Tropical Agricultural Research Vol. 21(2): 119 - 125 (2010)

Investigation of Farm Gate Cow Milk for Aflatoxin M_1

U.P.D. Pathirana, K.M.S. Wimalasiri[1], K.F.S.T. Silva[2] and S.P. Gunarathne[3]

Postgraduate Institute of Agriculture
University of Peradeniya
Peradeniya, Sri Lanka

ABSTRACT. *Importance of cow milk in human, especially in infant nutrition, is well known. Nevertheless, contamination of milk with aflatoxins is considered as a potential risk for human health. The aim of this study was to determine the levels of aflatoxin M_1 (AFM_1), metabolite of aflatoxin B_1 in raw cow milk in high milk producing areas in Sri Lanka. Aflatoxin M_1 levels were investigated by high performance liquid chromatography (HPLC) equipped with a fluorescence detector, monitoring at wave lengths 365 nm and 425 nm for excitation and emission, respectively. Eighty seven samples of raw milk were collected from randomly selected dairies in seven provinces in the country, and analyzed for AFM_1 using the Official Methods of Analysis of Association of Official Analytical Chemists (AOAC) International. The percentage recovery of AFM_1 was 85.2 ± 4.03 with respect to an artificially contaminated concentration of 48.6 ng/L.*

AFM_1 was detected in 33% of locally manufactured raw milk samples in concentrations ranging from 13.1 ng/L to 84.5 ng/L with a mean level of 40.2 ng/L. Percentage of contaminated samples (9.2%) exceeded the European Communities/Codex Alimentarius recommended limit of 50 ng/L. None of the milk samples from Western, Uva & Sothern provinces were contaminated at a detectable level of AFM_1. The results suggest a need to introduce safety measures for AFM_1 levels in liquid milk in local market under Prevention of Food Adulteration Act of Sri Lanka as well as to prescribe a limit of aflatoxin AFB_1 level in dairy cattle feedstuffs in order to minimize the health hazard risk in Sri Lanka.

INTRODUCTION

Aflatoxins (AF) are a group of mycotoxins mainly produced by common fungi, namely *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (JECFA, 2001). These fungi infect a wide range of agricultural commodities, especially cereals and oilseeds, both in the pre-harvest and post-harvest seasons (Galvano *et al*., 1996; Sarimehmetoglu *et al.,* 2004). The occurrence of Aflatoxin B1 (AFB_1) in feed material and their consumption has caused not only health hazards but also economic losses (Sarimehmetoglu *et al.,* 2004). Aflatoxins, even in small amounts, are biologically active compounds that pose potential toxic, carcinogenic, teratogenic and/or mutagenic effects in human as well as in farm animals (Galvano *et al*., 1996) due to poor quality feeds. Aflatoxin B1 is usually regarded as a potent liver carcinogen

[1] Department of Food Science & Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya 20400, Sri Lanka

[2] Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Peradeniya 20400, Sri Lanka

[3] Department of Farm Animal Production & Health, Faculty of Veterinary Medicine & Animal Science, University of Peradeniya, Peradeniya 20400, Sri Lanka

for most mammalian species including humans (Zinedine *et al*., 2007). Aflatoxin M_1 (AFM_1), the 4-hydroxylated metabolite of AFB_1 known as "milk toxin" is less toxic compared to its parent material (Galvano *et al.,* 1996; Galvano *et al.,* 2005). Aflatoxin M_1 is formed in the liver by means of cytochrome P450-associated enzymes. It is known as a hepatic carcinogenic metabolite found in milk of lactating animals consuming AFB_1 (JECFA, 2001; Manetta *et al*., 2005). International Agency for Research on Cancer (IARC) of World Health Organization defined AFB_1 as primary and AFM_1 as secondary group of carcinogenic compounds (Kaniou-Grigoriadou *et al.,* 2005).

Martins and Martins (2004) reported that about 1-2% of AFB_1 in animal feed is transformed to AFM_1 in milk with variations from animal to animal, from day to day and from one milking to the next. When the intake of AFB_1 is stopped, the AFM_1 concentration in the milk decreases to an undetectable level after 72 hours. Moreover, Galvano *et al*., (2005) reported that 0.3-6% of ingested AFB_1 is available as AFM_1 in milk. Many studies in the world reported the occurrence of AFM_1 in dairy products and evidence of potential hazardous human exposure, as milk is a key source of nutrients for humans (Galvano *et al*., 1996). This is especially significant for infants and children, who are potentially more sensitive and have less variety in their diets. Seasonal trend in milk contamination was noted as there were occurrence of low levels of AFM_1 during the spring and summer seasons. During these periods, the animals tend to consume more forage, roughage and pasture that are widely available than concentrate feed (Galvano *et al.,* 1996; Sarimehmetoglu *et al.,* 2004). Further, the climatic and storage conditions of the tropical and subtropical countries in the world are most favorable for the development and growth of aflatoxigenic fungi in food and feed stuffs. The geographical distribution and climatic variations within a country also can influence AFM_1 occurrence and contamination levels in milk (Galvano *et al.,* 1996). Sarimehmetoglu *et al.,* (2004) reported that AFM_1 is relatively stable in raw and processed milk products and is unaffected by pasteurization or processing into cheese. Even though AFM_1 is more frequent in powdered milk than in fluid milk in Argentina, results indicate that the incidence is not much serious (López *et al*., 2003). Being a metabolite, AFM_1 is stable under severe environmental conditions such as elevated temperatures and relative humidity. Human exposure to AFM_1 is due to the consumption of contaminated milk and dairy products of which daily intake could be highly variable in the world. Infants represent the most exposed population due to their high consumption of dairy products either as bovine milk and related by-products in their diet or from breast milk where the mycotoxin can be excreted. JECFA (2001) reported that the intake of AFM_1 from milk was 6.8 ng/person/day for the European diet, 3.5 ng/person/day for the Latin American diet, 12 ng/person/day for the Far Eastern diet, 0.7 ng/person/day for the Middle Eastern diet and 0.1 ng/person/day for the African diet. Thus, many countries have introduced regulations to control the levels of AFB_1 in feed and have proposed Maximum Permissible Levels (MPL) for AFM_1 in milk to reduce the risk.

Therefore, establishment of legal limit for AFM_1 in Sri Lanka is very important to protect consumers from hazards that may occur due to AFM_1 contamination. Currently, the legal limits are highly variable depending on the degree of the development and economic involvement of different countries (Galvano *et al*., 1996; Kaniou-Grigoriadou *et al.,* 2005). According to the U.S. Food and Drug Administration, AFM_1 in milk should not exceed 500 ng/L (US-FDA 2000; Manetta *et al*., 2005; Rastogi *et al*., 2004). Aflatoxin M_1 level in milk has been set more restrictively to 50 ng/L by the European Union (EU) for adult consumption (EU Regulation 466/2001; Rastogi *et al*., 2004), while in baby-food products this level cannot be greater than 25 ng/L (EU Regulation 466/2001).

There is little information about the occurrence of AFM_1 in milk and milk products in Sri Lanka. Wimalasiri *et al.,* (2005) found that there is high incidence of AFM_1 in 82% of the local market samples of raw cattle milk and milk powder. Rostogi *et al.*, (2004) reported that out of 87 milk and infant milk products in Indian market, almost 99% of the contaminated samples exceeded the European Community/Codex Alimentarius recommended limits, while 9% samples exceeded the prescribed limit of US regulations.

The consumption of cow milk is highly popular among people in Sri Lanka. Further, these products are largely consumed by children including infants who are more sensitive to mycotoxins than adults (Galvano *et al.,* 1996). Therefore, this study was carried out with the objective of investigating the presence of AFM_1 in bovine milk samples from 29 selected dairies using High Performance Liquid Chromatograph (HPLC) equipped with a fluorescence detector.

MATERIALS AND METHODS

Chemicals

PrepSep™ SPE-C_{18} cartridges (particle size 40-45 µm, pore size 60^0A and surface area 500 m^2g^{-1}) were purchased from Fisher Scientific (Pittsburgh, USA). HPLC grade acetonitrile was purchased from BDH (Poole, England) and other chemicals and solvents of analytical grade were supplied by Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) and HIMEDIA (Mumbai, India). AFM_1 stock standard solution with a concentration of 486 µg L^{-1} in acitonitrile was purchased from Biopure (Konrad Lorenz Strasse, Austria) and stored in the dark at 4 ^oC. A working solution of 4860 ng L^{-1} was daily prepared in acetonitrile:water (25:75 v/v) wrapped in aluminum foil and stored under the same conditions to prevent gradual break down of aflatoxins under UV light.

Instrumentation

Aflatoxin M_1 was analyzed using an HPLC (SHIMUDZU, Kyoto, Japan) system consisting of a solvent delivery module (LC-10AT), column oven (CTO-10AVP/10ALVP) maintained at 35±1.0 ^oC and a fluorescence detector (RF-10AXL) in which excitation and emission wavelengths were set at 365 nm and 435 nm, respectively. The eluate was passed through a Shim-pack CLC G-ODS 4 mm guard column followed by reversed phase C_{18} Shim-pack separating column (150 mm × 6.0 mm) and data manipulation and chromatograms were obtained using SHIMUDZU chromatopac (C-R7A plus).

Samples

A total of 87 raw cow milk samples were collected from 29 dairies, representing seven provinces out of nine provinces in Sri Lanka, namely Western, Central, North-Central, Uva, Wayamba, Southern and Sabaragamuwa provinces. These provinces are particularly important due to their considerable contribution to the national milk production. Each sample of 200 mL volume, immediately after collection, was transported to the laboratory in ice boxes at temperatures about 4±2 ^oC and then stored at -20 ^oC until analysis for AFM_1.

Sample preparation

The sample preparation was based on the method described by Manatta *et al.*, (2005), with slight modifications. A sample of milk was homogenized and centrifuged at 1700 g for 20 min. Then, 5 mL of the aqueous phase, diluted with an equal volume of deionized water, were purified on a SPE-C_{18} cartridge, after conditioning with acetonitrile (5 mL) followed by deionized water (10 mL). After applying the diluted samples and washing with water (10 mL), followed by acetonitrile:water (20:80, v/v) (20 mL), fats were removed by washing with *n*-hexane (2×5 mL). Aflatoxin M_1 was eluted with dichloromethane:acetone (95:5, v/v) (6 mL), the eluate was evaporated under a gentle stream of nitrogen and the residue dissolved in 1 ml of acetonitrile. Then an aliquot (20 µL) of the AFM_1 extract was analyzed by HPLC.

HPLC separation

The mobile phase used for the analysis has been acetonitrile:water (25:75, v/v). Isocratic elution of HPLC was performed at a flow rate of 1.0 mL min. Identification of AFM_1 was based on its retention time with respect to the standard. Further identity confirmation was carried out by pre and post-injection of standard for each 10 batch sample set. The calibration curve, of peak area versus concentration, was linear as expected and data were fitted by the least-squares method. The line of regression was used to compute the amount of the analyte in sample extracts by extrapolation, using external standard method. Standard solutions for calibration curve of AFM_1 were prepared by diluting stock solution with acetonitrile to obtain final concentrations in the range 30–486 ng L^{-1} (Fig. 1).

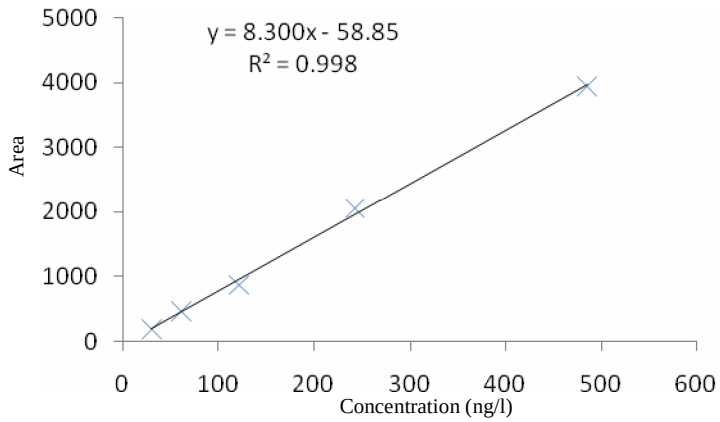

Fig. 1. Standard curve for AFM_1 reference standards

RESULTS AND DISCUSSION

Validation and Method Performance

The chromatogram shown in Fig. 2A illustrates the efficiency of the proposed method: i.e. there are no interferences in the region where AFM_1 is eluted. The retention time is about 13.0 min. The lower detection limit or the minimum level at which the analyte can be

reliably detected was found to be 10 ng L^{-1}. Recovery percentage of AFM_1 added to milk at a concentration of 48.6 ng L^{-1} was calculated to be 85.2%±4.03 by HPLC (Table 1)

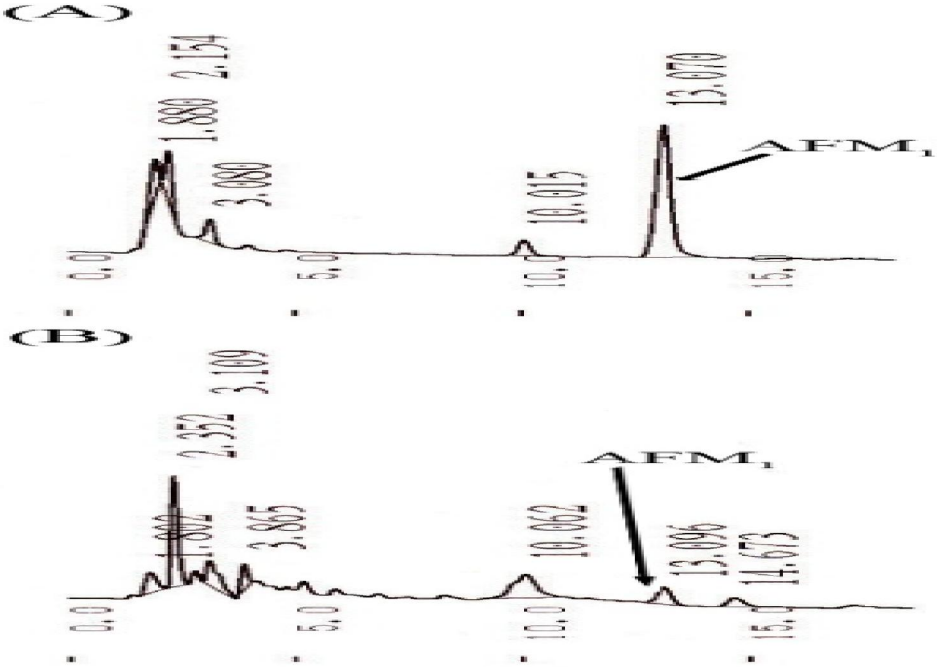

Fig. 2. Chromatograms of: (A) Reference standard (B) Sample matrix

Table 1. Recovery percentage of aflatoxin M_1 from artificially contaminated milk by HPLC

	Before aflatoxin M_1 addition (basal level ng/L)	Aflatoxin M_1 added (ng/L)	Expected Aflatoxin M_1 level (ng/L)	After aflatoxin M_1 addition (aflatoxin M_1 determined) (ng/L)	Recovery (%)
Sample 1	38.1 ± 3.4	48.6	86.7 ± 3.4	72.4 ± 7.1	83.5
Sample 2	35.6 ± 2.7	48.6	84.2 ± 2.7	69.3 ± 8.3	82.3
Sample 3	40.4 ± 7.8	48.6	89 ± 7.8	79.9 ± 5.8	89.8
Mean ± SD	38.0 ± 2.4	48.6	86.6 ± 2.4	73.9 ± 5.4	85.2 ± 4.03

Milk samples spiked at different concentrations and processed as described in methodology
All analysis were performed in duplicate for each sample.

Occurrence of AFM_1 in milk

The incidence of AFM_1 contamination in milk was not very high, since only 33.3% of all samples were positive (Table 2). However, eight samples out of 87 (9.2 %) were over the permisssible level of 50 ng L^{-1}, the regulatory limit of Europian Union (EU).

Zinedine *et al*., (2007) reported that the amount of AFB_1 present in feed depends on temperature and moisture, under which conditions some moulds, such as *A. flavus* and *A. parasiticus*, can easily grow in feed. These moulds easily grow on feed having moisture contents between 13% and 18% and environmental moisture between 50% and 60%.

Table 2. Incidence and levels of aflatoxin M_1 in milk samples as determined by HPLC

Province	Tested (No.)	Positive (%) (No.)	Samples					
			Frequency distribution No. (%)				Contamination	
			<10 ng L^{-1} [a]	10-20 ng L^{-1}	21-50 ng L^{-1}	≥50 ng L^{-1}	Range	Average [c]
Western	9	0 (0)	9 (10.3)					
Central	18	13 (14.9)	5 (5.7)	1 (1.1)	8 (9.2)	4 (4.6)		
North Central	15	5 (5.7)	10 (11.5)	3 (3.4)	2 (2.3)			
Uva	9	0 (0)	9 (10.3)					
Wayamba	15	7 (8)	8 (9.2)	1 (1.1)	3 (3.4)	3 (3.4)		
Southern	9	0 (0)	9 (10.3)					
Sabaragamuwa	12	4 (4.6)	8 (9.2)		3 (3.4)	1 (1.1)		
Total	**87**	**29 (33.3)**	**58 (66.7)**	**5 (5.7)**	**16 (18.4)**	**8 (9.2)**	**13.1 - 84.5**	**40.2**

[a] <10 ng/L – negative for aflatoxin M_1
[c] Mean of positive samples
All analysis were performed in duplicate for each sample

Milk samples representing Uva, Western and Sothern provinces were negative for AFM1 probably due to usage of quality concentrate feeding materials or minimal/non concentrate feeding of dairy cattle. Other provinces such as Central, North Central, Wayamba & Sabaragamuwa were positive for AFM_1 and this may be due to poor feed quality with respect to AFB_1 contamination. This confirms that the incidence of AFM_1 contamination is often higher in countries where cows are fed with high amounts of compound concentrate feeds.

CONCLUSIONS

Considering these results, it could be concluded that AFM_1 incidence in milk does not appear to be a serious public health problem in Sri Lanka at the moment. However, it is important to minimize exposure of milking animals to moldy feed contaminated with AFB_1 and take necessary precautions to prevent fungal contaminations/growth particularly during the storage of feed. It is important to establish the maximum permissible levels for AFM_1 in milk and milk products and AFB_1 in foods and feeds based on the ALARA (As Low As Reasonably Achievable) principles, which do not exist under Food Act of Sri Lanka. Seasonal variation of AFM_1 contamination in milk needs to be studied to assess the situation completely.

ACKNOWLEDGMENT

This research was funded by the National Science Foundation, Sri Lanka under the grant number RG/2006/AG/02.

REFERENCES

AOAC. (1995). Natural toxins. Official Methods of Analysis. (16th ed). Association of Official Analytical Chemists Inc, Virginia.

EU Regulation 466/2001 of 8 March 2001 (2001). Setting maximum levels for certain contaminants in foodstuff. Official J. of European Commission, L077, 11-12.

Galvano, F., Galofaro, V. and Galvano, G. (1996). Occurrence and stability of aflatoxin M_1 in milk and milk products: A worldwide review. J. of Food Protec. **59**, 1079-1090.

Galvano, F., Ritieni, A., Piva, G. and Pietri, A. (2005). Mycotoxin in the human food chain: The Mycotoxin Blue Book. (ed.) D. Diaz, Nottingham University Press, UK. pp. 187-224.

JECFA (2001). Fifty-sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Safety Evaluation of Certain Mycotoxins in Food. Food Additive Series, 47. World Health Organization, Geneva.

Kaniou-Grigoriadou, I., Eleftheriadou, A., Mouratidou, T. and Katikou, P. (2005). Determination of aflatoxin M_1 in ewe's milk samples and the produced curd and Feta cheese. Food Contr. **16**, 257-261.

López, C. E., Ramos, L.L., Ramadán, S.S. and Bulacio, L.C. (2003). Presence of aflatoxin M_1 in milk for human consumption in Argentina. Food Contr. **14**, 31-34.

Manetta, A.C., Giuseppe, L.D., Giammarco, M., Fusaro, I., Simonella, A., Gramenzi, A. and Formigoni, A. (2005). High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M_1 in milk and cheese. J. Chromat. A. **1083**, 219-222.

Martins, M. L. and Martins, H. M. (2004). Aflatoxin M_1 in yoghurts in Portugal. International J. of Food Microbiol. **91**, 315-317.

Rastogi, S., Dwivedi, P.D., Khanna, S.K. and Das, M. (2004). Detection of aflatoxin M_1 contamination in milk and infant milk products from Indian market by ELISA. Food Contr. **15**, 287-290.

Sarimehmetoglu, B., Kuplulu, O. and Celik, T. H. (2004). Detection of aflatoxin M_1 in cheese samples by ELISA. Food Contr. **15**, 45-49.

US-FDA (2000). Aflatoxin: Action levels for poisonous or deleterious substances in human food and animal feed. Industry Activities Staff Booklef, August 2000, CPG 527.400. http://www.fda.gov/ora/compliance ref/cpg/cpgfod/cpg527-400.html acess on:10 August, 2007.

Wimalasiri, K. M. S., Pathirana, U. P. D. & Liyanage, P. K. (2005). Occurrence of aflatoxin M_1 in raw milk & milk powder in the local market. Sri Lanka Association for the Advancement of Science, Proceedings of the 61st Annual session,12-17 December, 2005. pp.1.

Zinedine, A., González-Osnaya, L., Soriano, J. M., Moltó, J. C., Idrissi, L. & Mañes, J. (2007). Presence of aflatoxin M_1 in pasteurized milk from Morocco. Int. J. of Food Microbiol., **114**, 25-29.