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Development and Application of Solvent-free Extraction for the Detection of Aflatoxin M_1 in Dairy Products by Enzyme Immunoassay

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The official methods for the quantification of aflatoxin M_1 in dairy products (cheese and yogurt) include extraction into dichloromethane or chloroform, evaporation of the solvent, partitioning of the reconstituted residue with hexane, and subsequent analysis. To secure a rapid and inexpensive screen for aflatoxin M_1 contamination, a sensitive competitive ELISA, using a rabbit polyclonal antibody, was developed for measuring aflatoxin M_1 in milk and used in a comparative study for measuring the extraction efficiency of aflatoxin M_1 in aqueous or organic solvent buffers using yogurt samples. An aqueous sodium citrate solution was found to be suitable for extracting aflatoxin M_1, thus eliminating the need for organic solvents. The citrate extraction proved to be efficient (recovery ranged from 70 to 124%) in fortified samples of very different kinds of dairy products, including yogurt and six types of cheese. Fourteen yogurt and cheese samples were extracted with citrate solution and analyzed by ELISA. A good correlation was observed ($y = 0.95x - 0.59$, $r^2 = 0.98$) when the data were compared with those obtained through the official method, across a wide range of aflatoxin M_1 contaminations (10–200 ng/kg).

KEYWORDS: Aflatoxin M_1; extraction; cheese; yogurt; ELISA

INTRODUCTION

Aflatoxins are highly toxic and carcinogenic metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus*, which may contaminate a variety of agricultural commodities and animal foodstuffs. Aflatoxin M_1 is a major metabolite of aflatoxin B_1, the most common, and may be found in milk or milk products obtained from livestock that have ingested contaminated feed (*1*). Currently available research results demonstrate that aflatoxin M_1 can also be present in a wide range of milk-derived or products containing milk, such as cheese, yogurt, cream, and chocolate (*2, 4*), due to the stability of aflatoxins during processes involved in the preparation of such commodities.

The hepatotoxicity and carcinogenic effects of aflatoxin M_1 have been demonstrated, so it has been classified as a class 2B human carcinogen (*5*). To reduce the risk associated with the human consumption of contaminated milk, many countries have regulated the levels of aflatoxin M_1 in milk and, in particular, the European Union has set maximum permissible levels of 50 and 25 ng/kg for baby food (*6, 7*). Despite the fact that aflatoxin M_1 concentration not only does not decrease during dairy processes but could also be 2–4 times higher in cheese than those initially present in milk (*4, 8*), no specific limits have been set by the European Union for dairy products or other products containing milk. Some countries have indicated recommended limits, which vary from 250 to 450 ng/kg (*4, 9*).

Several analytical methods are currently available for aflatoxin M_1 determination, including high-performance liquid chromatography associated with fluorescence or mass spectrometric detection (*10–12*). Enzyme-linked immunosorbent (ELISA) methods have also been described (*10, 13–15*) and are widely employed as screening methods in routine analysis, mainly because of their simplicity and rapidity. Recent papers describe methods in which milk may be analyzed directly or after simple and limited pretreatment (*14, 15*). However, the analysis of dairy products still involves the time-consuming extraction of aflatoxin M_1, which, in addition, requires organic solvents. Validated protocols involve an extraction step with dichloromethane or chloroform, followed by evaporation of the solvent. The residue is reconstituted with an aqueous diluent (a buffered solution of skimmed milk) and analyzed according to the same methods developed for aflatoxin M_1 detection in milk or further treated by adding hexane and centrifuging it to eliminate fat before analysis (*4, 8, 16*).

To effectively monitor the occurrence of aflatoxin M_1 in food at low contamination levels, sensitive, reliable, and simple analytical methods are required. Therefore, this work describes the application of a sensitive quantitative ELISA, initially

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developed to determine aflatoxin M_1 in milk, to the widest variety of derived products, including yogurt (fromage frais, natural yogurt, and yogurt with fruit and flavors added), cream, ice cream, butter, chocolate, and all cheese categories, except from processed cheese. Six Italian cheeses, representing the same number of cheese categories, Robiola (fresh), Mozzarella (fresh, elastic), Certosa (soft), Asiago (semihard), Gorgonzola (blue), and Parmesan (hard), were considered.

In particular, the objective of this work was the identification of a rapid pretreatment protocol, which allows us to process several samples at the same time, and, mainly, a suitable aqueous extraction buffer to avoid hazardous waste disposal and to permit the widest possible application of the ELISA, which could be run easily for screening purposes requiring the minimum amount of laboratory equipment and authorization. Because aflatoxin M_1 is present in milk and milk-derived products essentially bound to proteins (*9*), the hypothesis was made that buffered solutions aimed at redissolving or resuspending the protein fraction of dairy products could carry the bound aflatoxin M_1 in the aqueous phase. An immunoassay able to measure aflatoxin M_1 in milk, that is, to measure the analyte even if bound to milk proteins, should also be capable of detecting aflatoxin M_1 in an aqueous suspension of milk proteins derived from dairy products. Therefore, several extraction buffers were compared to determine whether it was necessary to use an organic solvent for application.

MATERIALS AND METHODS

Materials. Aflatoxin M_1 (Oekanal standard solution), skimmed milk powder, 3,3′,5,5′-tetramethylbenzidine liquid substrate (TMB), bovine serum albumin (BSA), and Celite 545 were purchased from Sigma-Aldrich (St. Louis, MO). The aflatoxin M_1−BSA conjugate, the anti-aflatoxin M_1 antibody (rabbit polyclonal antiserum, affinity purified), and the goat anti-rabbit immunoglobulin horseradish peroxidase (HRP)-labeled antibody were kindly supplied by Generon srl (Modena, Italy). Certified reference milk powders were obtained from AIA (Rome, Italy). All other chemicals and microtiter plates were obtained from VWR International (Milan, Italy).

Sample Preparation. Samples were obtained from local markets and supermarkets and analyzed before their expiration dates. Cheese, butter, and chocolate samples (ca. 100 g) were roughly cut and mixed separately. Then they were thoroughly minced and homogenized in a kitchen mixer. Yogurt containing fruit and ice cream (ca. 100 g) were also homogenized in a kitchen mixer, whereas natural yogurt and cream were directly analyzed. All samples used in comparative studies were analyzed without fortification. Artificially contaminated samples were prepared by fortifying a 10 g portion of sample with aflatoxin M_1 dissolved in acetonitrile. Solvent was then evaporated, and contaminated samples were again homogenized and kept at 4 °C overnight before analysis.

Comparison of Extraction Buffers. Five natural yogurt samples were pooled and fortified by adding aflatoxin M_1 at a final concentration of 50 ng/L. Subsamples of 5 g were weighed from the pool and extracted with 5 and 10 mL of different solvents: ethanol mixed with water or 0.2 M phosphate/citrate buffer at pH 5.5, containing 1% (p/v) of BSA (P/CBSA) (organic solvent = 10 and 20%); 2-propanol mixed with water and P/CBSA (organic solvent = 5 and 10%); and 2-propanol mixed with P/CBSA pH 4, 5, 6, and 7 (organic solvent = 5%). After 15 min of stirring at room temperature, samples were centrifuged at 3200*g*, and supernatants were directly measured by the ELISA or diluted 1:2 with the reconstituted skimmed milk powder and analyzed. Each subsample was extracted in duplicate and analyzed in quadruplicate.

Sample Extraction and Cleanup. Yogurt and cream samples (2 g) were weighed in 50 mL conic tubes, 2 mL of a sodium citrate solution (7% w/v) was added, and the combination was maintained at 50 °C for 15 min under continuous stirring. The slurry was then cooled in an ice bath for 1 min and centrifuged at 3200*g* for 15 min. Alternatively, the hot slurry could be directly centrifuged in a refrigerated centrifuge (25 °C). The fatty semisolid upper layer was discarded, and the liquid serum was withdrawn and directly analyzed by the ELISA.

Extractions of cheese, butter, ice cream, and chocolate samples were carried out via the same protocol, except that 2 g of sample was extracted with 8 mL of the citrate solution.

Preparation, extraction, and the cleanup of samples for validation purposes was performed according to a validated method, indicated by the Italian Ministry of Public Health as the reference method to carry out a screening analysis of aflatoxin M_1 (*16*). Samples (2 g) and dichloromethane (20 mL) were homogenized and stirred for 5 min. Thereafter, the slurry was centrifuged (10 min at 3200*g*) and filtered through Whatman grade no. 43 filter paper; the filtrate was evaporated at 40–50 °C by means of a rotary evaporator. The residue was diluted with 0.5 mL of the reconstituted skimmed milk powder used to prepare aflatoxin M_1 standards of the calibration curve. Subsequently, 1 mL of hexane was added, the mixture was vigorously shaken and centrifuged. The upper semisolid layer was discarded, whereas the bottom aqueous layer was diluted 1:5 with the reconstituted skimmed milk and analyzed by ELISA.

Indirect Competitive ELISA Procedure. Antiserum was diluted 1:20000 (v/v) in P/CBSA. A volume of 75 μL of diluted antiserum was added to 75 μL of aflatoxin M_1 at concentrations ranging from 0 to 100 ng/L and incubated in duplicate in noncoated wells for 50 min. Aflatoxin M_1 standard solutions were prepared daily by diluting the Oekanal standard solution in a reconstituted skimmed milk powder. One hundred microliters of the mixture was transferred into coated wells and incubated for 15 min. After five washes, with a phosphate buffer containing 0.25 M NaCl and 0.05% Tween 20, 100 μL of a diluted goat anti-rabbit immunglobulins conjugate to HRP was used to detect immobilized rabbit antibody. The incubation time was 15 min, followed by three washes. Color development was obtained by a 20 min incubation with TMB (100 μL per well). One hundred microlitres of sulfuric acid (1 M) was used as a stop solution, and absorbance was recorded at 450 nm.

Aflatoxin M_1 concentrations were determined by interpolation on a linear calibration curve. Linearization of the calibration curve was performed by the logit-log transformation, by plotting the logit of the ratio (in percent) between the absorbance at each concentration of analyte (B) and the absorbance in the absence of analyte (B_0) against the log of analyte concentration. The best data fit was obtained by linear regression of the standard points.

HPLC Analysis. To evaluate the accuracy and to validate the method, a comparative study using both the developed extraction method followed by the ELISA detection and a HPLC reference procedure (*16*) was performed. Preparation, extraction, cleanup, and HPLC analysis were performed as previously reported (*4, 17*). Briefly, a 10 g aliquot of samples was submitted for blending with 5 g of Celite and 100 mL of dichloromethane using a Sonopuls Homogenizator HD 2070 (Bandelin Electronic, Berlin, Germany). The mixture was centrifuged and filtered, the filtrate was evaporated at 40–50 °C, and the residue was reconstituted in water/methanol (30:1, v/v) and partitioned with hexane. The aqueous phase was collected and submitted to an Afla M_1 immunoaffinity column cleanup (Vicam, Boston, MA). Matrix interference was washed off the column with 10 mL of water. Aflatoxin M_1 was eluted from the column with 5 mL of acetonitrile, which was then collected and concentrated to a residual volume of 0.5 mL prior to injection into the liquid chromatographic system. Each extract was injected in triplicate.

A liquid chromatographic system (LaChrom Elite, VWR-Hitachi, Darmstadt, Germany) equipped with a 100 × 4.6 mm i.d. C-18 Chromolith Performance column (VWR, Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile and water at a volume ratio of 25:75, delivered to the column at a rate of 1 mL/min. Detection was made by a spectrofluorometer, the excitation and emission wavelengths being set at 360 and 435 nm, respectively. Measured aflatoxin M_1 concentrations were corrected for the mean recovery value (77%), according to the validation report of the Italian Health Office (*16*).

RESULTS AND DISCUSSION

Optimization of the Enzyme Immunoassay. As a preliminary measure, the optimal coating antigen/antibodies ratio was optimized by comparing dose–response curves obtained using different combinations of antigen (0.1, 0.2, 0.5, and 1 μg/mL) and antibody dilutions (1:5000, 1:10000, 1:20000, and 1:40000 v/v). The lowest limit of detection (LOD) and the highest sensitivity (slope of the curve) were obtained by using a 0.2 μg/mL aflatoxin M_1−BSA solution as coating antigen and a 1:20000 (v/v) dilution of the antibody. The incubation times were also optimized by comparing dose–response curves. The preincubation between sample and antibody (in noncoated wells) was varied from 15 min to 2 h, and the incubation of the mixture in the presence of the competitor (in coated wells) was varied from 10 min to 1 h. The lowest LOD and highest sensitivity were obtained when a long preincubation (50 min) and a short incubation, in the presence of the competitor (15 min), were carried out. Finally, the reaction of the second antibody, a commercial goat anti-rabbit antibody labeled with HRP, was also optimized. Aflatoxin M_1 concentration was measured by means of the developed ELISA directly on milk.

The developed indirect competitive ELISA has a dynamic range of 10–100 ng/L, with an IC_{50} of 20 ng/L. The LOD, calculated as the concentration corresponding to the B_0 minus 3 standard deviation of the blank, was 5 ng/kg. Precision was determined by analyzing replicates of an aflatoxin M_1 contaminated raw milk sample (low level). It was first tested by measuring aflatoxin M_1 in milk powder and comparing obtained results with HPLC measurements ($y = 1.06\ x - 0.13$; $r^2 = 0.988$, $n = 11$). Aflatoxin M_1 concentrations in certified reference materials (milk powder) were also measured and proved the method to be accurate and precise. The accuracy, determined as the mean of recovery experiments on four certified reference milk powder samples, was 104 ± 10%; the within-assay precision was measured to be 8% ($n = 6$), and the between-assay precision was measured to be 13% ($n = 10$).

Relative cross-reactivity for aflatoxin B_1 was 33%. No other compounds were tested.

Comparison of Extraction Buffers. Initially, different aqueous–organic solvents were considered as extracting systems, such as methanol, ethanol, dioxane, 2-propanol, and acetonitrile from 10 to 30% (v/v). Their effects on the calibration curve were assessed, and all systems decreased the sensitivity of the assay, except methanol and ethanol below 20% and 2-propanol below 10%. Methanol mixtures were not further investigated because of solvent toxicity, whereas aqueous ethanol (10 and 20%) and aqueous 2-propanol (5 and 10%) were tested as aflatoxin M_1 extracting solvents. Fortified samples of natural yogurt, with aflatoxin M_1 contamination level corresponding to the European MRL (50 ng/L) (*6*), were used as a model system to investigate extraction recoveries and the matrix effect. Mixtures of solvents and phosphate/citrate-buffered solution were also evaluated (pH from 4 to 7), as the addition of protein and the variation of the ratio between the volume of extracting buffer and weight of the sample. Obtained recoveries were, in all cases, between 10 and 50%.

Therefore, a very different approach was investigated. According to the observation that aflatoxin M_1 is bound to whey protein in milk and derived products (*9*), buffered solutions aimed at extracting or resuspending proteins were considered, according to the hypothesis that aflatoxin M_1 would be coextracted with proteins. The buffer used to dilute the antibody in the ELISA (P/CBSA) was initially tested as the one that would ensure the lowest variation between the standard curve and sample extracts. A satisfactory 81% of mean aflatoxin M_1 recovery was obtained. Very similar results were observed when a phosphate/citrate buffer at pH 7 was used (mean recovery percentage of 86%). The addition of 1% of BSA and doubling the volume of extracting buffer did not significantly modify the recovery percentage (83–87%). Contrarily, a notable improvement was obtained by using a simple sodium citrate solution (7% w/v) and warming the solution to 50 °C, which allowed us to reach quantitative recovery (103%). In fact, the use of sodium citrate solutions is commonly accepted to homogenize and melt cheese for microbiological analysis (*19, 20*). Moreover, it is known that citrate increases solubilization of colloidal calcium phosphate, which is accompanied by increased hydration of casein micelles and increased dissociation of casein from micelles (*21*). Therefore, the final product of the extraction is a suspension of milk proteins in water; thus, the analyte, which is bound to proteins, is "extracted" in the sense that it is carried by proteins in the aqueous medium. The extracts become similar to the suspension obtained by reconstituting a skimmed milk powder in water, thus allowing the same standard curve optimized for milk to be used without observing matrix interference.

Table 1. Corrective Factors for Calculating Aflatoxin M_1 in Dairy Products As Determined by Citrate Extraction and ELISA Detection and Relative Limits of Detection of the Method for the Different Dairy Products Investigated

sample	mean water content[a] (%)	dilution due to extraction	total corrective factor	LOD (ng/kg)
yogurt and ice cream	80	1:1	1.8	10
cream	50	1:1	1.5	10
fresh and creamy cheese	70	1:4	4.7	25
soft, blue, and elastic cheese	50	1:4	4.5	25
butter, semihard, and hard cheese	30	1:4	4.3	25

[a] From ref *22*. Values were approximated to form five main categories

The extraction method was further optimized to apply it to different dairy products by varying the temperature, ratio of solvent to sample, and time and speed for the centrifugation. Flavored yogurt and cheese samples needed minor modifications of the protocol to be accurately measured. In particular, very colored yogurt samples, such as blackberry or coffee yogurt, could be accurately measured after a 1:2 dilution of extracts with citrate solution. Cheese samples should be extracted with an increased volume of citrate solution. Fresh and cream cheese could be extracted with a 2:1 ratio between the volume of citrate and the quantity of samples. Contrarily, soft and hard cheeses needed a higher dilution ratio (4:1); thus, a 4:1 dilution ratio was applied in all cases to standardize protocols for cheese.

Analytical Performance. The LOD was calculated considering the LOD of the ELISA for milk and multiplying it by a corrective factor, which takes into account the dilution made during extraction. In particular, apart from the solvent added, which is different for different matrices, the contribution of sample water content should also be counted. Therefore, dairy products were divided into five main categories, according to their mean water content (*22*), and corresponding corrective factors were assigned (**Table 1**). These factors were also used to calculate aflatoxin M_1 content in real samples by multiplying the aflatoxin M_1 concentration given by ELISA measurement by the corresponding corrective factor.

The precision of the method was determined by extracting and analyzing replicates of aflatoxin M_1 artificially contaminated

Table 2. Recovery of Aflatoxin M_1 from Artificially Contaminated Samples of Yogurt and Cheese As Determined by Citrate Extraction and ELISA Detection

sample	mean protein content (*22*) (%)	aflatoxin M_1 used for fortifying samples (ng/kg)	estimated aflatoxin $M_1 \pm$ SD (ng/kg)	recovery (%)
natural yogurt	3–4	0	18 ± 1.1	
		25	37 ± 0.3	76
		50	62 ± 5	88
		100	114 ± 4	96
skimmed yogurt	3–4	0	21 ± 0.4	
		25	39 ± 3	72
		50	60 ± 1	78
		100	112 ± 2	91
fromage frais	3–4	0	17 ± 3	
		25	40 ± 0.2	92
		50	60 ± 0.8	86
		100	112 ± 2	95
yogurt flavored with apricots	3–4	0	18 ± 0.1	
		25	37 ± 0.5	76
		50	65 ± 3	94
		100	109 ± 3	91
yogurt flavored with cherry[a]	3–4	0	43 ± 1	
		25	72 ± 8	116
		50	92 ± 1	98
		100	167 ± 9	124
fresh cheese (Robiola)	8–10	0	16 ± 2	
		50	67 ±	102
		100	113 ±	97
elastic cheese (Mozzarella)	18–20	0	47 ± 14	
		50	95 ± 2	96
		100	134 ± 11	87
soft cheese (Certosa)	15–16	0	34 ± 9	
		50	80 ± 5	92
		100	137 ± 8	103
semihard cheese (Asiago)	24–28	0	97 ± 3	
		50	142 ± 13	90
		100	188 ± 3	91
blue cheese (Gorgonzola)	18–20	0	55 ± 1	
		50	102 ± 1	94
		100	144 ±	89
hard cheese (Parmesan)	32–35	0	73 ± 5	
		50	108 ± 14	70
		100	146 ± 5	73

[a] sample extracts were diluted 1:2 with sodium citrate before analysis

samples (natural yogurt, Mozzarella, and Parmesan), which were fortified with an aflatoxin M_1 concentration of 25 (low level), 50 (medium level), or 100 (high level) ng/kg. The assay was carried out in six replicates on the day for the evaluation of within-assay precision and on six different days for the evaluation of the between-assay precision. The values of RSD% were calculated at each nominal concentration level and ranged from 4 to 17% for medium and high levels and from 15 to 22% for the low level, which fulfilled U.S. FDA requirements for the validation of bioanalytical methods according to FDA guidance (*18*).

To evaluate the accuracy of the extraction method, 12 fortified samples, representative of 12 categories of dairy products, fortified with an aflatoxin M_1 concentration of 25, 50, or 100 ng/kg, were extracted and analyzed. Because no blank samples were available, recovery was calculated by measuring aflatoxin M_1 concentration in the nonfortified and fortified samples. The difference in terms of aflatoxin M_1 concentration between fortified and nonfortified samples was compared to the fortification level. Results are summarized in **Table 2**. Recovery values ranged from 70 to 124%, thus indicating a good accuracy of the assay when applied to real samples of very different compositions.

Yogurt and Cheese Analysis. A limited cross-validation of the whole assay (extraction and ELISA determination), with a HPLC reference assay, was carried out by measuring aflatoxin M_1 concentration in three yogurt samples (two natural and one skimmed yogurt) and in three cheese samples (one Mozzarella and two Parmesan), representing very different contamination levels. Results are summarized in **Table 3**.

A more extensive cross-validation of the extraction method was performed by comparison with a reference extraction protocol indicated for immunoassay analysis (*16*), followed by analysis with the same ELISA method.

Table 3. Comparison of Citrate Extraction Followed by ELISA Detection and HPLC on Aflatoxin M_1 Quantization

sample	HPLC (ng/kg)	estimated concn of aflatoxin $M_1 \pm$ SD (ng/kg)
natural yogurt I	<20	18 ± 2.5
natural yogurt II	<20	18 ± 0.2
skimmed yogurt	<20	23 ± 5
Mozzarella	31	32 ± 2.5
Parmesan I	160	192 ± 12
Parmesan II	80	89 ± 6

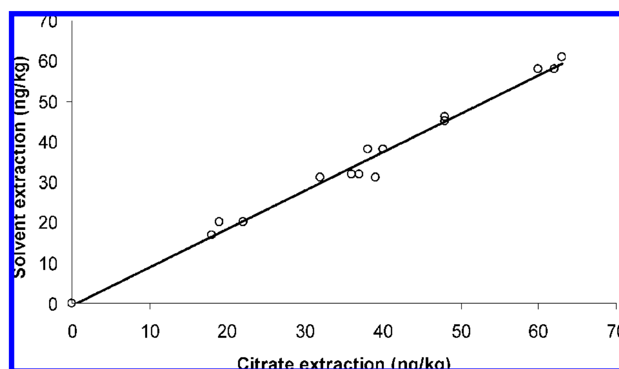

Figure 1. Correlation of results obtained by both citrate extraction and reference dichloromethane extraction for the aflatoxin M_1 detection by ELISA on yogurt and cheese samples. The linear regression analysis yielded a good correlation between methods ($y = 0.95x - 0.59$, $r^2 = 0.98$).

A total of 16 samples (4 yogurts and 12 cheeses, representative of all categories previously indicated) were extracted with the newly developed citrate method. For each sample, two extractions were performed on different days and the results were compared with those obtained using the reference extraction method.

Consistent results were obtained with the two methods: the linear regression analysis (**Figure 1**) yielded a good correlation between the methods ($y = 0.95x - 0.59$, $r^2 = 0.98$, $n = 16$). These results proved that the developed citrate method can be applied for the extraction of aflatoxin M_1 in dairy products of very different kinds at levels of regulatory relevance, with accuracy and precision comparable with those obtained with the reference method. In addition, the citrate extraction is relatively simple, rapid (processing of each sample requires 30 min, but several samples could be extracted at the same time, the number being limited only by the centrifugation step), and does not involve the use of organic solvents or other hazardous chemicals. Moreover, the citrate method is particularly easy to combine with immunochemical methods of analysis, where the presence of organic solvents may cause interference and generally worse performance. In addition, because the extracting solvent would be aqueous, the lipid fraction would remain undissolved, thus avoiding the need of further steps aimed at its elimination.

The citrate extraction was also applied to aflatoxin M_1 fortified samples of different kinds of dairy products (butter, industrial cream, handmade cream, vanilla ice cream, and white chocolate) giving, in all cases, an accurate estimation of the aflatoxin M_1 contamination. Therefore, the developed extraction method, associated with the immunoenzymatic assay, proved to be generally applicable for first level screening or routine quality control of all dairy products.

Aflatoxin M_1 has been detected in the large majority of analyzed samples (**Table 2**; **Figure 1**), thus confirming the potential risk associated with the presence of this toxic compound in food of wide human consumption. Yogurt and fresh cheese showed themselves to be of low contamination. Contrarily, levels well beyond the European MRL for milk were measured in semihard and hard cheeses. These findings are in accordance with previously reported observations (*4, 8, 23*) that aflatoxin M_1 concentration could be significantly higher in cheese than in milk. Moreover, a rough positive correlation between protein content and aflatoxin M_1 contamination could be established, and the same is true for ripening, which produces a decrease of water content and a concentration of proteins and aflatoxin M_1.

ABBREVIATIONS USED

BSA, bovine serum albumin; TMB, tetramethylbenzidine; HRP horseradish peroxidase; PBS, phosphate-buffered saline; P/CBSA, phosphate/citrate-buffered saline with 1% BSA added.

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