Binding of Aflatoxin M₁ to Different Protein Fractions in Ovine and Caprine Milk

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ABSTRACT

The affinity of aflatoxin M₁ toward the main milk protein fractions in ewe and goat milk was investigated by using an ELISA. This study took into account the possible effects of common dairy processes such as ultrafiltration, acidic or rennet curding, and production of ricotta from acidic or rennet whey. Treatments that allowed the separation of casein from whey proteins under conditions that do not alter the physical or chemical status of the proteins (such as ultracentrifugation) were used as a reference. None of the treatments used in typical dairy processes caused significant release of the toxin, in spite of the relevant changes they induced in the interactions among proteins. Only the combined heat and acidic treatment used for production of ricotta cheese altered the structure of whey proteins to the point where they lost their ability to bind the toxin. This study also showed that, regardless of the physical state of the sample, a commercial electronic nose device, in combination with appropriate statistical tools, was able to discriminate among different levels of sample contamination.

Key words: aflatoxin M₁, goat and ewe milk protein, dairy by-product, electronic nose

INTRODUCTION

Aflatoxins are the most studied group of mycotoxins produced by molds, especially *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin M₁ (AFM₁) is the main hepatic carcinogenic metabolite of aflatoxin B₁ (AFB₁) that is excreted in milk when lactating animals are fed AFB₁-contaminated feeds. Lafont et al. (1989) observed that AFM₁ has high genotoxic activity. To reduce the risk associated with AFM₁ uptake by humans, most developed countries have regulated the maximum levels of AFM₁ (50 ng/kg) allowed in milk and dairy products (Van Egmond, 1989). Aflatoxin M₁ is heat sta-

ble and is only inactivated at temperatures of at least 250°C (Ellis et al., 1991).

Aflatoxins in cheese have 3 main sources: the presence of AFM₁ in milk, as a consequence of feed contaminated with AFB₁ eaten by dairy cattle; synthesis of aflatoxin (B₁, B₂, G₁, and G₂) by fungi that grow on cheese (although the low level of carbohydrate does not make it a very suitable substrate; Zerfiridis, 1985); and the use of powdered milk contaminated with AFM₁ for cheese production (Blanco et al., 1998).

Many studies on bovine milk have demonstrated that AFM₁ binds to milk proteins, and in particular to CN, and that it is concentrated in the curd during manufacture of cheese (Brackett and Marth, 1982; Lopez et al., 2001). For these reasons, AFM₁ concentrations are 3- to 4-fold higher in cheese than in milk (Kiermeier and Buchner, 1977). Investigations on the affinity of AFM₁ toward different proteins in milk from other species are very limited. Information on the binding of AFM₁ to proteins in ovine and caprine milk is particularly important because most ovine and caprine milk is processed into cheese (Battaccone et al., 2003, 2005; Boggiano et al., 2006). The presence of AFM₁ in commercial ovine cheese was reported by Minervini et al. (2000); those authors reported AFM₁ amounts between 50 and 210 ng/kg; that is, at levels well above the current European limits.

In this study, we investigated AFM₁ distribution in the different protein fractions in milk and in protein fractions obtained during production of ovine and caprine cheese, with the aim of identifying a possible relationship between the physical status of the various proteins and AFM₁ binding. For this purpose, the AFM₁ content was measured by an ELISA procedure. The same samples were analyzed by a commercial electronic nose that in previous work was used as an innovative screening methodology for simple and rapid detection of AFM₁ in a large number of ovine milk samples (Benedetti et al., 2005).

MATERIALS AND METHODS

Materials

Aflatoxin M₁ was obtained from Sigma (St. Louis, MO). Because AFM₁ is photosensitive, the solutions

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were stored in aluminum foil-wrapped vials, and were not exposed to daylight or placed close to fluorescent light sources.

An ELISA-format Ridascreen aflatoxin M₁ kit (R-Biopharm GmbH, Darmstadt, Germany) was used to detect AFM₁ immunochemically.

Samples

Twenty-four raw milk samples were collected from several different groups of ewes and goats fed a formulated feed containing increasing amounts of aflatoxin B₁. Samples were immediately frozen after milking and stored at -20°C until analysis. Individual milks were then pooled according to their AFM₁ content as determined immunochemically. This gave 3 distinct pools of each milk with AFM₁ contents of 50, 100, and 150 ng/kg. A reference milk sample had an AFM₁ content lower than the detection limit of the ELISA assay.

The experiments on the animals followed the guidelines of the Council Directive of EC (European Community, 1986). The health of animals was monitored continuously before and during the experimental period.

Milk Treatment

Unless otherwise specified, the same procedures were used for caprine and ovine milk. Ultrafiltration was performed in an Amicon apparatus using a 10,000-Da cut-off membrane for milk samples and a 5,000-Da cut-off membrane for whey samples.

Separation of native CN micelles was obtained by ultracentrifugation at $80,000 \times g$ at 4°C for 45 min, using an SW 28 rotor in a Beckman L5-75 ultracentrifuge as described by Iametti et al. (1993). The precipitated CN fraction was resuspended in 50 mM phosphate buffer, pH 6.8, to a final volume identical to that of the starting milk. The supernatant was used as such for all determinations.

Acid coagulation of milks was performed by addition of 88% lactic acid to lower the milk pH to 4.3. Flocculated CN was separated by centrifugation at $3,000 \times g$ for 10 min. Enzymatic coagulation was performed on milk heated at 60°C for 15 min. After this treatment, milk was refrigerated to 35°C, and a 0.8% (wt/vol) solution of chymosin in water was added at a ratio of 1:25. After 30 min at 35°C, the curd was separated by using a suitable metallic mesh. Ricotta cheese was produced starting from the residual whey fraction from either enzymatic or acid coagulation. Whey samples from enzymatic coagulation were acidified to pH 5.6 with 2 N HCl before the heat coagulation step, which was carried out at 90°C for 15 min under stirring. After resting for 15 min, the resulting ricotta was separated by draining.

ELISA Procedure

Quantification of AFM₁ in all samples was carried out with a commercial competitive ELISA kit (Ridascreen aflatoxin M₁, R-Biopharm). A suitable sample aliquot was centrifuged at $2,800 \times g$ at 4°C for 10 min, and 0.1 mL of the supernatant was used for the ELISA determination, which was carried out as recommended by the kit supplier. Final absorbance was measured in a BioRad model 3550 Microplate Reader (BioRad, Richmond, VA).

Electronic Nose

Analyses were performed with a commercial electronic nose (model 3320, Applied Sensor Lab Emission Analyser; Applied Sensor Co., Linköping, Sweden). The instrument consisted of 3 parts: an automatic sampling apparatus, a detector unit containing the arrays of sensors and the controls for keeping them at the appropriate working temperature, and the hardware and software required for pattern recognition (Gardner and Bartlett, 1994; Shaller et al., 1999). The automatic sampling system allowed sample loading under controlled temperature.

The sensor array in the instrument used here was composed of 22 different sensors, namely, 10 metal oxide semiconductor field effect transistors (MOSFET), and 12 Taguchi-type sensors (metal oxide semiconductors, MOS; Benedetti et al., 2005). The MOSFET sensors were divided into 2 arrays of 5 sensors each, one operating at 140°C and the other at 170°C, whereas the 12 MOS sensors, mounted in a separate chamber, were kept at 400 to 500°C.

One milliliter of each sample was placed in a 40-mL Pyrex vial fitted with a silicon/Teflon septum. After a 20-min equilibration at 20°C, the measurement sequence was started. Operating conditions were flow rate: 1 mL/min; sampling time: 60 s; standby temperature: 20°C. The total cycle time for each measurement was 5 min. No sensor drift was experienced during the measurement period. Each sample was evaluated 3 times and the average of the results was used for subsequent analysis.

Data Analysis

The data obtained from the electronic nose for the ovine and caprine milk samples were analyzed by principal component analysis (PCA) performed with the SCAN software (v. 1.1; Minitab Inc., State College, PA).

Principal component analysis was used for explorative data analysis on the autoscaled data because it identifies orthogonal directions of maximum variance in the original data, in decreasing order, and projects

the data into a lower-dimensionality space formed of a subset of the highest variance components. The orthogonal directions are linear combinations (the principal components) of the original variables and each component explains in turn a part of the total variance of the data; in particular, the first significant component explains the largest percentage of the total variance, the second one, the second largest percentage, and so forth.

Cluster analysis performs agglomerative hierarchical clustering of objects based on distance measures of dissimilarity or similarity. The hierarchy of clusters can be represented by a binary tree called a dendrogram. A final partition (i.e., the cluster assignment of each object) may be obtained by cutting the tree at a specified level (Gardner and Bartlett, 1992).

RESULTS AND DISCUSSION

ELISA Detection of AFM₁

In this investigation, we addressed the binding of AFM₁ to the main protein fractions in ovine and caprine milk samples that underwent different physical or enzymatic treatments, performed in small, discontinuous batches.

We first explored whether laboratory-scale ultrafiltration was able to remove AFM₁ from ewe or goat milk, because this physical technique has been used for the selective separation of some toxic compounds from milk (Kosikowski and Jimenez-Flores, 1985). The membranes used for this study had a nominal cutoff of 10,000 Da, thus retaining all proteins in milk, as confirmed by SDS-PAGE analysis (not shown).

The distribution of AFM₁ between the retentate and permeate fractions of ewe and goat milk samples is shown in Figure 1 as a function of the amount of toxin in the starting milk. Figure 1 shows that more than 80% of the AFM₁ is retained in the protein-containing phase. Almost identical trends are observed for ewe and goat milks. From a practical standpoint, these results indicate that ultrafiltration does not represent a suitable method for removal of AFM₁ from contaminated ovine and caprine milks.

To better clarify whether the affinity toward AFM₁ is related to the physical properties of milk proteins, and in particular to the micellization state of CN, we studied whether CN fractions separated by different methods had a different AFM₁-binding abilities. Treatments used in this study encompassed the range from nondenaturing isolation procedures (i.e., ultracentrifugation; Iametti et al., 1993) to procedures that are known to affect the micellar organization and interprotein interactions, such as renneting or acid coagulation.

Figure 1. Distribution of aflatoxin M₁ (AFM₁) between the retentate and permeate fraction of goat and ewe milk is plotted as a function of the AFM₁ concentration in the original milk.

The partition of AFM₁ between the 2 protein fractions separated by ultracentrifugation from ovine milk is shown in Figure 2. Almost identical results were ob-

Figure 2. The distribution of aflatoxin M₁ (AFM₁) between the protein fractions separated by ultracentrifugation from ewe milk is given as a function of the AFM₁ concentration in the original milk.

Figure 3. The distribution of aflatoxin M_1 (AFM₁) between the 2 fractions separated by acidic or enzymatic (rennet) curdling is given as a function of the AFM₁ concentration in the original milk.

tained with caprine milk, indicating a change in the distribution of the toxin when the toxin content in the starting milk was higher. Binding of AFM₁ to native CN shows saturation-like behavior. In view of the modest increase in free AFM₁ even at the highest concentrations used here (as indicated by ultrafiltration data), it seems that nonprecipitable proteins bind AFM₁ only after the toxin saturates the binding sites on the native, precipitable CN.

The results presented above do not provide information about the structural features of CN that are most relevant to the binding of AFM₁, and about the possible effects of enzymatic or chemical modifications involved in cheese making. The amount of AFM₁ found in the protein fractions separated after enzymatic or acid curdling of the 2 milks is shown in Figure 3. These results suggest that the modification induced by curdling may affect the affinity of AFM₁ toward CN. In most cases, no indication of preferential partition of the toxin between the curd and whey phases was evident. In ovine milk, renneting did not alter the relative affinity of CN toward AFM₁. In this case, binding to curd pro-

teins showed saturation behavior close to that observed for the native CN isolated by ultracentrifugation.

Whey is often used in Italy for the production of ricotta cheese. Given the relatively high amounts of AFM₁ in the residual whey fractions from either milk, we investigated the relative distribution of AFM₁ in ricotta cheese and in the residual liquid fraction (called "scotta"). Very low amounts of AFM₁ (sometimes below the detection limits of the ELISA used in this study) were found in ricotta cheese prepared from milk and from both whey types. This indicates that AFM₁ did not bind to protein fractions in ricotta cheese, and that the structural modifications of whey proteins induced by the acid or thermal treatments used for ricotta production prevented binding of AFM₁ to the involved proteins.

Electronic Nose

As previously demonstrated, the electronic nose is well suited to the routine detection of AFM₁-contaminated milk samples and can be successfully applied for rapid screening of AFM₁ contamination in ovine milk

Figure 4. Typical sensor responses for milk samples containing increasing amount of aflatoxin M_1 , and for curd, whey, and by-products obtained by enzymatic or acidic curdling of ewe and goat milk.

samples (Benedetti et al., 2005). The electronic nose is only sensitive to molecules in the headspace, which implies that AFM_1 must be present in the headspace even in conditions where significant protein binding occurs, and that binding of AFM_1 to milk proteins is characterized by relatively high dissociation constants. In previous studies, the presence of AFM_1 in the headspace of naturally contaminated and AFM_1 -spiked ovine milk samples was confirmed by mass spectrometry analysis (Benedetti et al., 2005). As pointed out by

those authors, this observation does not rule out the possibility that the electronic nose senses other AFM_1 -derived molecules (including other AFB_1 catabolites) present in the headspace along with unmodified AFM_1 . In the frame of this study, the electronic nose was used to gather further information on the stability of protein- AFM_1 complexes in the various samples considered in the first part of this report, including the effects of the various treatments considered above on the interaction between AFM_1 and proteins.

Figure 5. Principal component (PC) analysis score plots for ewe and goat milk products. In both panels, samples are identified as follows: acidic curd (○); rennet curd (●); acidic ricotta (□); rennet ricotta (■); acidic scotta (▽); and rennet scotta (▼). For each datapoint, values identify the aflatoxin M₁ content in the original milk (ng/kg).

Figure 4 shows typical sensor patterns for milk samples containing increasing amounts of AFM₁, and includes data on curd, whey, ricotta, and scotta obtained by enzymatic or acidic curdling of ewe and goat milk. The individual bars within each sample column (e.g., milk 50) represent the signals of the 23 different sensors of the electronic nose. Each bar represents the mean value obtained from the analysis of 3 different samples. The differences in the signal patterns are clearly visible,

as is their dependence on the presence of AFM₁. When comparing the data presented in Figure 4 with the distribution data in the previous section, it appears that the results obtained with the sensor array closely parallel those obtained with the more laborious ELISA assays. Of particular relevance is the fact that the electronic nose supports immunochemical evidence for the absence of AFM₁ in all the ricotta samples, regardless of the origin of the whey used for their production.

Figure 6. Classification dendrograms (cluster analysis) for ewe and goat milk products.

The sensor responses were collected and elaborated by PCA, which was performed on unscaled data to achieve a partial visualization of the set in a reduced dimension. The first 2 principal components (PC1 and PC2) represented 99.1% (ewe) and 99.5% (goat) of total variance, and their score plots (Figures 5A and 5B) allowed a separation of the samples according to the presence of AFM₁. Samples were distributed along PC1 from the left to the right part of the plots, as a function of the amount of AFM₁ in the samples.

To identify some groups among ewe and goat samples, a hierarchical cluster analysis was applied to the sensor responses. Figure 6 presents dendrograms showing the results of the application of cluster analysis obtained using the Ward method and Euclidean distance. The resulting clusters perfectly separate 2 groups within each dendrogram. The first group comprises AFM₁-free samples (including ricotta prepared from contaminated whey). The second group in each dendrogram can be further divided into a cluster that includes all the milk, cheese, and whey samples containing the toxin, whereas another cluster is formed by almost all the scotta samples derived from contaminated whey.

Thus, the electronic nose can reliably distinguish between AFM₁-contaminated and AFM₁-free samples not only in milk, but also in dairy products and by-products (whey, ricotta, and scotta), regardless of the physical status of the proteins in the system, and whether they underwent treatments capable of affecting their native structure.

CONCLUSIONS

This study indicates that there is no simple physical method to remove AFM₁ from ovine and caprine milk. Neither ultrafiltration, nor acidic or enzymatic treatments were able to influence the toxin's interaction with CN or whey proteins. Only the combined action of heat and low pH (as used in ricotta production) was able to denature whey proteins to a point where they lost their AFM₁-binding capacity.

This study also indicates that the physical status of the various protein components of ewe and goat milks does not influence the capability of sensor arrays (electronic nose) to detect the presence of AFM₁. Although the sensor response in the electronic nose does not need and does not provide information on the nature of the compound under investigation (only its digital fingerprint), changes in the digital fingerprint provide reliable information regarding the presence of AFM₁ regardless of the physical status of the sample. In spite of previous observations about the presence of AFM₁ in the headspace of AFM₁-contaminated samples, the

nature of other possible species present in these samples to which the sensor array may respond needs clarification through the application of suitable analytical tools such as mass spectrometry (alone or in combination with other separation techniques).

The classical performance descriptors used in analytical chemistry (specificity, accuracy, sensitivity, and the possible occurrence of false positives and false negatives) do not apply to the electronic nose, because sensor arrays only sense electrical signals on a hot semiconductor surface, as noted above. Use of the electronic nose makes sense only in combination with suitable statistical tools, and this combination may represent a very practical and quick tool for screening purposes. This work confirms that cluster analysis of the electronic nose data may offer substantial help in creating clusters that allow recognition of samples at different contamination levels.

Cluster analysis may be used for selecting those samples in a given lot that require accurate quantitative analysis by chemical or immunochemical methods (Manetta et al., 2005; Micheli et al., 2005; Sørensen and Elbæk, 2005; Cavaliere et al., 2006). These methods are expensive and time consuming, but are capable of offering accurate information at the molecular level.

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