September 2015 and December 2016 was almost as high as in 2013. In this comprehensive study, it was also reported that the variability of climate conditions which affect the AFB1 contamination in feed and thus AFM1 contamination in milk for years, justified the continuous surveillance and updating of risk assessment (41). In Egypt, 120 raw milk samples were examined to determine the AFM1 levels in different seasons in 2016 and 2017. For the two years, the proportions of samples that exceeded the legal limits were reported as 21.6% and 18.3%, respectively. Despite the seasonal changes that affect the production of AFM1, it has been reported that AFM1 levels were significantly higher ($P \leq 0.001$) in the samples collected in winter compared to those collected in summer (42).

Conclusion

In this study, the use of the HPLC method provided fast results with high precision. As a result of the study, we detected that the AFM1 levels in raw milk samples were in accordance with the European Commission and Turkish Food Codex limits (<50 ng/L) and pose no risk for public health. According to previous results presented in the literature, in Cyprus, the detected AFM1 levels are lower than other countries, and it can be observed that the AFM1 levels have significantly decreased over the years as a result of the milk and dairy product producers training provided. However, we detected different concentrations of AFM1 in the samples we examined. The incidence of AFM1 contamination was particularly high in autumn and winter due to the rainfall and moisture. Therefore, dairy production must be closely monitored and inspected, particularly during these seasons.

The limitations of the study may be that the milk and dairy product producers producing unregistered milk could not be identified.

Regular trainings and seminar programs should be provided for milk and dairy product producers, animal breeders, dairy processing facility owners and employees to raise awareness of the potential health problems caused by aflatoxins and their consequences. Public health will be protected by implementing Good Agricultural Practice, (GAP) and increasing awareness of the producers and consumers.

In addition, forming databases and detecting any possible changes in AFM1 levels in foods may be a useful step towards managing any potential emergencies.

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