DOI 10.1002/mnfr.200500224 Mol. Nutr. Food Res. 2006, *50*, 300 – 305

# Survey of the occurrence of Aflatoxin M1 in ovine milk by HPLC and its confirmation by MS

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During the period of October – July 2000, 240 samples of dairy ewes milk, obtained from farms of Enna (Sicily, Italy), were checked for Aflatoxin $M_1$ ($AFM_1$) by HPLC using a fluorimetric detector. The limit of detection and the limit of quantification were 250 ng/L for $AFM_1$. All the positive milk samples for $AFM_1$ were confirmed by LC-MS. $AFM_1$ was detected in 81% of milk samples, ranging from 2 to 108 ng/L. Three samples were over the legal limits (50 ng/L). Mean contamination of samples obtained from stabulated ewes was higher than that from grazing ewes (35.27 *vs*. 12.47 ng/L). Furthermore, samples collected in the period September – October showed higher contamination than samples collected during the other months (42.68 *vs*. 10.55 ng/L). Both differences are related to the administration of compound feed. Based on current toxicological knowledge we concluded that the $AFM_1$ contamination levels recorded in ewe milk did not present a serious human health hazard. However, as ewe milk is exclusively used to produce cheese due to its higher protein content, and also considering the preferential binding of $AFM_1$ to casein during coagulation of milk, a potentially high concentration effect could occur, thus the surveillance of contamination levels should be more continuous and widespread.

**Keywords:** Aflatoxin $M_1$ / Italy / Ovine milk / Ovine cheese / Survey

Received: October 25, 2005; revised: December 20, 2005; accepted: December 21, 2005

## 1 Introduction

Aflatoxins (AF) are a group of highly toxic secondary metabolic products of some *Aspergillus* spp. (*A. flavus, A. parasiticus* and the rare *A. nomius)* that easily occur on feeds and foods during growth, harvest, or storage. Aflatoxin $B_1$ ($AFB_1$), in the AF group, is the most potent hepatotoxin with a large variety of biological effects, such as carcinogenicity, teratogenicity and mutagenicity in humans and farm animals and it is included in the group 1B by International Agency for Research on Cancer [1, 2].

Aflatoxin $M_1$ ($AFM_1$) and Aflatoxin $M_2$ ($AFM_2$) are the hepatic hydroxylated metabolites of $AFB_1$ and Aflatoxin $B_2$ ($AFB_2$), respectively. $AFB_1$ *per se* is not genotoxic but is bioactivated by liver metabolism trough hepatic microsomal cytochrome P450 in the attempt to increases its hydrophilic properties and excretion in urine or milk. $AFM_1$ is found in milk and milk products obtained from livestock that have ingested $AFB_1$-contaminated feed [3]. The carcinogenicity of $AFM_1$ is about ten times less than that of $AFB_1$ [4 – 6] and for these reasons has been included in the class 2B by International Agency for Research on Cancer [2]. The dangerousness of mycotoxins and the unavoidability of their contamination led public authorities to adopt severe control policies. The Joint Expert Committee on Food Additives (JEFCA) of the World Health Organization and of the Food and Agriculture Organization has recently evaluated the hazard of the most significant mycotoxins (*e.g.* OTA, fumonisins, deoxynivalenol, T-2 toxin, patulin, and zearalenone) and established the Provisional Maximum Tolerable Weekly Intake (PMTWI) and the Provisional Maximum Tolerable Daily Intake (PMTDI). A different approach was applied for genotoxic mycotoxins such as $AFB_1$. As $AFB_1$ has carcinogenic properties, assuming that

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**Abbreviation: AF**, Aflatoxin(s)

a no-effect concentration limit cannot be established for genotoxic compounds, any dose will have a proportional effect. Thus, JECFA did not establish a PMTWI or PMTDI, but recommended that its level in food should be As Low As Reasonably Achievable (ALARA). To reduce AF exposure risk, European Community established maximum consented limits for $AFB_1$ in animal feed (5 μg/kg) [7] amended with Commission Directive 2003/100/EC of 31 October 2003 [8] and a legal limit of 0.025 μg/kg for $AFM_1$ in infant foods milk and milk products (Commission Regulation (EC) N. 683/2004) [9].

In lactating animals the conversion rate of $AFB_1$ to $AFM_1$ ranges between 0.5 and 6% [10]. Variability is due to different factors such as individual response, $AFB_1$ intake level, stage and order of lactation [10]. Literature data about $AFB_1$ carry over in dairy ewes milk are very scarce. The first experimental study was recently conducted by Battacone *et al*. [11], who reported that milk $AFM_1$ concentration was significantly affected by the $AFB_1$ dose. The authors concluded that a single dose of $AFB_1$ to lactating dairy ewes showed that $AFM_1$ excretion in milk is lower than that observed for the dairy cows. On the contrary, continuous administration of $AFB_1$ up to 128 μg for 14 days showed that the concentration of $AFM_1$ in milk was significantly related to the $AFB_1$ dose, but no $AFM_1$ was detected in the milk 3 days after last administration. Evidence of potential hazardous human exposure to $AFM_1$ from dairy products arises from several studies [12, 13]. Moreover, as milk is the main nutrient for growing young, who are potentially more sensitive and have less variety in their diets, the occurrence of $AFM_1$ in milk and milk products is a serious problem of food hygiene [14].

Here, we show the $AFM_1$ occurrence in several dairy ewes breeding from Enna, a Sicilian province (Italy), where ovine milk and cheeses are largely produced. Ancillary for the first time the chemical identification of $AFM_1$ in ewe milk was obtained by LC-MS/MS in order to avoid any doubts about its chemical identification.

## 2 Materials and methods

### 2.1 Sampling

During the period October – July 2000 a monthly sampling of dairy ewe milk from 24 farms from Enna province, Sicily, Italy has been conducted. Two hundred-forty milk samples (100 mL, taken by milk including the portions derived from both milking) were immediately refrigerated and subsequently analyzed by chromatographic approach. Detailed nutritional information has been recorded by each farmer about the kind and composition of the animal diet.

### 2.2 Analysis for $AFM_1$

Milk samples (40 mL) were added and vortex-mixed with 1 g of sodium chloride and then centrifuged to Relative Centrifugal Force (RCF) = $3082 \times g$ ($RCF = 1{,}118 \times 10^{-5} \times r \times rpm^2$, where $r$ = rotor radius expressed in centimeters = 17.2 cm and rpm = 4000). After centrifugation, the upper cream layer was completely removed by aspirating through a Pasteur pipette. The skimmed milk was separated by cream part and 25 mL of this sample was loaded onto ImmunoAffinity Columns (IAC) produced by Vicam (Afla $M_1^{TM}$ columns, MA, USA). The IAC was eluted following technical sheet of Vicam and AF were collected with one mL of pure methanol. All the purified extracts were filtered through a 0.20 μm syringe filter (Millipore, Bedford, MA, USA) prior to injection (20 μL) onto the HPLC column. The samples were dried by vacuum system (RC10.10. and RTC60, Jouan, Nantes, France) and resolved with 0.1 mL of methanol to concentrate the samples 250 times.

### 2.3 Reagents

$AFM_1$ and $AFM_2$ standards were from Sigma Chemical Company (St. Louis, MO, USA). Water, for the HPLC mobile phase was produced in a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and HPLC-grade solvents were from Merck (Darmstadt, Germany).

### 2.4 Standards preparation

#### 2.4.1 AF stock standard solution

$AFM_1$ and $AFM_2$ from a commercial supplier were shipped in a sealed ampoule. Of pure $AFM_1$ and $AFM_2$ standards 0.1 mg was transferred to a volumetric flask and re-suspended in 1 mL of pure methanol to obtain a final concentration of 100 mg/L. It was diluted with methanol and immediately they were labeled, frozen and stored at dark and −20°C.

#### 2.4.2 Blank sample

The blank sample was prepared by extracting uncontaminated portion milk where AF levels were under limit of detection of fluorimetric detector. It was necessary to evaluate any background matrix interferences due to co-extracted compounds that contaminate reagents, apparatus or other.

#### 2.4.3 AF dilution standard solutions

All the working AF solutions were prepared by appropriate dilutions with pure methanol of the stock standard solution and the dilutions were representative of the range analyzed.

 

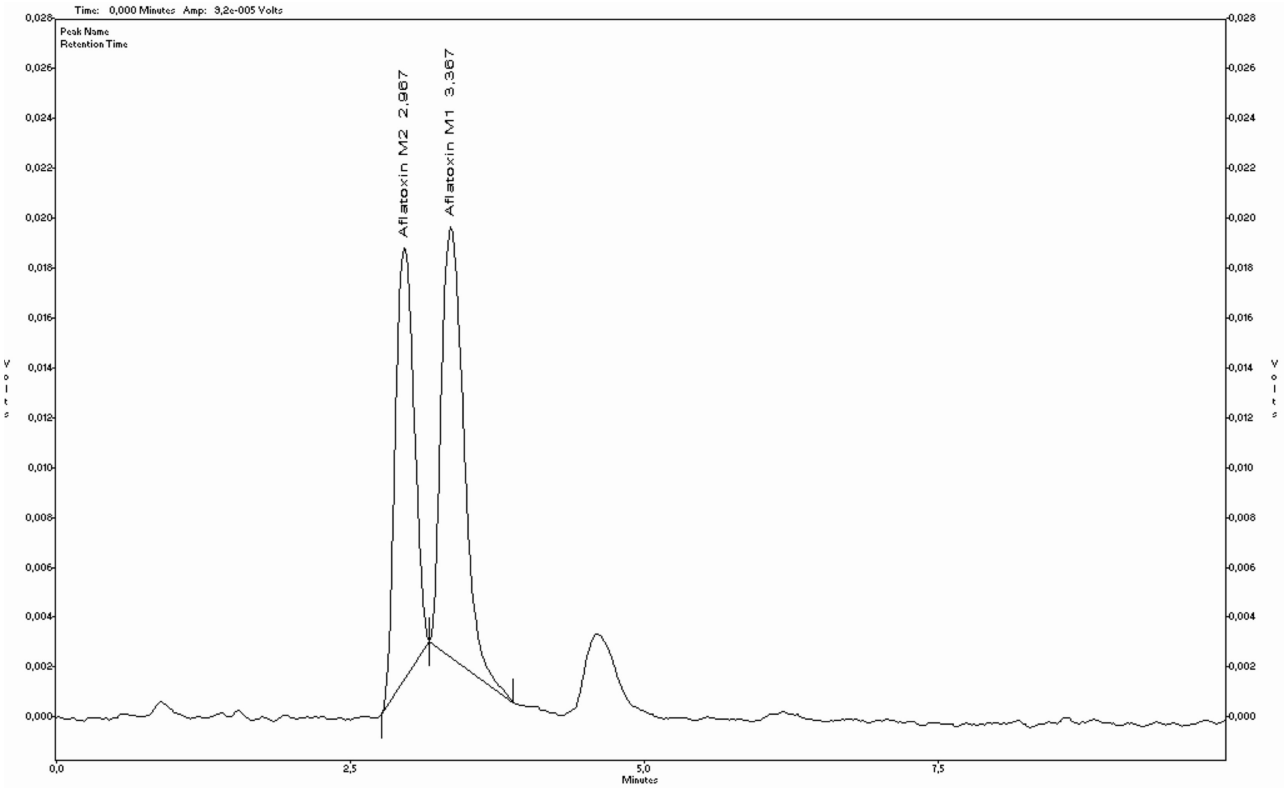


**Figure 1.** HPLC-fluorescence detector peaks of 25 ppb Aflatoxin $M_1$ and $M_2$ standards.

### 2.5 HPLC equipment

HPLC analyses were performed using LC-10AD pumps and a fluorescence detector model RF-10Axl (Shimadzu, Japan), set at an excitation wavelength of 360 nm and emission wavelength of 440 nm. Data acquisition and handling were done by a system control SLC10A with Shimadzu software Class VP version 4.3. A Bio-Sil C18 HL ($150 \times 4.6$ mm, 5 µm, Bio-Rad) column was used. Isocratic HPLC conditions require constant flow of 1 mL/min and $CH_3CN–H_2O–CH_3OH$ (25 : 50 : 25 v/v) as eluent system. Eluent was freshly prepared and filtered (0.22 µm, Millipore) before use.

Mycotoxin HPLC identification was performed by comparing retention times, fluorimetric spectra of the purified samples and co-injection with AF standards.

Each chromatographic run was 10 min and retention times recorded were 2.99 and 3.39 min for $AFM_2$ and $AFM_1$, respectively (RSD 1.3%). These retention times were obtained with ten consecutive injections of the working solutions (ten for each AF) within the same day with HPLC with fluorimetric detector and a mix of $AFM_1$ and $AFM_2$ to 25 ppb run is showed in Fig. 1. The calculated instrumental LOD (S/N = 3) and LOQ (S/N = 5) for $AFM_1$ and $AFM_2$ were 250 ng/L and 500 ng/L, respectively. These limits were obtained with AF working solution diluted with pure methanol to concentration 250 and 500 ng/L.

To quantify AF in the samples the calibration curves for $AFM_1$ and $AFM_2$, ranging from 1 to 500 ng/L, were built. All chromatographic results were statistically examined, and the RSD were calculated [IUPAC Compendium of Chemical Terminology, http://makeashorterlink.com/?A16D56 CEB].

### 2.6 Recovery experiments

Extraction protocol was tested to determine recovery efficiency for $AFM_1$ and $AFM_2$ at three different contamination levels: 200, 50 and 1 ng/L. The three different contamination levels were obtained spiking 1 L of fresh milk with 200, 500 and 100 µL of $AFM_1$ and $AFM_2$ standard solution at 1000, 100 and 10 ng/L, respectively. Each test was performed three times, and the value shown is the average of three HPLC measurments. All of the residue data shown were corrected for calculated recovery.

Recovery averages for $AFM_1$ were 68.31% at 1 ng/L, 75.53% at 50 ng/L and 90.06% at 200 ng/L (RSD 6.68, 10.19 and 21.92%, respectively). On the contrary, the results

 

for $AFM_2$ were considered insufficient: 25.16% at 1 ng/L, 12.10% at 50 ng/L and 18.34% at 200 ng/L (RSD 27.38, 9.46 and 21.53%, respectively). Due to unsatisfactory recovery levels, data on $AFM_2$ occurrence are not shown.

### 2.7 MS analysis

Perkin-Elmer LC series 200 pumps connected to an API-3000 triple quadruple mass spectrometer (Applied Biosystems, Toronto, Canada) were used. The method required a turboionspray source with positive capillary voltage of +5500 V and source temperature of 450°C. For both AF, declustering potential (42 to 58 V) and collision energy (26 to 42 V) were optimized infusing with an external pump (Harvard Apparatus, Holliston, MA, USA) a standard solution containing 10 μg/mL of $AFM_1$ and $AFM_2$ at a flow of 6 μL/min. Full-scan spectra were acquired from 100 to 500 amu with a step size of 0.1 amu and a dwell time of 0.1 ms in order to select the most abundant *m/z* value. The molecular ions *m/z* 329.1 for $AFM_1$ and 331.1 for $AFM_2$ were selected.

To optimize chromatographic separation conditions a Synergi column (Phenomenex, USA) MAX-RP 80Å 50 × 2.00 mm, 4 μm, was used with a constant flow of 200 μL/min. The selected time program required $H_2O$ (A) and MeOH/$CH_3CN$ (90/10 v/v) (B) as starting eluent mix. Then solvent B rises to 20% in 10 min and from 11 to 13 min rises to 100% to come back to 80% in other 2 min. MS/MS spectra were obtained selecting with collision-activated dissociation and fragmenting a selected parental ion. The instrument *m/z* scale was calibrated with the ions of the ammonium adducts of polypropylene glycol. The main spectrometer parameters (molecular weight, retention time, daughter ions, declustering potential and collision energy) for investigation of AF by LC/MS/MS are briefly summarized in Table 1.

**Table 1.** MS parameters used to investigate AF by LC/MS/MS[a)]

| Compound | $[MH]^+$ | $t_r$ (min) | daughter ions (*m/z*) | DP (V) | CE (V) |
|---|---|---|---|---|---|
| $AFM_1$ | 329.1 | 5.43 | 273.1 | 50 | 33 |
| | | | 258.9 | | 32 |
| | | | 309.1 | | 26 |
| $AFM_2$ | 331.1 | 4.88 | 273 | 50 | 30 |
| | | | 285.1 | | 31 |
| | | | 259.1 | | 31 |

a) $[MH]^+$ = precursor ion; $t_r$ (min) = retention time; MS/MS ions (*m/z*) = daughter ions, DP (V) = declustering potential; CE (V) = collision energy.

Under these conditions, $AFM_2$ and $AFM_1$ average retention times were 4.88 and 5.43 min, respectively (RSD 1.8%) and were obtained with ten consecutive injections of the working solutions (10 ng/L for each AF) within the same day. These retention times enhanced the chromatographic resolution of the AF peaks from matrix interferences. In Fig. 2 LC/MS/MS run of a mix of $AFM_1$ and $AFM_2$ at 10 ppb concentration is reported.

The calculated instrumental LOD and LOQ for $AFM_1$ and $AFM_2$ were 250 ng/L (S/N = 3) and 500 ng/L (S/N = 5), respectively.

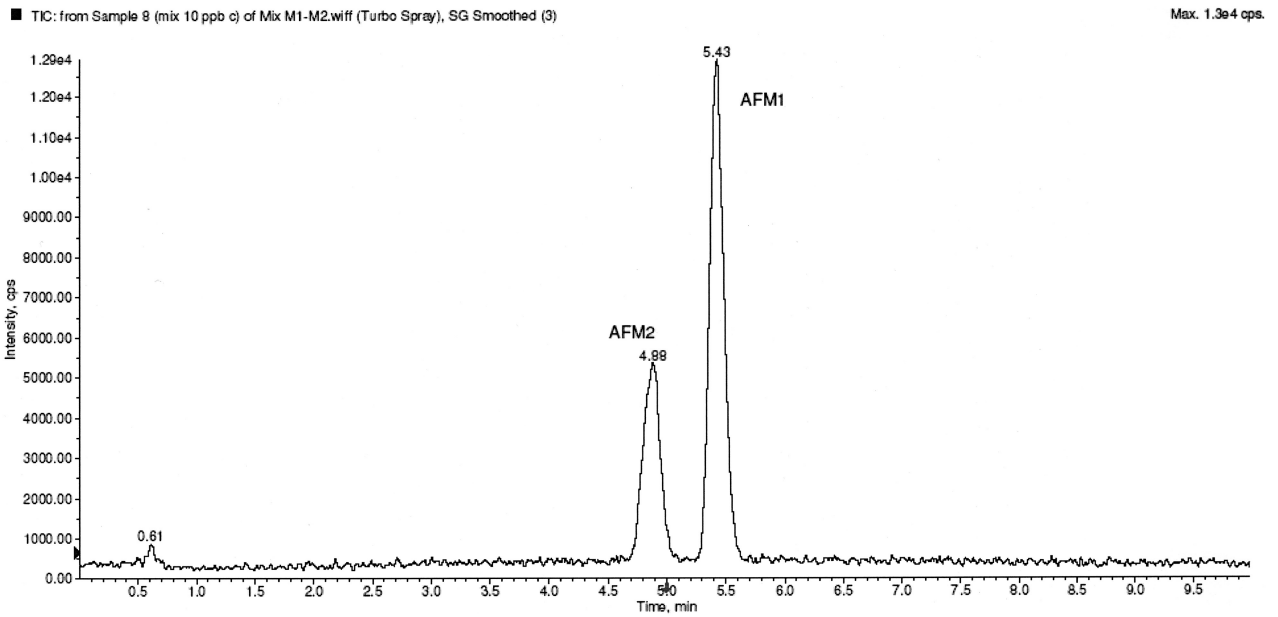


**Figure 2.** LC/MS/MS peaks of 10 ppb Aflatoxin $M_1$ and $M_2$ standards.

 

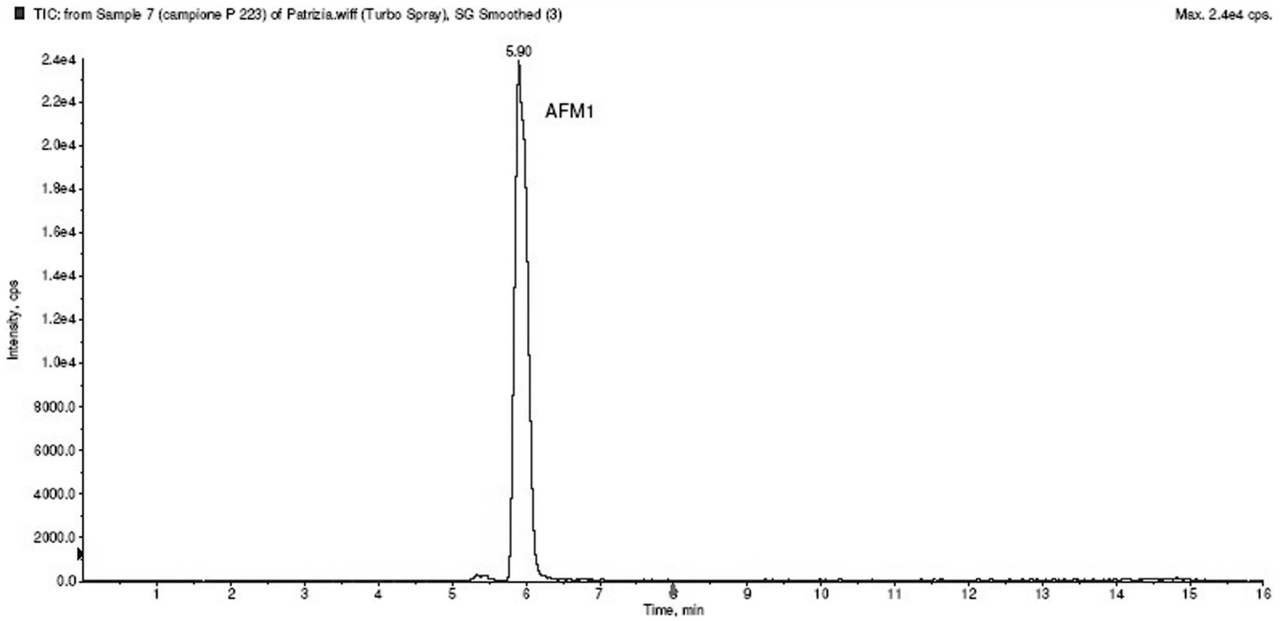


**Figure 3.** Chromatogram of ewe milk $AFM_1$ contaminated sample.

## 3 Results and discussion

All samples were analyzed by LC/MS/MS using molecular peak ions and potassium and sodium adducts while the $AFM_1$ quantification was performed by HPLC-fluorescence detector. Chromatogram of ewe milk $AFM_1$-contaminated sample is shown in Fig. 3 where a co-eluted $AFM_1$ peak at 5.90 min corresponding to peak fragmentation pattern and retention time of $AFM_1$ can be recognized. The use of LC/MS/MS allowed to achieve confirmation of $AFM_1$ chemical structure and, at the same time, to avoid immunoaffinity prepurification of samples, thus being also cost and time effective.

$AFM_1$ incidence in milk was severe as 81% of samples were positive (Table 2), however, the contamination levels have been quite modest. In fact, $AFM_1$ was detected at low level (1–10 ng/L) in 79% of the samples and ranging from 10 to 50 ng/L in the 20% of the samples and only three samples were above the legal limits (50 ng/L).

The meaningful data consist in the relation among different levels of contamination and the sample provenience; in fact, $AFM_1$ contamination of milk samples from stabulated ewes was higher than of those from grazing ewes (35.27 *vs*. 12.47 ng/L). In addition, a significant difference was observed between the mean $AFM_1$ contamination levels observed during the September–November period and the remaining period of the year (42.68 *vs*. 10.55 ng/L). Both differences can be easily accounted for by the administration of compound feed. Indeed, stabulated ewes received daily compound feed for the whole survey period. Grazing ewes received no or low amount of compound feed, except for the September–November period, when poor pastures are usually available.

**Table 2.** $AFM_1$ (ng/L) occurrence in dairy ewes milk

| Samples | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Frequency distribution | | | | Contamination | |
| Tested | Positive | < 1 ng/L | 1–10 ng/L | > 10–50 ng/L | > 50 ng/L | Range[a] | Average[b] |
| *n* | *n* (%) | *n* (%) | | | | | |
| 240 | 195(81) | 45 | 145 (60) | 47 (20) | 3 (1) | <1–108 | 15.36 |

a) Min–max.
b) Mean of positive samples.

We are not aware of other surveys on the $AFM_1$ content of ewe milk, so that comparison of results is not possible. However, our results are very similar to those observed for cow milk. Indeed, natural occurrence of $AFM_1$ in cow milk is well reported in literature by numerous papers recently reviewed by Galvano *et al*. [13]. In their conclusions, the authors evidenced that: (i) the incidence of $AFM_1$ contamination is often higher in commercial milk than in raw farm milk, because of the dilution of uncontaminated bulked milk by a few contaminated samples. (ii) For the same reason, high $AFM_1$ contamination levels in commercial milk seldom occur. (iii) Seasonal effect on contamination is related to the reduced availability of pastures and forages with consequent administration of compound feed. (iv) The occurrence of $AFM_1$ in cow milk and milk products is widespread, even if contamination levels do not seem to be a serious health hazard according to the current scientific fund

 

of information. We believe that the same considerations are suitable for ovine milk, too.

At any rate, the enormous discrepancy between the amount of literature data on $AFM_1$ contamination of cow and ewe milk appears unjustified. It is a fact that cow milk is a staple food largely consumed worldwide also by its numerous derivative. However, data on $AFM_1$ in ewe milk would be necessary for many reasons. Ewe milk is not consumed directly but is widely used, singly or mixed to other animal milk, to produce cheese. It is notorious that $AFM_1$ distribution in milk is not homogeneous. In particular, the preferential binding of $AFM_1$ to casein during milk coagulation leads to a remarkable concentration effect. For cheese from cow milk, this concentration is 2.5- to 3.3-fold higher in soft cheese and 3.9- to 5.8-fold higher in hard cheese [15].

No data are available on the concentration effect for ovine cheese, but it is presumable that it could be more pronounced in cheese from ewe milk, which have the highest protein content among the diverse milk used to produce commercial cheese. Obviously, this hypothesis should be experimentally verified and, in this respect, studies are actually in progress in our laboratory.

## 4 Concluding remarks

According to results reported for cow milk, this surveillance indicates a high incidence along with low levels of $AFM_1$ contamination in ovine milk. Mean contamination level of $AFM_1$ recorded in the present survey should be classified as a no serious human health hazard. Nevertheless, since ewe milk is exclusively used for cheese production with unverified concentration effects, also during ripening, and considering that ovine cheeses are fairly consumed in Italy, any definitive toxicological conclusion is premature. Thus, until experimental data on the concentration effects will be available, surveillance must be continuous and widespread, also because $AFB_1$ recurs in feeds over long periods based on the overall climate and it may or may not be present in a particular year depending on the weather in the respective period.

*Authors are very grateful to Dr. Patrizia Dell'Aquila for her technical assistance and to Dr. Rosalia Ferracane for her contribution to LC/MS/MS aspects of research.*

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