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A survey on the Aflatoxin M1 occurrence and seasonal variation in buffalo and cow milk from Southern Italy

Antonella De Roma [a, *], Carmela Rossini [a], Alberto Ritieni [b], Pasquale Gallo [a], Mauro Esposito [a]

[a] Istituto Zooprofilattico Sperimentale del Mezzogiorno, 80055, Portici, Napoli, Italy
[b] Department of Pharmacy, University of Napoli Federico II, Napoli, Italy

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ABSTRACT

This study evaluates the aflatoxin M1 (AFM1) contamination in 804 samples of raw milk from cow and buffalo, collected randomly in Campania and Calabria regions of Southern Italy over a two years period.

The competitive enzyme linked immunosorbent assay (ELISA) method was used to analyze AFM1 in the samples. AFM1 levels result above the CCβ value of 0.004 μg/kg in 51 (12.3%) cow milk samples and in 28 (7.2%) buffalo milk samples. Positive results from screening analysis were confirmed by high performance liquid chromatography with fluorimetric detection (HPLC-FLD) after a procedure of centrifugation, extraction and immunoaffinity column clean-up of milk. Only one cow milk sample exceeded the maximum limit (0.05 μg/kg) set by the European Regulation.

The occurrence of AFM1 contamination was significantly ($p < 0.05$) higher in cold season, particularly fall, than in warm season, principally spring.

Our results indicate that feedstuff used in the buffalo and cow farms were not highly contaminated with aflatoxins, determining a good quality of the analyzed milk. Therefore, the AFM1 contamination of the milk does not represent a serious public health problem in both regions in Southern Italy.

1. Introduction

Aflatoxins are a group of heterocyclic compounds produced by some species of filamentous fungi, particularly by some strains of *Aspergillus flavus* and *Aspergillus parasiticus* that can contaminate a wide range of food and agricultural commodities such as cereals, seeds, grain, silage (Mahmoudi, 2014; Mohammadi, 2011). Aflatoxin M1 (AFM1) presence in milk results primarily from the conversion of aflatoxin B1 deriving from contaminated feedstuffs and that is metabolized in the mammal liver by microsomial cytochrome P450. After AFM1, the principal 4-hydroxylated metabolite, is formed, it is subsequently excreted in the milk of lactating cows or buffaloes.

Aflatoxin M1 is very stable towards normal milk processing methods such as pasteurization and ultra-high temperature heating or other procedures of the dairy industry, and if present in raw milk, the AFM1 molecule cannot be inactivated and may persist into final products for human consumption (Brackett & Marth, 1982; Manetta et al., 2009; Oruc, Cibik, Yilmaz, & Kalkanli, 2006; Pietri, Bertuzzi, Fortunati, & Gualla, 2003).

High levels of AFM1 in milk and other dairy products are considered undesirable because of its toxic properties. High genotoxic activity of AFM1 was observed (Lafont, Siriwardana, & Lafont, 1989) and also DNA damage resulting in chromosomal anomalies, gene mutation and cell transformation (Prandini et al., 2009), thus the International Agency for Research on Cancer classified AFM1 as human carcinogen in group 1 (IARC, 2002).

Several surveillance or monitoring plans have been carried out in Italy from Veterinary Authorities for controlling AFM1 contamination and its occurrence in milk and dairy products (Bellio et al., 2016). There is considerable manufacturing of dairy products derived from buffalo milk and cow milk in Campania and Calabria regions and some of the major sources for milk production are located in the area of Caserta, where there are many water buffaloes rearing in the farms.

In this study, we report the results of monitoring the AFM1 contamination in buffalo and cow milk from these regions during different seasons between 2015 and 2016.

* Corresponding author. Chemistry Department, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, 80055, Napoli, Italy.
E-mail address: antonella.deroma@izsmportici.it (A. De Roma).

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Analyses were performed by using the ELISA screening test; the positive samples were analyzed by HPLC with fluorimetric detection to confirm the presence of AFM1.

2. Materials and methods

2.1. Sampling

The milk samples were randomly collected between 2015 and 2016 from buffalo's and cow's farms or cheese factories located in the Campania and Calabria regions of Southern Italy. Samples (1000 mL each) were put into glass bottles and sent in ice box to the laboratory where they were stored at −20 °C until chemical analysis.

2.2. ELISA screening method for analysis of AFM1

The qualitative analysis of AFM1 in the milk samples was performed by competitive enzyme immunoassay using Euroclone-Aflatoxin M1 Elisa kit (Quantitative EuroClone Aflatoxin M1).

For the preparation of the sample, 50.0 g of milk were chilled to 10 °C for 15 min and then centrifuged at 3000 × g for 10 min to separate fat. The upper, creamy layer was completely removed by aspirating through a Pasteur pipette and the lower phase was directly used in the ELISA test. ELISA test procedure was carried out according to the instructions of manufacturer, therefore 200 μL of standard solutions (prepared at concentrations of 4, 10, 25 and 50 ng/L) and 200 μL of samples were added into separate wells of micro-plate and incubated at room temperature (25 °C) in the dark for 30 min. The liquid was then poured out and the wells were washed three times with 300 μL of washing buffer. In the next step of the procedure, 200 μL of the enzyme conjugate solution were added to the wells, mixed gently by shaking the plate manually and incubated at room temperature in the dark for 15 min. After this, wells were washed three times with the washing buffer solution. Afterwards, 200 μL of substrate/chromogen were added, mixed gently and incubated in the dark at room temperature for 15 min. Finally, the reaction was stopped by adding 50 μL of the stop reagent solution into the wells and the absorbance was measured at $\lambda = 450$ nm by means of the ELISA reading apparatus. The AFM1 content was calculated from the standard curve. The detection capability (CCβ) was based on the acceptance of 5% false compliant results for a given concentration level (0.05 μg/kg) by analyzing 20 blank fortified samples.

2.3. HPLC confirmatory method for analysis of AFM1

Samples (50.0 g) were warmed to 37 °C in a water bath and afterwards were centrifuged at 3500 rpm for 15 min. The upper fatty layer was removed, and the remaining aqueous phase was passed through Whatman No. 4 filter paper, then the filter was washed twice with ultrapure water (2 × 5 mL). The filtered portion was then loaded onto the AFM1 immuno-affinity column (IAC) and passed at a rate of approximately 1–2 drops/second. The IAC was washed four times with 3 mL ultrapure water and AFM1 was eluted by 2 mL acetonitrile/methanol (3:2, v/v) at a rate of approximately 2–3 drops/second, collecting sample in a test tube. Finally, 100 μL of the eluate were injected into the HPLC.

Instrumental analyses were performed by means of an HPLC system Waters mod e2695 equipped with a fluorescence detector 2475 FLR (Waters, Ireland) and a Synergi Polar-RP column 250 mm × 4.6 mm × 4μm (Phenomenex, Italy). The mobile phase was water/acetonitrile/methanol (52:18:30, v/v) in isocratic mode at a flow rate of 1.0 mL/min with the column oven set at 25 °C. The injection volume was 100 μL. The total run time of each analysis was 15 min and the retention time of AFM1 under these conditions was approximately 8.0 min.

For the detection of AFM1, excitation and emission wavelengths were set at 360 nm and 440 nm, respectively. Identification of sample peak was based on the retention time of standard peaks.

2.4. Chemicals and reagents

The solvents methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Italy), purified water was obtained by a Milli-Q water system (Millipore, MA, USA).

Other chemicals and used supplies included an AFM1 standard (Orsell SRL, Modena, Italy) and AFM1 Immunoaffinity columns (IAC) (VICAM, Watertown, MA, USA). The AFM1 standard was purchased in acetonitrile at a concentration of 10 μg/mL.

2.5. Statistical analysis

Statistical methods were used in this study to evaluate differences in the AFM1 occurrence in milk during different seasons; *t*-test and ANOVA were applied to data. The groups were considered statistically significant different at $p < 0.05$.

3. Results and discussion

3.1. Method performance

Determination of AFM1 concentration in the samples was carried out by external standard calibration curve. The validation of the method was performed evaluating the performance criteria set out in the European Regulation EC/519/2014 laying down the methods of sampling and analysis for the official control of the mycotoxins levels in foodstuffs (EC, 2014).

The linearity of the method was verified in the range of 0.1–5.0 μg/L, corresponding to AFM1 concentration values between 0.004 and 0.200 μg/kg. Linear least square regression was applied in order to calculate five-point calibration curve plotting peak area *vs* AFM1 concentration. The value of r^2 was higher than 0.99, demonstrating good linearity. The limit of quantification (LOQ = 0.004 μg/kg) was calculated by analyzing in triplicate several blank samples fortified with AFM1 at decreasing concentrations in order to define the lowest concentration level determinate with a 5/1 signal-to-noise ratio. Precision of the analytical method, in terms of repeatability, was assessed through the analysis of the blank sample spiked at concentrations of 0.025, 0.050, 0.075 μg/kg, in at least five replicates. Five replicates for each level were also analyzed by another operator with diverse lots of reagents and solvents, on different days (15), to calculate the within laboratory reproducibility. Trueness was then calculated as the mean recovery percentage during an intra-laboratory study by analysing six replicates of spiked milk samples at 0.025, 0.050 and 0.075 ng/g, and compared with the interval of acceptability provided by the European Regulation EC/519/2014 (EC, 2014) in all cases; the recovery values obtained, ranging between 80 and 107%, are compliant to the required performance criteria for quantitative methods of mycotoxin analysis (range of 60–120%). Specificity was assessed by verifying the absence of interfering peaks at around ±2.5% of the AFM1 retention time in the chromatogram of twenty aflatoxin-free samples. For analytical quality assurance measures, a procedural blank and a spiked sample were included in each batch of samples.

3.2. Buffalo milk

From January 2015 to December 2016, a total of 804 samples of