A ONE-YEAR SURVEY ON AFLATOXIN M1 IN RAW MILK

M. SCHIRONE[1], P. VISCIANO[1]*, A.M.A. OLIVASTRI[2], R. TOFALO[1], G. PERPETUINI[1] and G. SUZZI[1]

[1]Facoltà di BioScienze e Tecnologie Agro-Alimentari e Ambientali, Università di Teramo, Via C.R. Lerici 1, 64023 Mosciano Sant'Angelo, Teramo, Italy

[2]Azienda Sanitaria Unica Regionale Marche - Ascoli Piceno, Italy

* Corresponding author: Tel. +39 0861 266911, Fax +39 0861 266940, email: pvisciano@unite.it

ABSTRACT

In the year 2012, 288 raw milk samples were collected from six different dairy cow farms and analyzed for the presence of aflatoxin M_1 (AFM_1) using the ELISA technique. The AFM_1 levels ranged from 5 to 25 ng/kg and the highest concentrations were found in autumn, with a significant difference ($p<0.05$) between February and November. The EU legal limit of 50 ng/kg has never been exceeded. Even if the results of the present study show a low risk for AFM_1, its occurrence in dairy products has to be regularly monitored due to their importance as foodstuffs for people and children above all.

- Keywords: *Aspergillus*, carry-over, ELISA -

INTRODUCTION

Nowadays food industry has the responsibility to develop and implement a Hazard Analysis and Critical Control Point (HACCP) system aiming at identifying and preventing important hazards to food safety. The presence of aflatoxins (AFs) in dairy products is one of the most important critical control points to be checked in raw milk supplies. Aflatoxins are secondary metabolites mainly produced by three species of *Aspergillus* including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (CRAPPY, 2002). Even if eighteen AFs have been identified, only four out of them have been found in food and feed, i.e. AFB_1, AFB_2, AFG_1 and AFG_2 (HESHMATI and MILANI, 2010). The toxic effects of these compounds can be both acute, thus causing hepatitis, oedema or hemorrhagic necrosis, and chronic, thus resulting in liver, lung and kidney carcinomas as well as immunosuppression (WILLIAMS *et al.*, 2004). In particular, AFB_1 shows different toxic activities, including teratogenicity, mutagenicity and carcinogenicity (MCLEAN and DUTTON, 1995). Therefore, the International Agency for Research on Cancer (IARC) has included AFB_1 in group 1 as a human carcinogen (IARC, 1993). Animals eating contaminated feed rapidly adsorb and transfer AFB_1 to the liver, where it is metabolized into the 4-hydroxylated derivate AFM_1 and excreted through faeces and urine (POLONELLI *et al.*, 2011). Consequently, AFM_1 may be secreted in mammalian milk by means of a carry-over process, within 12-24 h after the ingestion of AFB_1 (KAV *et al.*, 2011). AFM_1 exhibits a high level of genotoxic activity due to its possible accumulation and linkage to DNA (SHUNDO and SABINO, 2006). It can cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in *in-vitro* mammalian cells, insects, lower eukaryotes and bacteria (PRANDINI *et al.*, 2009). For that reason, AFM_1 has been included in group 1 (IARC, 2002) and removed from group 2B (i.e. as a possible human carcinogen).

Sources of AFs contamination in animal foodstuffs may vary geographically, with prevalence in areas with favorable environmental and climatic conditions. *Aspergillus flavus* and *A. parasiticus* colonize plants when still in the field, mainly when damaged; the highest AFs production occurs at temperatures between 20° and 30°C. In particular, *A. parasiticus* prefers a soil environment and can be found more commonly on peanuts, while *A. flavus* is better adapted to an aerial environment and colonizes cotton and corn (PRANDINI *et al.*, 2009). These molds can also colonize products in post-harvest if not adequately stored. However, the relationship between the amount of AFB_1 ingested by animals and the quantity of AFM_1 in milk is quite variable, as many factors – such as the individual variability among animals, the presence of udder infections, and the lactation period (at the beginning the carry-over is 3.3-3.5 times greater compared to the advanced lactation) – can affect carry-over (VAN EGMOND, 1989). The maximum limit for AFM_1 concentration in food varies in the legislation of different countries (GODIČ TORKAR and VENGUŠT, 2008). The European Community has prescribed a limit of 50 ng/kg in raw milk, heat-treated milk and milk for the manufacture of milk-based products (EC, 2006) while, according to US regulations, the action level of AFM_1 in milk should not be higher than 500 ng/kg (GHANEM and ORFI, 2009). AFM_1 is relatively stable in raw and processed milk products and is not affected by pasteurization or cheesemaking processes performed in dairy industry (KAV *et al.*, 2011). It has been reported that AFM_1 concentration in dairy products can be 3 to 4 times higher than in milk, as it is associated with milk proteins (BATTACONE *et al.*, 2003).

The present study aims at detecting the AFM_1 levels in bovine raw milk designed to a dairy factory located in the Marche region, central Italy. The preventive action used by such factory to control this hazard will also be discussed. The samples were analyzed by means of an enzyme-linked immunosorbent assay (ELISA) that is the most representative method for the fast screening analysis of AFs.

MATERIALS AND METHODS

A total of 288 samples of raw milk collected from six different suppliers (named 1 to 6) located in the Marche region, central Italy, were examined over the year 2012. Raw milk samples from each farm were provided to that dairy factory four times in a month and were transported in tanks at 0-4°C. All samples were analyzed in duplicate. Such raw milk samples (10 mL) were at first centrifuged at 3,500 *g* for 10 min at 4°C, then the upper cream layer was completely removed. A sample unit of 100 μL was used for the quantitative analysis of AFM_1 using the commercial kit RIDASCREEN (R-Biopharm, Germany). Such kit includes microtiter plates coated with capture antibodies, AFM_1 standard solutions used for the construction of the calibration curve, peroxidase-conjugated AFM_1, substrate (urea peroxidase), chromogen (tetramethylbenzidine) and stop reagents 1 N sulfuric acid. The test procedure was performed according to HESHMATI and MILANI (2010). The evaluation of AFM_1 was obtained dividing the absorbance values of the standards and the samples by the absorbance value of the first standard (zero standard), then multiplying the result by 100 (percentage of maximum absorbance). The adsorption was inversely proportional to the AFM_1 concentration in samples. The limit of quantitation according to the kit was 5 ng/kg.

A statistical analysis was carried out by GraphPad InStat Version 3.0, GraphPad Software (San Diego, California, USA). All the obtained data were assessed for normality by means of Kolmogorov-Smirnov test. Since the values were not normally distributed, non-parametric tests were applied. The differences among the values obtained from the six different suppliers and among the milk samples collected over 12 months were evaluated by Kruskal-Wallis Test (non-parametric ANOVA). When the *p* value was lower than 0.05, the Dunn's Multiple Comparisons Test was used.

RESULTS AND CONCLUSIONS

The mean AFM_1 concentrations in four collections (analyzed in duplicate) of raw milk over a month (for a total of 12 months) from each dairy farm are reported in Table 1. The levels ranged from the limit of quantitation (5 ng/kg) to a maximum of 25 ng/kg, with the highest values observed in the months of September, October and November. However, no sample exceeded the maximum levels (50 ng/kg) set for AFM_1 in milk by EU legislation (EC, 2006). No significant difference ($p>0.05$) was also observed among the AFM_1 concentrations in samples from the different suppliers, while a significant difference ($p<0.05$) was noticed only between the values obtained in February and November.

According to the HACCP plan implemented in the dairy factory of the present study, AFM_1 content is regularly monitored four times in a month but, when it results to be higher than 10 ng/kg, the supplier is contacted (as a preventive action) and analyses of raw milk from the matching dairy farm are repeated at the next supply. In this study (Table 2), 68.4% of the samples contained AFM_1 in the range of 5-10 ng/kg, while 27.1% was in the range of 11-19 ng/kg, exceeding the above mentioned preventive limit (10 ng/kg). Moreover, the dairy factory has set an internal system of corrective actions when AFM_1 content exceeds 20 ng/kg, defined as action limit. In the present study, the action limit was exceeded only in 4.5% of the samples collected from some dairy farms in different months (i.e. January, September, October, November and December), with values ranging from 20 ng/kg to a maximum of 25 ng/kg. In that case the supply of milk from the dairy farm is suspended until concentrations return to regular values. Whereas, if such value exceeds 50 ng/kg (the maximum level by law), the positive sample is analyzed by means of the HPLC as confirmatory assay, and milk has then to be intended as "Category 2 material" according to the EU regulations on animal by-products (EC, 2009). However, as a preventive measure, the HPLC procedure is routinely performed every four months.

Table 1 - Concentrations of AFM_1 (ng/kg) and range (in parentheses) for each six suppliers in the year 2012.

Supplier	January	February[a]	March	April	May	June	July	August	September	October	November[b]	December
1	ND	ND	12.5±2.9* (5.0-15.0)	10.0±3.3 (5.0-15.0)	ND	7.5±2.9 (5.0-15.0)	ND	9.10±3.2 (5.0-15.0)	7.5±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	11.8±4.8 (5.0-22.0)	10.0±3.6 (5.0-18.0)
2	8.8±4.3 (5.0-20.0)	ND	6.8±2.0 (5.0-12.0)	9.3±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	ND	10.0±3.3 (5.0-15.0)	13.4±2.8 (5.0-17.0)	15.8±0.9 (15.0-18.0)	15.2±4.1 (5.0-25.0)	9.3±2.9 (5.0-15.0)	10.0±3.3 (5.0-15.0)
3	13.0±5.5 (5.0-24.0)	ND	ND	7.5±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	ND	ND	ND	17.3±0.9 (15.0-18.0)	10.4±4.3 (5.0-19.0)	11.0±4.1 (5.0-19.0)	7.5±2.9 (5.0-15.0)
4	6.8±2.0 (5.0-12.0)	7.0±2.6 (5.0-15.0)	ND	ND	9.0±3.2 (5.0-15.0)	6.8±2.0 (5.0-12.0)	ND	8.0±2.6 (5.0-15.0)	10.8±3.9 (5.0-18.0)	10.6±4.7 (5.0-23.0)	14.0±6.1 (5.0-25.0)	12.8±3.0 (5.0-16.0)
5	ND	ND	9.3±2.9 (5.0-15.0)	ND	7.6±3.4 (5.0-18.0)	ND	7.5±2.9 (5.0-15.0)	9.2±3.3 (5.0-16.0)	17.8±1.2 (15.0-20.0)	17.6±1.7 (15.0-22.0)	15.8±4.5 (5.0-23.0)	10.0±3.3 (5.0-15.0)
6	ND	ND	7.5±2.9 (5.0-15.0)	ND	8.8±4.9 (5.0-24.0)	ND	ND	10.0±4.1 (5.0-15.0)	ND	ND	7.5±2.9 (5.0-15.0)	9.3±4.9 (5.0-22.0)

*These data are expressed as mean ± standard error; ND= $AFM_1 \leq 5$ ng/kg; a,b ($p<0.05$)

Table 2 - Distribution of AFM_1 in raw milk samples.

Months	Range of AFM_1 concentrations (ng/kg)		
	5-10 Number of samples (%)	11-19 Number of samples (%)	20-25 Number of samples (%)
January	20 (83.4)	2 (8.3)	2 (8.3)
February	23 (95.8)	1 (4.2)	-
March	17 (70.8)	7 (29.2)	-
April	19 (79.2)	5 (20.8)	-
May	16 (66.7)	8 (33.3)	-
June	22 (91.7)	2 (8.3)	-
July	21 (87.5)	3 (12.5)	-
August	15 (62.5)	9 (37.5)	-
September	9 (37.5)	14 (58.3)	1 (4.2)
October	9 (37.5)	12 (50.0)	3 (12.5)
November	12 (50.0)	8 (33.3)	4 (16.7)
December	13 (54.2)	10 (41.6)	1 (4.2)

- = no sample.

In Figure 1 the mean content of AFM_1 in milk per month was reported, without considering the different suppliers.

In the present study, the overall contamination levels of AFM_1 in milk samples were lower than those reported by other authors (HAN *et al.*, 2013; HUSSAIN and ANWAR, 2008; RAHIMI and AMERI, 2012; TAJIK *et al.*, 2007). These differences could be due to several factors, including different analytical techniques, samples size, season of the year, livestock management, and dairy processing systems. Moreover, the AFM_1 levels in milk seemed to be significantly influenced by the geographical region. The outcomes of some studies carried out in Italy showed an AFM_1 concentration range of 2-90 ng/kg (NACHTMANN *et al.*, 2007) and < 23 ng/kg (GALVANO *et al.*, 2001). In 2003, the risk of mycotoxins was brought to public attention following the indication of the presence of unusual amounts of AFM_1 in milk, in northern Italy in particular. At the beginning controls aimed at checking that the levels in milk did not exceed the limit established by law, but special monitoring plans were coordinated for milk and feed towards the end of 2003 due to an alarming amount of positivity in the self-check plan carried out on milk. Maybe the positive levels found in feed at the end of 2003 were the consequence of particularly unusual climatic conditions (high temperatures and drought lasting more than four months) that characterized the summer in the year 2003 (DECASTELLI *et al.*, 2007). Such approach – i.e. paying particular attention to the correlation in milk-feed monitoring procedures – could be considered particularly valid in order to find contaminated batches starting from controls on milk. In fact, many countries in Europe have shown relatively low levels of AFM_1 contamination in milk samples as a result of stringent rules on AFB_1 in dairy cattle feed (TRUCKSESS, 2006). In the present study AFM_1 concentrations were not very high and that result could be due to the feeding practices in dairy cow farms. The lower limits adopted by this dairy factory could be particularly effective, above all when the Italian Ministry of Health established an increase in milk analyses in order to detect AFM_1 following a series of notifications on AFB_1 in maize of European origin by the Rapid Alert System for Food and Feed (RASFF) since the last maize harvest in autumn 2012 (ANONYMOUS, 2012). In order to control AFM_1 levels in milk it is necessary to reduce AFB_1 contamination of feed for dairy cattle by preventing fungal growth and AFB_1 formation in agricultural commodities. That purpose can be achieved through some agricultural practices, such as the choice of hybrids, seeding time and density, suitable ploughing and fertirrigation, and stricter chemical or biological controls. Cereals harvested with the lowest possible moisture and conservation moisture close to or less than 14% are necessary to reduce contamination risks. Furthermore, kernel mechanical

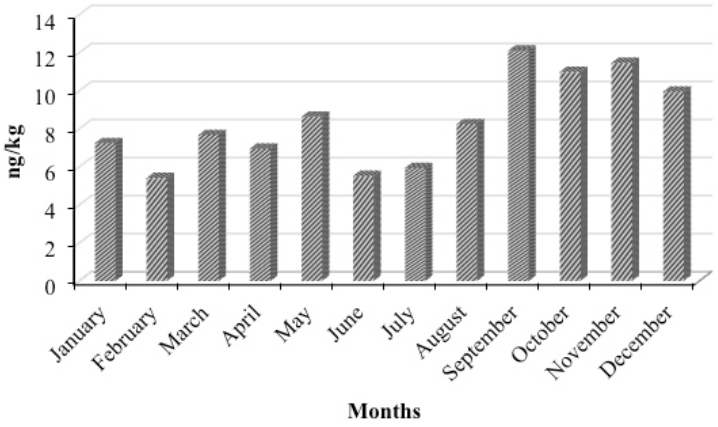

Fig. 1 - Mean content of AFM_1 in milk per month.

damage, grain cleaning practices and conservation temperature are also factors which need to be carefully controlled (PRANDINI *et al.*, 2009).

A marked seasonal variation in AFM_1 levels in milk has been previously reported (KAMKAR, 2005; RAHIMI and AMERI, 2012; RUANGWISES and RUANGWISES, 2010). It has been reported that AFs levels in feed are higher in rainy than in dry seasons. Moreover, the use of high amounts of contaminated concentrates is more frequent in cold months (KAMKAR *et al.*, 2011). Although no significant differences were observed in AFM_1 levels among the different months, except between February and November, the results of the present study shows that the mean concentrations in raw milk samples collected in autumn were higher than in other seasons. Such variation may be a result of toxin accumulation when storage occurs in hot and humid conditions. Many authors (BLANCO *et al.*, 1988; LOPEZ *et al.*, 2003; KAMKAR, 2005) reported on a higher number of yeasts, moulds and consequently on a higher concentration of mycotoxins in ensiled feed, mostly used in autumn or winter. Also DASHTI *et al.* (2003) observed that the contamination levels in the samples from local companies were higher in winter than in summer. That could be explained by the prolonged storage required for feed, which would provide favorable conditions for fungi to grow; or by the use of contaminated feed for the animals in winter, in addition to other factors such as temperature and relative humidity, agricultural products used as animal feed as well as seasonal effects from the country of origin of feed. Two other studies showed similar results – i.e., AFM_1 contamination is higher in winter than in summer. The first study was conducted in five regions of Iran on ninety-eight samples of raw milk analyzed in order to observe the possible presence of AFM_1. All samples resulted positive for AFM_1 with an overall mean level of 53 ng/L. The levels of AFM_1 were also higher in winter and spring than in summer and autumn (TAJKARIMI *et al.*, 2007). The second study was carried out in Sarab City, Iran, and showed that 76.6% of 111 raw milk samples was positive, with AFM_1 levels ranging between 15 and 280 ng/L. The lowest AFM_1 levels (24 ng/L) were found in August and the highest (118 ng/L) in December (KAMKAR, 2005).

In conclusion, the occurrence of AFM_1 in milk intended for human consumption is a critical control point to be steadily monitored in dairy products. Controls of the supply chain from feedstuffs for lactating cows to milk production represents the key to guarantee the safety of the end product, due to the large variation in the content of AFB_1 in animal feed and consequently of AFM_1 in milk. Even if the risk of a high AFM_1 content appears limited, it is certainly of great interest to implement a valid system of regular monitoring in order to have always safe raw materials. Establishing more restrictive limits, as those chosen by the dairy factory, taken as a case-study in the present paper, could be a good approach to achieve a better food quality for consumers.

ACKNOWLEDGEMENTS

The authors thank Francesca Rosati, University of Teramo, for her support in editing the English text.

REFERENCES

Anonymous 2012. Rapid Alert System for Food and Feed (RASFF). Available at: http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.print.htm.

Battacone G., Nudda A., Cannas A., Cappio Borlino A., Bomboi G. and Pulina G. 2003. Excretion of aflatoxin M1 in milk of dairy ewes treated with different doses of aflatoxin B1. J. Dairy Sci. 86: 2667-2675.

Blanco J.L., Dominguez L., Gómez-Lucia E., Garayzabal J.F., Garcia J.A. and Suarez G. 1988. Presence of aflatoxin M1 in commercial ultra-high temperature treated milk. Appl. Environ. Microbiol. 56: 1622-1623.

Crappy E.E. 2002. Update of survey, regulation and toxic effects of micotoxins in Europe. Toxicol. Lett. 127: 19-28.

Dashti B., Al-Hamli S., Alomirah H., Al-Zenki S., Abbas A.B. and Sawaya W. 2009. Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. Food Control 20: 686-690.

Decastelli L., Lai J., Gramaglia M., Monaco A., Nachtmann C., Oldano F., Ruffier M., Sezian A. and Bandirola C. 2007. Aflatoxins occurrence in milk and feed in Northern Italy. Food Control 18: 1263-1266.

EC. 2006. Regulation (EC) No 1881/2006 of the European Parliament of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364/1-24.

EC. 2009. Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). Official Journal of the European Union, L 300/1-33.

Galvano F., Galofaro V., Ritieni A., Bognanno M., De Angelis A. and Galvano G. 2001. Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy: second year of observation. Food Addit. Contam. 18 (7): 644-646.

Ghanem I. and Orfi M. 2009. Aflatoxin M1 in raw, pasteurized and powdered milk available in the Syrian market. Food Control 20: 603-605.

Godič Torkar K. and Vengušt A. 2008. The presence of yeasts, moulds and aflatoxin M1 in raw milk and cheese in Slovenia. Food Control 19: 570-577.

Han R.W., Zheng N., Wang J.Q., Zhen Y.P., Xu X.M. and Li S.L. 2013. Survey of aflatoxin in dairy cow feed and raw milk in China. Food Control 34: 35-39.

Heshmati A. and Milani J.M. 2010. Contamination of UHT milk by aflatoxin M1 in Iran. Food Control 21: 19-22.

Hussain I., Anwar J., Munawar M.A. and Asi M.R. 2008. Variation of levels of aflatoxin M1 in raw milk from different localities in the central areas of Punjab, Pakistan. Food Control 19: 1126-1129.

Hussain I. and Anwar J. 2008. A study on contamination of aflatoxin M1 in raw milk in the Punjab province of Pakistan. Food Control 19 (4): 393-395.

IARC, International Agency for Research on Cancer. 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monograph on the evaluation of carcinogenic risks to humans. Lyon, France, pp. 1-599.

IARC, International Agency for Research on Cancer. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC monograph on the evaluation of carcinogenic risks to humans. Lyon, France, pp. 1-556.

Kamkar A. 2005. A study on the occurrence of aflatoxin M1 in raw milk produced in Sarab city of Iran. Food Control 16: 593-599.

Kamkar A., Khaniki G.R.J. and Alavi S.A. 2011. Occurrence of aflatoxin M1 in raw milk produced in Ardebil of Iran. Iran. J. Environ. Health Sci. Eng. 8 (2): 123-128.

Kav K., Col R. and Tekinsen K.K. 2011. Detection of alatoxin M1 levels by ELISA in white-brined Urfa cheese consumed in Turkey. Food Control 22: 1883-1886.

Lopez C.E., Ramos L.L., Ramadán S.S. and Bulaciol C. 2003. Presence of aflatoxin M1 in milk for human consumption in Argentina. Food Control 14: 31-34.

McLean M. and Dutton M.F. 1995. Cellular interactions and metabolism of aflatoxin: an update. Pharmacol. Therapeut. 65: 163-192.

Nachtmann C., Gallina S., Rastelli M., Ferro G.L. and Decastelli L. 2007. Regional monitoring plan regarding the presence of aflatoxin M1 in pasteurized and UHT milk in Italy. Food Control 18: 623-629.

Polonelli L., Giovati L., Maiani W., Conti S., Sforza S., Calabretta A., Casoli C., Ronzi P., Grilli E., Gallo A., Masoero F. and Piva G. 2011. Vaccination of lactating dairy cows for the prevention of aflatoxin B1 carry over in milk. PLoS One 6 (10): e26777.

Prandini A., Tansini G., Sigolo S., Filippi L., Laporta M. and Piva G. 2009. On the occurrence of aflatoxin M1 in milk and dairy products. Food Chem. Toxicol. 47: 984-991.

Rahimi E. and Ameri M. 2012. A survey of aflatoxin M1 contamination in bulk milk samples from dairy bovine, ovine, and caprine herds in Iran. Bull. Environ. Contam. Toxicol. 89: 158-160.

Ruangwises N. and Ruangwises S. 2010. Aflatoxin M1 contamination in raw milk within the central region of Thailand. Bull. Environ. Contam. Toxicol. 85: 195-198.

Shundo L. and Sabino M. 2006. Aflatoxin M1 in milk by immunoaffinity column cleanup with TLC/HPLC determination. Braz. J. Microbiol. 37: 164-167.

Tajik H., Rohavi S.M.R. and Moradi M. 2007. Detection of aflatoxin M1 in raw and commercial pasteurized milk in Urmia, Iran. Pakistan J. Biol. Sci. 10 (22): 4103-4107.

Tajkarimi M., Aliabai F.S., Nejad M.S., Pursoltani H., Motallebi A.A. and Mahdavi H. 2007. Seasonal study of aflatoxin M1 contamination in milk in five regions in Iran. Int. J. Food Microbiol. 116 (3): 346-349.

Trucksess M.W. 2006. Mycotoxins. J. AOAC Int. 89(1): 270-284.

Van Egmond H.P. 1989. Aflatoxin M1: occurrence, toxicity, regulation. In: Van Egmond H.P. (Ed.), Micotoxins in dairy products, Elsevier Applied Science, London and New York, pp. 1155.

Williams J.H., Phillips T.D., Jolly P.E., Stiles J.K., Jolly C.M. and Aggarwal D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr. 80: 1106-1122.

Paper Received February 14, 2014 Accepted August 6, 2014