Evaluation of analytical assays efficiency to detect aflatoxin M₁ in milk from selected areas in Egypt and South Africa

Mulunda Mwanza,*¹ Ahmed Abdel-Hadi,†‡ Ahmed M. Ali,§ and Mary Egbuta*

*Department of Animal Health, Faculty of Agriculture and Technology, Mafikeng Campus, North West University, Private Bag X2046, Mmabatho 2735, South Africa

†Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut Branch, 71524 Assiut, Egypt

‡College of Applied Medical Sciences, Medical laboratories Department, Majmaah University, Kingdom of Saudi Arabia

§Chemistry Department, Faculty of Science, Al-Azhar University, Assiut Branch, 71524 Assiut, Egypt

ABSTRACT

Recently, methods to analyze aflatoxin M₁ (AFM₁) in milk and dairy products have been developed for both screening purposes (i.e., rapid, economical, and simple methods) and for confirmation by accurate, reproducible, and sensitive quantification. The aim of this study was to evaluate the efficiency of different rapid kits and techniques available on the market by using different analytical methods: thin layer chromatography (TLC), immunoaffinity column, AFM₁ immunochromatographic strip, and ELISA; some samples were also submitted to HPLC for comparison of results. One hundred thirty-eight samples were collected from rural subsistence and commercial dairy farms in selected areas of Egypt and South Africa and analyzed for the presence of AFM₁. The results obtained by AFM₁ immunochromatographic strip indicated the lowest frequency of occurrence, with a detection incidence of 20.45% in Egyptian samples and 16% in South African samples. Aflatoxin M₁ was detected by ELISA in 65 (73.9%) Egyptian milk samples, with a range of 8.52 to 78.06 ng/L, and in 34 (68%) South African milk samples, with a range of 5 to 120 ng/L. A higher incidence of AFM₁ in Egyptian milk samples was shown by TLC (81.8%) compared with ELISA (73.9%). Samples analyzed by ELISA in South African milk samples demonstrated satisfactory correlation when compared with HPLC coupled with Coring cell (an electrochemical cell for the derivatization of AFM₁). Among the positive samples, 18 of the Egyptian samples (20.45%) positive by ELISA had levels of AFM₁ above the European Union (EU) regulatory limit (50 ng/L), whereas 65 samples (73.9%) were above the Egyptian regulatory limit (0 ng/L). Six of the South African samples (12%) tested by ELISA were above the South African (50 ng/L) and EU regulatory limits. The mean concentra-

tion of AFM₁ was 25.79 ng/L in Egyptian samples and 17.06 ng/L by ELISA and 39 ng/L by HPLC in South African samples. These contamination levels would not represent a serious public health hazard according to EU legislation.

Key words: aflatoxin M₁, milk, thin layer chromatography, HPLC, ELISA

INTRODUCTION

Aflatoxin contamination in animal feed and human food remains a major concern; aflatoxins have carcinogenic properties and are found worldwide, especially in warmer and humid climatic regions (Whittaker et al., 1991). The most common aflatoxins are aflatoxins B₁ and B₂, which contaminate mainly cereals, and aflatoxin M₁ (AFM₁), which is a hydroxylated metabolite of AFB₁ that is excreted in milk by cows fed a diet naturally contaminated with aflatoxin B₁ (Sørensen and Elbæk, 2005). Reference is frequently made to aflatoxin B₁, which is the most prevalent and the most toxic form (Whitlow et al., 1998). Aflatoxin M₁ has been demonstrated to be cytotoxic on human hepatocytes in vitro, and its acute toxicity in several species is similar to that of aflatoxin B₁; liver cancer has been related to dietary intake of aflatoxins (Henry et al., 2001; Makun et al., 2012). Yousef and Marth (1989) extensively reviewed information on the stability of AFM₁. Studies have shown no significant changes in AFM₁ concentration after heat processing (pasteurization or boiling) or UHT processing (Patel et al., 1981; Galvano et al., 1996). Aflatoxin M₁ is, in general, stable in cheese, yogurt, pasteurized milk, fat-free or whole milk, and ice cream. In addition, it resists some sterilization processes, pasteurization, and direct heating processes (Mwanza et al., 2013).

South African and Egyptian agriculture is mostly divided into commercial and small-scale farming; 95% of the rural population relies on small-scale farming. There is no clear boundary between these 2 types of farming and activities; for example, farmhouse cheese

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¹Corresponding author: Mulunda.Mwanza@nwu.ac.za

making is not strictly a formally regulated activity. Due to the potential health hazard of aflatoxins, and AFM₁ in particular, it is important to monitor and regulate the levels of AFM₁ in milk and milk products through control of animal feed quality to ensure consumer safety (Whitlow et al., 2010). Milk and derived products can consequently be implicated in the spread of aflatoxins. Therefore, most countries have established maximum residue levels of AFM₁ in milk, ranging from the 50 ng/L established by the European Union (EU; European Commission, 2006) to the 500 ng/L established by US Food and Drug Administration (US FDA, 2012). South Africa has established an acceptable level of AFM₁ in milk and milk products of 50 ng/L. However, Egypt has decided on a zero-tolerance strategy to ensure maximum consumer health protection at the expense of milk and cheese producers (EOSQC, 1990; Amer and Ibrahim, 2010). However, this target (0 ng/L) will be difficult to achieve as long as aflatoxin contamination in animal feed remains an issue.

Several methods of extraction and detection have been used or developed for detection of AFM₁ in milk and dairy products during the past decade. It is important to consider the type of matrix (fresh, stored, pasteurized milk, liquid or powder milk, cheese) because this can affect the results (Chen et al., 2005). In addition, most commercial kits or rapid tests are designed for a specific matrix, which makes the extraction of mycotoxins and AFM₁ from different matrices challenging and costly. The detection of AFM₁ in milk or milk products remains a challenge because of the typically very low concentrations. Therefore, sensitive methods are needed for extraction and detection. The most common analytical methods used for AFM₁ determination are thin layer chromatography (TLC), HPLC, and ELISA. Among these, TLC is often used for routine screening because it has many advantages: it is cheaper than HPLC methods and does not require extensively trained operators; however, it is less accurate than HPLC (Gilbert and Anklam, 2002). Due to the low cost and ease of use, TLC is the preferred detection method in developing countries. Recently, several groups have used ELISA for detection of AFM₁, which is not only a suitable tool for quick and sensitive analysis with high sample throughput (Lee et al., 2004), but is also cost effective and fast and requires only a small sample volume for analysis (Sherry, 1997). Most groups have used A kit made by R-Biopharm (Darmstadt, Germany; Sarimehmetoglu et al., 2004), part of the RIDAScreen range of diagnostics tests. With all ELISA techniques, a positive result needs to be verified by HPLC because no ELISA method has been given AOAC International approval (Henry et al., 2001). Another interesting application using antibodies is the use of an immuno-

matographic strip for detection of AFM₁. Wang et al. (2011) established a rapid competitive direct ELISA and a gold nanoparticle/immunochromatographic strip method for detecting AFM₁ in milk and milk products. The test uses monoclonal antibodies conjugated to colloidal gold particles that yield a visual pink band on the strip upon reaction with immobilized aflatoxin M₁-BSA. Although this technology is a sensitive and rapid assay (the whole analytical procedure can be completed in 10 min because no sample treatment is required), it can only give a positive or negative result. Several kits for rapid testing are available on the market but lack proper validation. Therefore, the aim of this study was to analyze and evaluate the occurrence of AFM₁ in milk samples from both commercial and subsistence farms in semi-arid areas of Egypt and South Africa and to evaluate the efficiency of different rapid kits and techniques available on the market to determine the best rapid test.

MATERIALS AND METHODS

Sampling

In this study, 138 fresh milk samples were collected randomly from dairy farms between April and October 2013 in selected areas of Egypt (Governorate of Assiut) and South Africa (Ngaka Modiri Molema District). Fresh milk samples were obtained directly from cows in sterile containers, transported in cooler boxes to the respective laboratories, and stored in freezers at -20°C until further analysis.

The research was approved after review by the National Research Foundation in South Africa and the Academy of Science in Egypt.

Aflatoxin M₁ Extraction Procedures

In this study, the method used by Mwanza et al. (2013) was used for AFM₁ extraction using an immunoaffinity column (Biopharm R) as follows: 5 g of salt (NaCl) was added to 50 mL of the milk sample and mixed. The mixture was centrifuged at $2,000 \times g$ for 10 min and the skim portion was filtered through a micro fiber filter (Vicam/Waters, Milford, MA). Ten milliliters of the filtered skim milk was passed through the immunoaffinity column at a rate of 1 to 2 drops per second until air came through the column. The column was washed twice with 10 mL of methanol:water (10:90) solution. Aflatoxin M₁ was then eluted with 2 mL of HPLC-grade methanol:acetonitrile (80:20) at a rate of 1 to 2 drops per second. Extracts were dried using nitrogen gas and stored in the freezer at -20°C until further analysis by HPLC and ELISA.

AFM₁ Detection and Quantification in Milk Samples by TLC

To confirm the presence of AFM₁, milk samples were extracted according to AOAC official method (AOAC International, 2008; method no. 980.21). One hundred milliliters of milk sample was centrifuged at $3,500 \times g$ at 10°C, and the fatty layer was removed. Fifty milliliters of defatted milk sample, 10 mL of salt solution, and 120 mL of CHCl₃ were mixed in a 250-mL separator (STS 200 system, Separator Technology Solutions, Melbourne, Australia) for 60 s. The lower CHCl₃ layer was drained into a 125-mL Erlenmeyer flask. Then, 20 µL of AFM₁ standard (100 µg/mL) and 20 µL of the extracts were dispensed on TLC plates and 3 different solvent systems—dichloromethane:acetone:propan-2-ol (85:10:5), diethyl ether:methanol:water (94:4.5:5), and dichloromethane:acetone:methanol (90:10:2)—were used for the detection of AFM₁ on TLC (Mwanza et al., 2013). In this study, the detection limit of AFM₁ by TLC was 350 ng/L.

Determination of AFM₁ Using a Rapid Detection Method (Strip Kits)

Aflatoxin M₁ strip test kits (AuroFlow) were purchased from Bioo Scientific (cat. no. 1101-01; Bioo Scientific, Austin, TX). The assay was carried out by adding 200 µL of milk sample, the positive control (milk spiked with 1,000 ng/L of AFM₁), and the negative control (supplied by manufacturer) into reaction wells containing lyophilized gold particles. Subsequently, after incubation of the plate of reaction wells for 4 min at ambient room temperature, the strip was dipped into the wells vertically. After 4 min, the strip was removed and placed on a horizontal surface with the unmarked side facing up. The strip was allowed to develop color for 1 min after removing it from the well, and the test results were determined visually (Figure 1). The detection limit of this strip was 500 ng/L.

ELISA

Commercial ELISA kits (RIDAScreen aflatoxin M₁, cat. no. R1101) were purchased from R-Biopharm. The detection limit for milk samples was 5 ng/kg, with recovery rates of 95% for milk. All milk samples were prepared and defatted using the method outlined in the ELISA kits, as briefly described here. Twenty milliliters of liquid milk was centrifuged at $3,500 \times g$ at 10°C. The fatty layer was removed and 100 µL of the defatted milk was applied directly in the ELISA kit for AFM₁ determination.

HPLC Analysis

The HPLC analysis of AFM₁ was done according to Bakirci (2001) and Manetta et al. (2005) using a UHPLC system (Shimadzu, Kyoto, Japan) consisting of liquid chromatograph (LC 20A) fitted to degasser (DGU 20A3), auto sampler (injection; SIL 20A), communications bus module (CBM 20A), column oven (CTO 20A), photodiode array detector (SPD M20A)

Figure 1. Illustration of aflatoxin M₁ (AFM₁) strip test results. The assay used a competitive colloidal gold-based format. A T-line (test) signal intensity that is stronger than that at the C-line (control) indicates a negative result; a T-line signal intensity that is equal or less intense than that at the C-line indicates the presence of 0.5 µg/L or greater of AFM₁ in milk. Color version available online.

Table 1. Mean recovery of mycotoxin from milk by HPLC

Mycotoxin standard ¹	Spiked concentration (ng/10 mL of milk)	Mean ($\pm$ SD) recovery (ng)	Percentage recovery (%)
Aflatoxin M ₁ immunoaffinity column	100	9.8 ($\pm$ 1.3)	98
Aflatoxin M ₁ SPE (C ₁₈ column)	100	65 ($\pm$ 5.4)	68

¹Immunoaffinity column was from Bio-Pharm, ASA (Darmstadt, Germany); SPE (solid-phase extraction) column was from ThermoScientific (Waltham, MA).

and fluorescence detector (RF 10AXL), all connected to a computer with Intel Core Duo processor and Microsoft XP (Microsoft Corp., Redmond, WA). The analysis of AFM₁ was done using a fluorescence detector (RF 10AXL) coupled to a reverse phase C₁₈ column (Waters, Milford, MA) and a Coring cell (CoBrA cell; DR Weber Consulting, Germany), an electrochemical cell for the derivatization of AFM₁. The mobile phase was composed of HPLC-grade methanol:acetonitrile:water (20:20:60) containing 119 mg of potassium bromide and 100 μ L of nitric acid. Extracts were redissolved in 1 mL of HPLC-grade methanol, and 20 μ L of the diluted solution was injected in the HPLC. The HPLC conditions were as follows: excitation at 362 nm and emission at 440 nm, with a flow rate of 1 mL/min. Standard AFM₁ solutions of 0.5, 1, 2, 5, 10, and 20 ng/mL were prepared in methanol (HPLC grade), and linearity was achieved with a regression of 0.9987. The mean limit of detection was 0.01 ng/mL.

Recovery and Statistical Analysis

For recovery validation, milk samples were spiked with AFM₁ at a single concentration of 100 ng/10 mL as shown in Table 1.

Data obtained from this study were analyzed and compared by *t*-test in Excel (Microsoft Corp.). Mean values were deemed to be significantly different if the level of probability was ≤ 0.05 .

RESULTS

Table 2 compares the occurrence of AFM₁ in tested milk samples (88 from Egypt and 50 from South Africa) using different analytical assays. The frequency of AFM₁ contamination in 88 milk samples from Egypt was 20.45% (18 samples) by AFM₁ strip; 81.8% (72 samples) by TLC; and 73.9% (65 samples) by ELISA. In the 50 samples from South Africa, frequency of AFM₁ contamination was 16% (8 samples) by AFM₁ strip; 68% (34 samples) by HPLC; and 68% (34 samples) by ELISA. Of the 4 methods used for analyzing AFM₁ in milk samples, the AFM₁ strip had the lowest frequency of occurrence with a detection incidence of 20.45% in Egyptian samples and 16% in South African samples. The lowest frequency of occurrence of AFM₁ obtained by AFM₁ strip indicates the much lower sensitivity of this method compared with the other methods.

Among positive samples, 18 of the Egyptian samples (20.45%) by ELISA had AFM₁ concentrations above the EU regulatory level (50 ng/L), whereas 65 samples (73.9%) had concentrations above the Egyptian regulations (0 ng/L). Six of the South African samples (12%) tested by ELISA had concentrations above the South African and EU regulatory limits. The mean concentration of AFM₁ in Egyptian samples was 25.79 ng/L (range: 8.52–78.06 ng/L), and that in South African samples was 17.06 ng/L (range 5–120 ng/L) by ELISA and 39 ng/L (range 8–93 ng/L) by HPLC (Table 3). The standard curve for AFM₁ detection by ELISA is

Table 2. Comparison between aflatoxin M₁ (AFM₁) strip, thin-layer chromatography (TLC), ELISA, and HPLC results for detection of AFM₁ in Egyptian and South African milk samples from rural and commercial farms

Country	Test run	No. of samples	Positive, no. (%)	Mean (ng/L)	SD
Egypt	AFM ₁ strip ¹	88	18 (20.45)	—	22.76
	TLC		72 (81.8)	—	
	ELISA		65 (73.9)	25.79	
South Africa	AFM ₁ strip	50	8 (16)	—	22.0
	ELISA		34 (68)	17.06	
	HPLC		34 (68)	39	

¹Aflatoxin M₁ strip test kits (AuroFlow) were purchased from Bioo Scientific (cat. no. 1101-01; Bioo Scientific, Austin, TX)

Figure 2. Standard curve of aflatoxin M₁ (AFM₁) by ELISA.

depicted in Figure 2. Absorption was inversely proportional to the AFM₁ concentration in the samples. The calibration curve was virtually linear in the 0 to 80 ng/L range. The peak for AFM₁ by HPLC was obtained at a retention time of 7.68 min (Figure 3), but, as expected, the retention time moved under the influence of experimental conditions such as mobile phase and HPLC room temperature variations. Eighteen positive Egyptian samples (20.45%) and 8 positive South African samples (16%) were identified by using the strip analysis. The positive and negative controls were evaluated and the results are shown in Figure 1.

DISCUSSION

Aflatoxin and aflatoxin M₁ contamination in food and milk remains poorly monitored and studied in developing countries and not much is known about the levels of aflatoxins in milk from semi-arid areas of South Africa

and Egypt. In addition, several kits for rapid detection and quantification of AFM₁ in milk have been developed for the milk industry market for the detection and quantification of AFM₁ in milk. These kits and methods must be validated to ensure their scientific use in developing countries for the monitoring of milk quality in regard to AFM₁ contamination. This is the first study to evaluate the occurrence of AFM₁ in milk samples in semi-arid areas of 2 African countries with climatic and environmental variations (Egypt and South Africa) and to evaluate possible health risks for consumers in regard to regulatory limits. Milk and dairy products play an important role in a healthy human diet because many people, especially children, frequently include them in their diets (Baskaya et al., 2006). Parallel to the increasing amount of milk and dairy product consumption, studies on the presence of AFM₁ in milk and milk products have been increasing globally as well as in Egypt and South Africa.

Table 3. Aflatoxin M₁ levels in Egyptian and South African milk samples from rural and commercial farms as determined by ELISA and HPLC¹

Country	Test	No. of samples	S < 25 ng/L (%)	25 < S < 50 ng/L (%)	S > 50 ng/L (%)	Range (ng/L)
Egypt	ELISA	88	22 (25)	25 (28)	18	8.52–78.06
South Africa	ELISA	50	22 (44)	6 (12)	6 (12)	5–120
	HPLC	50	22 (44)	5 (10)	7 (14)	8–93

¹S = number of positive samples.

Results obtained in this study showed a higher incidence of AFM₁ in Egyptian milk samples (73.9%) than in South African milk samples (64%) by ELISA. However, the AFM₁ concentration range was smaller in Egyptian milk samples (8.52–78.06 ng/L) than in South African milk samples (5–120 ng/mL). These differences are probably due to climatic and environmental variations and the type and quality of feed given to cows. In general, our results are in agreement with previous studies, which reported a high incidence of AFM₁ and a low concentration in milk and dairy products in developing countries from North and South Africa (Tomerak et al., 2011; Dutton et al., 2012; Mwanza et al., 2013). In Egypt, the contamination of milk by AFM₁ ranges from 40 to 66% (Salem, 2002; Motawee et al., 2009). In South Africa, the frequency of contamination with AFM₁ is 86% in rural subsistence milk samples and 100% on commercial dairy farms by HPLC (Dutton et al., 2012; Mwanza et al., 2013).

Most rapid screening methods rely on antibodies (immunological assays) to detect mycotoxins and differ according to how the antibodies are used in the assay. In our study, we used 2 basic techniques: ELISA and immunochromatographic strips. These analytical methods gave similar incidences but the AFM₁ strip method gave a much smaller range of contamination than the ELISA, HPLC, and TLC methods (Table 1). The red color of the test line in the strip disappeared with the positive control sample, and the red color of the control line in the strip disappeared with the negative control. All samples with AFM₁ levels from 5 to 50 ng/L by ELISA were found to have 2 red lines (test line and control line) on the AFM₁ strip membrane. This

finding suggests an inconclusive result in the strip assay (Figure 1) and such results were recorded as negative samples in our study. However, all milk samples with AFM₁ concentrations varying between 50 and 120 ng/L gave positive results. These results would be considered false-positive if the EU regulation (50 ng) were considered but would be classified as false negative if the US limit (500 ng/L) were considered. We concluded that the AFM₁ rapid test was not an ideal tool for milk quality control as it might give false-positive results when compared to other methods such as ELISA and HPLC. Protein and fat contents of milk may influence test results in various ways: sample flow can be altered (e.g., fat content strongly affects viscosity) and any of the milk components can interact specifically or nonspecifically with immuno-reagents used in the assay (Anfossi et al., 2011).

In Egyptian milk samples, the results obtained by TLC indicated a higher incidence of contamination (81.8%) compared with that detected by ELISA (73.9%). This may be due to the use of an immunoaffinity column as a clean-up method of milk samples before detection by ELISA. The ELISA method may not be fully reliable due to cross-reaction interferences, especially at concentrations <50 ng/L, leading to false-positive or false-negative results (Stark, 2010). This might also explain differences between ELISA and TLC results in Egyptian samples. Reports have shown that the detection and quantification of AFM₁ can be done by separation using TLC or HPLC coupled with fluorescence detector (Dragacci et al., 2001). In addition, in the current study, the TLC detection limit (350 ng/L) was lower than that of the strips (500 ng/L) and

Figure 3. Illustration of a chromatogram of aflatoxin M₁ standard at 2 ng/mL (40 mL injection) on HPLC coupled to a fluorescence detector connected to the Coring cell (CoBra cell; DR Weber, Germany). Color version available online.

higher than that of HPLC (10 ng/L). These results are in line with the results obtained by Filazi et al. (2010), who reported a much lower detection limit (20 ng/L) for AFM₁ by TLC.

A drawback of the TLC method is that precise determination of mycotoxin concentrations is challenging (Mwanza et al., 2012). A possible explanation of this is that a large amount of an unknown compound had some affinity to the antibodies directed against AFM₁, thus saturating the binding sites on the immunoaffinity column and reducing the binding capacity for AFM₁, resulting in decreasing elution and detection of AFM₁ and lowering the amount of the AFM₁ extracted (Mwanza et al., 2013). This problem can be solved by using either TLC or HPLC with detection in the Co-BrA cell (Coring cell). Shundo and Sabino (2006) found no significant difference between the TLC and HPLC methods when analyzing of 107 samples of raw, pasteurized, and UHT milk commercially available in São Paulo and Marília (Brazil) for the presence of AFM₁ after clean-up using an immunoaffinity column.

South African milk samples analyzed by ELISA demonstrated a satisfactory correlation with samples analyzed by HPLC coupled with a Coring cell. Over the last 20 yr, the importance and application of immunoassays, especially ELISA, has grown significantly. The ELISA method is not only suitable for quick and sensitive analysis with high sample throughput (Lee et al., 2004), but it is also cost effective and fast, and requires only a small sample volume for analysis (Sherry, 1997). Mwanza and Dutton (2014) reported that coupling HPLC with Coring cell improved detection of AFM₁ at lower concentrations.

CONCLUSIONS

Although several methodologies for detection and quantification of AFM₁ have been developed, emerging technologies for AFM₁ detection are at various stages in the progression to useful analytical tools. Although TLC, HPLC, and ELISA are sufficiently developed for field use, immunochromatographic strips are not able to detect small quantities of AFM₁ in milk samples (i.e., below the detection limit). Despite these disadvantages, immunochromatographic strips continue to advance. Strips can only be used to detect samples with high levels of AFM₁ contamination because of their high detection limit (500 ng/L). The data obtained by comparison of analytical methods should be useful to scientists who need to select one analytical method to detect AFM₁ in milk samples. We recommend combining a screening test (immunochromatographic strip, TLC, or both) with a confirmatory test such as HPLC

to ensure milk with low or absent levels of aflatoxins to ensure consumer safety.

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