

Aflatoxin M₁ in bulk-tank raw milk produced in a low risk area

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Abstract

The present study was designed to determine the levels of aflatoxin M₁ (AFM₁) and its seasonal variation in bulk tank cow milk collected from dairy farms located on the Italian island of Sardinia. Cow bulk tank milk samples (No 356) were collected from 178 farms twice a year, during the winter and summer seasons. All samples were analysed for AFM₁ with a commercial ELISA kit. Confirmation of AFM₁ positive samples (>10 ng L⁻¹) was accomplished by using high-performance liquid chromatography (HPLC) with a prior clean-up step using immunoaffinity columns. AFM₁ was found at levels ≥ 5 ng L⁻¹ in 112 samples (31.5%) but only one exceeded the maximum EC level (50 ng L⁻¹). There was no seasonal influence on the aflatoxin content of the milk samples analysed.

INTRODUCTION

Aflatoxins (AFs) are a group of mycotoxins produced by mycetes of the *Aspergillus* genus (Goto et al., 1996; Bennet and Klich, 2003). Their hepatotoxic, genotoxic, carcinogenic, teratogenic, mutagenic, immunosuppressive and antinutritional effects are well documented (IARC, 1993; Williams et al., 2004; Wangikar et al., 2005). The toxic activity of AFs is due to their capacity to interact with nucleic acids, nucleoproteins and protein syntheses. Aflatoxin B₁ (AFB₁) is known as the most powerful hepatocarcinogen in mammals. Epidemiological studies have found a positive correlation between AFB₁ dietary exposure and increased liver cancer risk in humans chronically infected with hepatitis B virus (WHO-IARC, 1985; Bean et al., 1989; Creppy, 2002). The development of mycetes on vegetables in the field or after crop harvesting is linked to the production of AFB₁. Dairy animals fed a diet containing AFB₁ excrete aflatoxin M₁ (AFM₁) into the milk (Ionghde et al., 1964). AFM₁ is a metabolic hydroxylation product originating from a cytochrome P450-mediated hepatic biotransformation of AFB₁. The toxicity of AFM₁ is about one order of magnitude less than AFB₁ (Creppy, 2002; Pong et al., 1971) and it is classified as a probable human carcinogen [group 2B of the IARC] (IARC, 1993; Smith et al., 1995). The carry over of AFM₁ into the milk is influenced by numerous factors, including the species, the individual variability, the stage of lactation, the milking time, the production level and udder health. The carry over

ratio (AFM₁ excreted in milk/AFB₁ ingested) has been found in cattle to be 0.17 - 3% (Sieber et al., 1978; Veldman et al., 1992), with peaks of about 6% at the beginning of lactation (Pitet, 1998). AFM₁ could be detected in milk 12 or 24 hours after the ingestion of AFB₁ (Stoloff, 1977) and disappeared 4 days after AFB₁ was removed from the diet (Whitlow et al., 2000). The AFM₁ concentration in the bulk tank milk is quite different at the farm level and is usually lower in bulk milk, due to the dilution occurring during the collection of milk from different farms (Piva et al., 1987). The concentration of AFM₁ in milk is not appreciably reduced by heat treatment [pasteurization, UHT] (Gelosa and Buzzetti, 1994; Galvano et al., 1996; JECFA, 2001). In European countries the weighted mean concentration of AFM₁ in milk (based on a large number of analysed milk samples) is 0.023 mg Kg⁻¹ and the intake from milk is estimated to be 6.8 ng/person per day in the European-type diet (Creppy, 2002). The legal limits vary significantly from country to country and they are influenced by economic factors (van Egmond, 1989; Stoloff et al., 1991). The European Commission (EC) maximum admissible limit for AFM₁ in milk products is 50 ng Kg⁻¹ (Commission Regulation EC No. 1881/2006) while the limit established by the Codex Alimentarius is 500 ng Kg⁻¹ (Codex Alimentarius Commission, 2001). The limits for infant formulae, infant milk and follow-on milk are more restrictive, 25 ng Kg⁻¹ in the EU (Commission Regulation EC No. 1881/2006) and 10 ng Kg⁻¹ in Switzerland. The present study was designed to determine the levels of AFM₁

in bulk tank cow milk and its seasonal variation.

MATERIALS AND METHODS

SAMPLING

The research was carried out on samples of raw tank cow milk from 178 dairy farms located on the Italian island of Sardinia. The milk samples were collected from intensive or semi-intensive dairy farms twice per year, during the winter and summer seasons. A sample of raw bulk-tank milk (100 mL) was collected from each farm. The samples were refrigerated at +4 °C and then transferred to the laboratory for analysis.

AFLATOXIN M ANALYSIS (ELISA METHOD)

A commercial ELISA kit (r-Biopharm, AG, Darmstadt, Germany) was used for quantitative analysis of the AFM₁. The pH of each sample was measured to establish its suitability for analysis (pH≥6.5). The samples were centrifuged at 3500 x g at + 10 °C for 10 min to remove the fat. AFM₁ standards, blank (against air) and milk samples were analysed twice with microtiter plates coated with antibodies for the AFM₁. The plates were then incubated in the dark for 60 min at + 25 °C. After a first washing, 100 µL of the conjugated-peroxidase AFM₁ was added to microwells and incubated in the dark at + 25 °C for 60 min. The non-bound conjugated AFM₁ was removed at the end of incubation, during the second washing. In the next step, 50 µL of enzyme substrate (urea peroxide) and 50 µL of chromogen (tetramethylbenzidine) were added to each well. The plates were incubated for a further 30 min in the dark and then 100 µL of 1M H₂SO₄ were added to stop the reaction. The absorbance was measured at 450 nm using a Sunrise microplate spectrophotometer reader (TECAN GmbH, Grödig, Austria). The detection limit of the ELISA kit for AFM₁ in the milk was 5 ng L⁻¹. AFM₁ levels in samples were obtained by data analysis using RIDA SOFT WIN software (r-Biopharm AG, Darmstadt, Germany) by plotting the B/B values versus their corresponding concentration interpolated from the calibration curve (cubic spline fits) obtained by analysing five internal standards, ranging from 5 to 80 ng L⁻¹. The yield (%) and the coefficient of variation (CV%) of each internal standard was evaluated. The % yield was expressed as actual yield/theoretical yield x 100. The CV% was expressed as the ratio between the standard deviation (SD) and the average.

AFLATOXIN M ANALYSIS (HPLC METHOD)

HPLC (High Performance Liquid Chromatography)

confirmation analysis (Ioannou-Kakouri et al., 1995) was carried out on the positive samples (> 10 ng L⁻¹). 70 mL of each milk sample was centrifuged at 4000 g at + 4 °C for 15 min to separate the fat, which was then removed with a sterile spatula. 50 mL of each fat-free milk sample was passed through an immunoaffinity column (VICAM, Watertown, MA, USA) with a flow rate of 1-2 drops/sec. Each immunoaffinity column was then washed with 10 mL of deionised water (Milli Q, Millipore S.p.A., Vimodrone, MI) with a flow rate of 1-2 drops/sec. The AFM₁ was slowly eluted (flow rate of 1 drop/2-3 sec) from the column with a mixture of acetonitrile-methanol (3:2) and water (2 mL) and the final extract collected in an amber-coloured vial. The extract was evaporated at + 50 °C under a nitrogen stream and reconstituted in 100 µL of mobile phase. 10 µL of this solution was injected in a C18 column. The mobile phase (consisting of 24% acetonitrile, 63% water and 13% methanol) in isocratic conditions was pumped at a flow rate of 1 mL min⁻¹. The HPLC equipment used for AFM₁ determination was an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany), equipped with the auto sampler LAS G1313A and fluorescence detector FLD G1321A, with excitation and emission wavelengths of 365 nm and 435 nm, respectively. A Zorbax SB-C18 column (Agilent Technologies Inc., Santa Clara, CA, USA), 4.6 x 250 mm with particle size 5 µm in diameter, was used. The AFM₁ standard (10 µg mL⁻¹ in acetonitrile) was purchased from Supelco (Bellfonte, PA, USA) and stored at + 4 °C. The calibration curve was determined by injection of five standard solutions of AFM₁ in acetonitrile at concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 µg L⁻¹. The retention time for aflatoxin M₁ was 8.2 min. The sensitivity limit of this method was ≤10 ng L⁻¹.

DATA ANALYSIS

The results for the concentrations of AFM₁ in the sampling period were analysed by GLM (Statgraphics Plus 5.1; StatPoint, Inc. Herndon, Virginia). The differences between the average concentrations of AFM₁ were obtained by ELISA and HPLC and analysed by linear regression. The correlation between the HPLC and the ELISA estimates was tested by a simple linear regression analysis.

RESULTS AND DISCUSSION

The average % yield of the internal standards was determined for each ELISA test plate. It was: 95.6% for 5 ng L⁻¹ standard (CV 2.2%), 108.4% for 10 ng L⁻¹ standard (CV % 5.4), 100.8% for 20 ng L⁻¹ standard (CV 5.9%), 97.7%

for 40 ng L⁻¹ standard (CV 5.3%) and 117.9% for 80 ng L⁻¹ standard (CV 2.4%). AFM₁ contamination was not detected in 244 (68.5%) of the 356 milk samples (Table 1). In 112 (31.5%) of the samples, the concentration of AFM₁ was >5 ng L⁻¹, equivalent to (mean ± sd) 15.9±8.6 ng L⁻¹. In only one sample did the concentration levels of AFM₁ exceed the maximum EU limit of 50 ng L⁻¹. The AFM₁ content in milk from 99 (55.6%) farms was below the sensitivity of the ELISA method in both samples while in 46 (25.8%) farms it was positive to AFM₁ in only one of the two samples. The AFM₁ content in the milk from these farms was 14.3±10.5 ng L⁻¹, ranging from 5.1 to 69.8 ng L⁻¹. In 33 (18.5%) farms both samples were positive. The contamination of AFM₁ in milk samples from these farms was 17.1±6.8 ng L⁻¹ (5.1-29.4 ng L⁻¹). The AFM₁ content was less in the samples in which contamination was found in only one of the two samples (ns). In winter the AFM₁ content was >5 ng L⁻¹ in 58 (32.6%) of the 178 samples examined, with concentrations of 15.5±9.8 ng L⁻¹. In summer the AFM₁ content was >5 ng L⁻¹ in 54 (30.3%) of the samples, with concentrations of 16.5±6.9 ng L⁻¹. No difference was detected in the AFM₁ content of samples taken in winter and summer (ns). Intensive breeding of dairy cattle is characterised by standardised feed, conservation of feed and the use of feed supplements. The season did not influence the contamination of the milk in the area where we conducted our research. This is because the animals had limited access to pasture and because the feed supplements were mainly from outside sources. In different geographical situations and raising systems the season has a marked effect on contamination levels (Applebaum et al., 1982; Blanc and Karleskind, 1981; Kamkar, 2005). The analytical sensitivity was 10 ng L⁻¹ for the HPLC assay and 5 ng L⁻¹ for the ELISA assay. The HPLC method has confirmed the result obtained by ELISA method. The data showed an overestimation of AFM₁ content by ELISA. The ratio of ELISA: HPLC values (>10 ng L⁻¹) ranged from 0.5 to 1.8 with a mean of 1.4±0.3. Analysis of the data by linear regression has showed a correlation coefficient R²=0.74 and standard error of 5.1.

Figure 1

Table 1: Distribution of cow milk samples (356) and their production in relationship to AFM concentrations

Season	Farms n	Samples* n (%)	AFM ₁ ^b ng/l	Distribution n (%)						
				<5	≥5-10	>10-20	>20-30	>30-40	>40-50	>50
Winter	178	58 (32.6)	15.5±9.8	120 (67.4)	18 (10.1)	26 (14.6)	13 (7.3)	-	-	1 (0.6)
Summer	178	54 (30.3)	16.5±6.9	124 (69.7)	12 (6.7)	25 (14.0)	16 (9.0)	1 (0.6)	-	-
Total	178	112 (31.5)	15.9±8.6	244 (68.5)	30 (8.4)	51 (14.3)	29 (8.1)	1 (0.6)	-	1 (0.6)

CONCLUSIONS

There are low concentrations and only limited incidence of AFM₁ contamination of cow's milk produced in a low risk areas in Italian island of Sardinia. These findings agree with others in the literature (De Santis et al., 2000; Viridis et al., 2001; Roussi et al., 2002; Kamkar, 2005). In these conditions, the most important and frequent cases of contamination occur only when there are particularly critical climatic conditions or when large quantities of contaminated foodstuffs are fed to the animals. The latter observation highlights the importance of Good Agricultural Practice and Good Storage Practice when producing animal feed, and also of farms checking and evaluating feed from outside sources. HACCP plans are being developed to control the risk all along the feed supply chain. The effectiveness of the control measures must be stringently monitored and verified by checking contamination levels of AFM₁ in the milk (FAO, 2003). ELISA is a simple method useful for rapid screening of dairy milk. Although less specific, the ELISA was shown to be faster, more economical and more sensitive than HPLC and a useful first step in identifying positive samples for confirmation by HPLC.

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