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Development and Validation of an Ultrasensitive Chemiluminescent Enzyme Immunoassay for Aflatoxin M_1 in Milk

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A fast and ultrasensitive chemiluminescent enzyme immunoassay for aflatoxin M_1 in milk samples has been developed and validated. The method is an indirect competitive type format involving the immobilization of an aflatoxin M_1−bovine serum albumin conjugate on 384 well black polystyrene microtiter plates and the use of a secondary antibody labeled with horseradish peroxidase detected with a luminol-based substrate. Aflatoxin M_1 standard solutions were prepared in milk-based buffer, and milk samples were analyzed without any cleanup procedure. The limit of quantification was 1 ppt, the coefficient of variation was below 9% for both intra- and interassay precision, and the recovery ranged from 96 to 122%. The method is specific, and other aflatoxins do not significantly cross-react with the antibody. Twenty-four milk samples were analyzed, and a good correlation was observed ($y = 0.98x + 1.71$, $r^2 = 0.98$, $n = 24$) when the data were compared with a reference high-performance liquid chromatography method with a fluorescent detector. The developed method is suitable for an accurate, sensitive, and high-throughput screening of aflatoxin M_1 in milk samples with a reduction of costs and increased detectability, as compared with previously developed immunoassays.

KEYWORDS: Enzyme immunoassay; chemiluminescence; aflatoxin M_1; milk

INTRODUCTION

Aflatoxins are highly toxic mycotoxins produced by three species of *Aspergillus* (*Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*), which may contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favorable to mold growth.

Aflatoxin M_1 is a hydroxylated metabolite of aflatoxin B_1, formed when ruminants ingest feed contaminated with aflatoxin B_1. Aflatoxin M_1 is then excreted in milk (*1*, *2*), and because it is relatively stable during milk pasteurization or other thermal treatments, it can also be present in milk-derived dairy products, such as cheese and yogurt (*3*, *4*).

Because of its hepatotoxic and carcinogenic activity (*5*), aflatoxin M_1 contamination represents a risk for human health, especially for children, who are the major milk consumers. Aflatoxin M_1 legal limits, ranging from 0 to 1 μg/kg, have been established in various countries worldwide, and in particular, the European Union limit for aflatoxin M_1 in milk is 50 ppt (ng/kg) (*6*). Aflatoxin M_1 is frequently present in commercial milk samples and dairy products, and various milk samples containing aflatoxin M_1 levels greater than the legal limit were recently detected, thus causing "alert" among the European Union (*7*), the World Health Organization, and the Food and Agriculture Organization (*8*).

Several methods for aflatoxin M_1 determination have been developed, and high-performance liquid chromatography (HPLC) with a fluorescent detector and enzyme-linked immunosorbent assays are mainly used in routine analysis (*9*, *10*). For an effective screening and monitoring of aflatoxin M_1 in foodstuffs at ppt levels, analytical methods combining simplicity with high detectability and analytical throughput are required. This can be achieved by means of immunological methods in conjunction with a highly sensitive detection of the label. Previously developed enzyme immunoassays for aflatoxin M_1 analysis using conventional colorimetric detection with chromogenic substrates (*11*, *12*) were allowed to reach detection limits not lower than 5−10 ppt in milk. In addition, they usually require a 60−120 min incubation time, as well as several analytical steps, which limits their extensive use for a rapid aflatoxin M_1 screening (*13*, *14*).

Enzyme labels detected by chemiluminescent (CL) substrates, such as the luminol/peroxide/enhancer system for horseradish

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peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase, represent the most sensitive detection system in immunoassay development (*15*, *16*). In addition, the CL signal detection can be performed immediately after substrate addition, thus shortening the overall analytical procedure when compared with conventional colorimetric assays. In the present work, a fast and ultrasensitive CL enzyme immunoassay for aflatoxin M_1 has been developed, based on the use of an anti-aflatoxin M_1 rabbit polyclonal antibody characterized by a very high affinity constant. An aflatoxin M_1−BSA conjugate was synthesized and immobilized on 384 well black polystyrene microtiter plates for developing an indirect competitive type format immunoassay, in which a secondary anti-rabbit IgG labeled with HRP and a luminol/peroxide/enhancer CL system were used.

MATERIALS AND METHODS

Materials. Aflatoxins M_1, M_2, B_1, B_2, G_1, and G_2, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), Freud's complete adjuvant (FCA), goat anti-rabbit IgG-peroxidase conjugate, bovine nonfat-dried milk, certified reference materials BCR 285 and 282 (aflatoxin M_1 in whole milk powder, 0.76 and 0.05 μg aflatoxin M_1/kg, respectively), bovine serum albumin (BSA), and Tween 20 were all obtained from Sigma Chemical Co. (St. Louis, MO).

Black 384 and 96 well or transparent 96 well polystyrene microtiter plates (Labsystems, Helsinki, Finland) were used for the CL or the colorimetric method, respectively. An enhanced (luminol/peroxide/enhancer) CL system (SuperSignal ELISA Femto) (Pierce, Rockford, IL) was used for the measurement of HRP activity. Buffers were as follows: Coating buffer (0.05 M carbonate/bicarbonate buffer solution pH 9.6); blocking solution [0.01 M phosphate-buffered saline (PBS), pH 7.2−7.4, containing 2% nonfat-dried milk]; incubation buffer (PBS, pH 7.2−7.4, containing 3% nonfat-dried milk); washing buffer (PBS with 0.05% Tween 20). HPLC grade acetonitrile was purchased from Carlo Erba (Milan, Italy), and deionized water was purified on a Milli-Q system (Waters, Milford, MA).

Instrumentation. Hapten/protein densities analysis was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS), using a Voyager De Pro (Applied Biosystems, Foster City, CA) instrument equipped with a pulsed-N_2 laser operating at 337 nm (*17*, *18*). Reagents were dispensed in microtiter plates using a Multidrop 384 dispenser (Labsystems). Microtiter plates were incubated under shaking at 37 °C using a Wellwarm shaker−incubator (Labsystems).

A chemiluminescence microtiter reader Luminoskan Ascent (Labsystems), which allows measurement of the CL signal from a whole 96 or 384 well microtiter plate within few minutes, was used. As an alternative, the CL signal was measured using a LB 981 imaging device (EG&G Berthold, Pforzheim, Germany), which employs a highly sensitive, back-illuminated, cooled CCD camera (*19*). A Multiskan EX spectrophotometric microtiter reader (Labsystems), provided with 492 nm filter, was used for absorbance measurements.

A Varian Star 9010 HPLC system (Varian, Palo Alto, CA) equipped with an isocratic μ-flow pump and coupled to a Varian Fluorichrom scanning fluorescence detector was used for method validation. Data quantification was performed using a Varian Vista 402 integrator.

Aflatoxin M_1−BSA Conjugate (Antigen). Aflatoxin M_1 has no reactive groups for direct coupling to a protein; therefore, it was first converted to aflatoxin M_1-*O*-carboxymethyl-oxime (aflatoxin M_1-oxime) according to the method described by Chu et al. (*20*). The antigen was prepared by conjugating aflatoxin M_1-oxime to BSA in the presence of EDAC, using the method previously described by Chu et al. (*21*) for the preparation of BSA-aflatoxin B_1-oxime conjugate.

The aflatoxin M_1 to BSA conjugation ratio was determined by MALDI-TOF, by comparing the molecular weight of the standard BSA with that of the aflatoxin M_1−BSA conjugate (*22*). The matrix (sinapinic acid) was prepared at a concentration of 10 mg/mL in 1:1 (v/v) water−acetonitrile (+0.1% trifluoroacetic acid). Protein samples were typically 10−50 pmol/μL in water−acetonitrile (2:1, v/v). Sample and matrix solutions were mixed in equal volumes, and then, 2 μL of the resulting solution was transferred to the target plate and left to dry at room temperature. Spectra were recorded at threshold laser irradiance for 50−150 shots in the linear mode at 25 kV, using an external linear calibration with BSA standard solution. Protein conjugate concentration was determined using a Bradford assay.

Antibody Production and Characterization. Polyclonal antibodies against aflatoxin M_1−BSA were produced in rabbit following a previously described protocol (*23*). Briefly, 100 μg of aflatoxin M_1−BSA conjugate was subcutaneously injected as an emulsion in saline solution and Freund's complete adjuvant for each immunization. Antiserum with adequate titer, affinity, and specificity were obtained 3−4 months after the first immunization. Any anti-BSA present was eliminated by precipitation by adding BSA (0.3%) to the rabbit sera. The IgG-rich fraction was isolated by salting-out precipitation with sodium sulfate, following a slight modification of the method described by Axen et al. (*24*). In brief, 180 mg of Na_2SO_4 was added to 1 mL of anti-BSA free rabbit serum, and the mixture was stirred at 25 °C for 45 min and centrifuged at 4000*g* for 15 min. The supernatant was carefully removed, and the pellet was resuspended in 1 mL of PBS containing 18% Na_2SO_4, and the mixture was vortexed for 40 s and centrifuged at 4000*g* for 15 min. These steps were repeated 2−3 times, and then, the pellet was resuspended in 1 mL of PBS and extensively dialyzed against PBS. The antibody was stored at −20 °C in PBS containing 100 μL/mL trasylol. Antibody specificity was defined by evaluating the extent of cross-reactivity (CR) with structurally related compounds, such as aflatoxins M_2, B_1, B_2, G_1, and G_2, using an indirect competitive binding assay.

Samples. *Control Milk Samples.* Control milk samples were prepared at three aflatoxin M_1 concentration levels: lower (1 ppt), middle (10 ppt), and upper (100 ppt) levels. In particular, 1 mL aliquots of pooled noncontaminated milk samples (i.e., with aflatoxin M_1 content lower than the detection limit of the developed immunoassay) were spiked with the appropriate amount of aflatoxin M_1. Briefly, the standard solution of aflatoxin M_1 (10 μg/mL in methanol) was diluted 1:10000 v/v with noncontaminated milk in order to achieve a 1000 ppt aflatoxin M_1 in milk stock solution. Aflatoxin M_1 fortified milk samples were then obtained by serial dilutions in noncontaminated milk.

Milk Samples. A total of 24 milk samples of different kinds (whole fat, half-skimmed, and light) provided by "Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise" (Teramo, Italy), with aflatoxin M_1 concentration previously evaluated by a validated reference HPLC method, were analyzed. Certified reference materials (aflatoxin M_1 0.76 and 0.05 μg/kg), provided as powdered milk, were suspended in distilled water (0.1 g/mL) and dissolved by stirring.

Milk samples were centrifuged at 3500*g* for 10 min at 4 °C, the upper fat layer was completely removed, and the aqueous layer (middle portion) was directly used for the analysis. For an automatic procedure, the sampling system was set up to collect the appropriate middle portion of the centrifuged sample.

Aflatoxin M_1−BSA Immobilization. The developed method is a solid phase indirect competitive immunoassay, involving immobilization of the aflatoxin M_1−BSA on 384 well polystyrene microtiter plates (96 well conventional microtiter plates were also used and the reagent volume was 5-fold higher). In particular, the wells of the microtiter plate were coated with 20 μL of 150 ng/mL aflatoxin M_1−BSA in coating buffer. The plates were incubated overnight at 4 °C. After the plates were washed three times with washing buffer, 60 μL of blocking solution was added to each well and the plates were further incubated at 4 °C for 2 h. The solution was discarded, and the plates were washed and vacuum-dried, and then, they were either used immediately or sealed with drying materials and stored at 4 °C until use.

Enzyme Immunoassay Procedure. To perform the assay, 20 μL/well of sample or aflatoxin M_1 standard solution (aflatoxin M_1 concentration in the range from 0.2 to 5000 ppt in incubation buffer) was added in duplicate to the antigen-coated plate followed by the addition of 20 μL/well of the anti-aflatoxin M_1 polyclonal antibody diluted 1:60000 (v/v) in washing buffer (primary antibody). The wells were incubated with gentle shaking for 30 min at 37 °C. During incubation, a competition took place between the free analyte and the solid phase-bound antigen for binding to antibodies. After they were

washed four times with washing buffer, the plate was incubated for 30 min in the dark at 25 °C with 20 μL/well of HRP-labeled anti-rabbit IgG (secondary antibody) diluted 1:4000 (v/v) in washing buffer.

For the colorimetric method, the HRP activity of the tracer bound to the solid phase was measured by adding 100 μL/well of a chromogen [1,2-phenylenediamine (0.5 mg/mL), H_2O_2 30% (0.25 μg/mL) in 0.1 M citrate buffer, pH 5.0]. After incubation in the dark at 25 °C for 20 min, the enzymatic reaction was stopped with 4 N H_2SO_4 (100 μL/well), and the absorbance at 492 nm was recorded.

For the CL method, the peroxidase activity was measured using a CL substrate (30 μL/well). The light emission, which becomes stable within 2−3 min, was measured by means of a microtiter plate luminometer reader using a 1 s integration time. Alternatively, a CCD-based luminograph was used, which allows simultaneously measuring the CL signal from a whole 384 or 96 well microtiter plate within 1 min.

Calculations. Aflatoxin M_1 concentrations were determined by interpolation on the calibration curve, where the bound enzyme activity, expressed as the ratio between signal at each concentration of analyte (B) and the bound activity in the absence of analyte (B_0), was plotted against the log of analyte concentration. For each experiment, a calibration curve was determined by a nonlinear regression analysis of the standards' data using the following equation:

$$Y = Y_O + (100 - Y_O)/(1 + 10^{[(b-x)c]})$$

where $Y = B/B_0$, Y_O = bottom asimptote (the ∞ dose), x = log of analyte concentration (ppt), b = log of analyte at midpoint of the curve (ppt), and c = slope.

Linearization of the calibration curve was performed by plotting the bound enzyme activity, expressed as the logit of the ratio (in percent) between B and B_0, against the log of analyte concentration. The best data fit was obtained by linear regression of the standard points. The milk aflatoxin M_1 concentration was expressed as pg/mL (ppt) of milk.

HPLC Analysis. To evaluate the accuracy and to validate the method, a comparative study using both the developed enzyme immunoassay and HPLC was performed. For HPLC analysis, 5 mL of the aqueous layer of the centrifuged milk samples was diluted 1:1 (v/v) with water and submitted to a cleanup procedure using 3 mL C-18 SPE columns (Baker J. T., Phillipsburg, NJ) preconditioned with 2.5 mL of acetonitrile and 5 mL of water. The solution was applied to the cartridge, and then, the column was washed with 10 mL of water, followed by 20 mL acetonitrile/water 10:90 (v/v) and 10 mL of *n*-hexane. Aflatoxin M_1 was eluted with 2 × 3 mL of dichloromethane/acetone 95:5 (v/v), and then, the solvent was evaporated under a nitrogen stream. The fluorescent hemi-acetal aflatoxin M_1 derivative (aflatoxin M_{2a}) was obtained by the addition of 250 μL of trifluoroacetic acid followed by 15 min of stirring in the dark (*25, 26*). The solution was subsequently dried by evaporation under gentle nitrogen stream, and the residue was dissolved with 200 μL of mobile phase and analyzed by HPLC.

Analysis of aflatoxin M_1 was performed on a 250 mm × 4.6 mm i.d., 5 μm, Supelcosil LC-18 column (Superchrom, Milan, Italy). The column was thermostated at 40 °C, and the sample injection volume was 20 μL. The mobile phase consisted of acetonitrile/water 20:80 (v/v), and the flow rate was 1.2 mL/min. Aflatoxin M_1 was eluted at a retention time of 5.6 min and detected by setting the fluorescence detector at 353 and 423 nm. All analyses were performed in duplicate. The limits of detection (LOD) and quantification (LOQ) of the HPLC method were 5 and 15 ppt, respectively.

RESULTS AND DISCUSSION

Aflatoxin M_1−BSA Characterization. The average molar ratio between aflatoxin M_1 and BSA in the aflatoxin M_1−BSA conjugate, as determined by the molecular weight obtained by MALDI-TOF mass spectrometry, was 6 mol of aflatoxin M_1 for 1 mol of BSA. This indicated that the aflatoxin M_1−BSA conjugate was characterized by a relative high hapten density, thus by high antigenicity. In addition, mass spectrometry analysis showed the presence of a little amount of unconjugated BSA, suggesting a high reaction efficiency. The unreacted aflatoxin M_1-oxime has been completely removed by extensive dialysis of the reaction mixture using a cellulose membrane with a cutoff of 14000 Da.

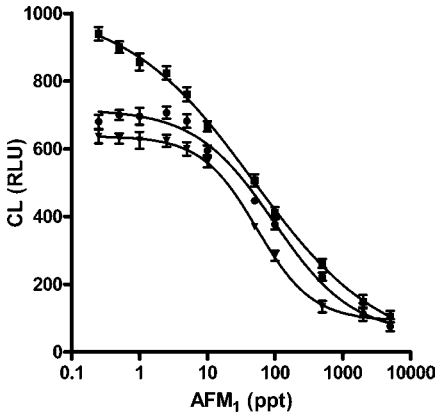

Figure 1. Calibration curves obtained using various dilutions of anti-aflatoxin M_1 polyclonal antibody (■, 1:60000; ●, 1:40000; and ▼, 1:30000 v/v). The aflatoxin M_1–BSA conjugate was immobilized at the optimal concentration (150 ng/mL), and the curves are expressed as relative light units (RLU) against the log of aflatoxin M_1 concentration (ppt).

The immobilization of an antigen monolayer on the solid phase is crucial to obtain a good analytical performance of the assay, in particular to minimize the nonspecific and uncontrolled binding. For this reason, the synthesis of the aflatoxin M_1−BSA was optimized by increasing the initial concentration of aflatoxin M_1 in the reaction mixture in order to achieve a high aflatoxin M_1 to BSA molar ratio, thus obtaining a molecule that would work as a powerful immunogen and allow proper antigen immobilization. In fact, at a relatively high conjugation ratio, few BSA and a high number of aflatoxin M_1 molecules are available at the well surface for antibody binding, with low steric hindrance. This facilitates the antibody binding to aflatoxin M_1, thus improving the kinetics of antibody−antigen reaction, being the phenomenon less affected by diffusion of the antibody to the antigen-coated well surface. The aflatoxin M_1−BSA derivative characterization by MALDI-TOF analysis was crucial to achieve the above-reported analytical performance and for a good assay standardization.

Optimization of the Enzyme Immunoassay. *Aflatoxin M_1−BSA and Anti-Aflatoxin M_1 Antibody Concentration.* Preliminary experiments were performed to assess the optimal coating antigen/antibodies ratio. Aflatoxin M_1−BSA and anti-aflatoxin M_1 antibody concentrations were optimized by comparing dose−response curves obtained using different combinations of antigen (50, 100, and 150 ng/mL) and antibodies dilutions (1:30000, 1:40000, and 1:60000 v/v), according to an optimized experimental design. A satisfying compromise between the lowest LOD, the highest sensitivity (slope of the curve), and the widest linear dynamic range was obtained by using a 150 ng/mL aflatoxin M_1−BSA solution in the immobilization step and a 1:60000 (v/v) dilution of the antibody (**Figure 1**).

Comparison between Colorimetric and CL Detection. The optimized CL enzyme immunoassay was compared with a conventional colorimetric method. By comparing the dose−response curves shown in **Figure 2**, it can be observed that the CL method provided a lower detection limit with respect to the colorimetric assay (0.25 and 5 ppt, respectively). In addition, the CL method provided a lower midrange of the calibration curve, i.e., the concentration giving 50% of the response (30 ±

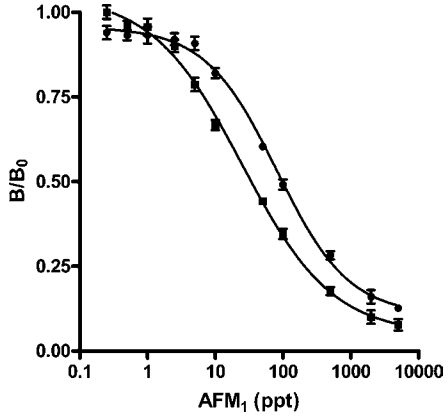

Figure 2. Representative calibration curves obtained in milk-based buffer with the immunoassay method for aflatoxin M_1 using the colorimetric (●) and CL (■) detection. The curves are expressed as fraction of bound enzyme activity (B/B_0) against the log of aflatoxin M_1 concentration (ppt). Each curve point and the SD associated with it are the result of an averaging of 10 standard curves obtained in six different days.

2 and 82 ± 5 ppt, respectively), as well as a higher dynamic range of linearity. The fact that the CL detection offered an improved analytical performance in terms of detectability and dynamic range is not surprising, due to the superior characteristics of the detection system, being the CL signal linear for up to 6 orders of magnitude (*27*). Moreover, because the detectability of HRP enzymatic activity is at least 100−1000 times higher, the CL system is particularly suitable for microsystems characterized by very small reading volumes (e.g., 30 μL, as used in the developed method). A further advantage obtained by using the CL detection is the rapidity of the assay, since the CL signal can be measured immediately after substrate addition, while the colorimetric assay requires a 20−30 min incubation step, as well as an enzyme activity blocking step, prior to signal detection. Indeed, thanks to the glow type emission kinetics of the enhanced CL substrate, the steady state light emission is reached 2−3 min after substrate addition and it is maintained for at least 15 min, thus allowing easy handling and standardization of the experimental conditions. The CL signal can be recorded either with a CCD imaging device or with a conventional photomultiplier tube-based microtiter plate reader. The imaging device allows simultaneously measuring the light signal in the 384 wells of the microtiter plate, and with the same imaging instrument, it is possible to perform simultaneous measurement in up to four 384 well microtiter plates; in addition, thanks to the use of correction optics (flat field correction lenses), no major effects on the light path and geometry have been observed. The light output can be measured using 1 min exposure time. On the other hand, the microtiter plate reader offers better performance in terms of detectability (10 times higher than the CCD imaging), but it requires longer measurement times. Usually a 1 s acquisition time for each well is necessary, which results, also considering the time required to slide the wells under the photomultiplier tube, in approximately a 6 min total measurement time for a 384 well microtiter plate.

Enzyme Immunoassay Format. CL detection allows the use of 384 well microtiter plates with an assay volume of 20 μL, which is at least five times lower than that used in the conventional 96 well microtiter plate format (*28*). A 5-fold reduction in antibody, labeled probe, and CL cocktail volume reduces the costs of the assay, while maintaining the same analytical performance.

Table 1. CR% of Some Aflatoxin M_1 Related Compounds with the Anti-Aflatoxin M_1 Antibody

aflatoxin	CR%[a]
aflatoxin M_1	100
aflatoxin M_2	4
aflatoxin B_1	2
aflatoxin B_2	1
aflatoxin G_1	0.5
aflatoxin G_2	0.5

[a] CR% = (ppt of aflatoxin M_1 that leads to 50% binding inhibition/ppt of aflatoxin that leads to 50% binding inhibition) × 100.

Because of the very small volume of samples and reagents used in the analysis and to the high density of wells of the microtiter plate format, the use of an automated system to dispense samples and reagents is essential to control the analytical steps and the reproducibility of the overall analytical procedure. In addition, more compact devices can be used. The combination of a small incubation volume with rapidity and sensitivity of the CL detection of the probe allowed the development of a sensitive and low cost immunoassay, with which more than one hundred samples may be analyzed in the same analytical session, thus representing an attractive method for high-throughput screening (HTS).

Analytical Performance. *Antibody Specificity.* The antibody specificity was defined by evaluating the extent of CR% with aflatoxin M_1 structurally related compounds, such as aflatoxins M_2, B_1, B_2, G_1, and G_2. In particular, for each compound, a calibration curve was produced using the same analytical conditions, and then, the CR% was calculated for each compound as follows:

$$\mathrm{CR\%} = \frac{a}{b}100$$

where b is the concentration of tested compound corresponding to $B/B_0 = 0.5$ and a is the concentration of aflatoxin M_1 corresponding to the same B/B_0 value.

The antibody used is highly specific for aflatoxin M_1. Indeed, all other tested aflatoxins do not significantly cross-react (**Table 1**), since they are able to cause a potential interference only if present at a concentration 10−100 times higher than aflatoxin M_1.

Matrix Effect. To evaluate milk matrix effect, aflatoxin M_1 calibration curves produced in either buffer (PBS), skimmed milk, or full-cream milk were compared. In addition, serial dilutions (2−16-fold with PBS) of a milk sample spiked with aflatoxin M_1 at a high concentration (100 ppt) were assayed by interpolation on a calibration curve produced in buffer and the observed concentration values were compared with the expected concentrations. Because a significant matrix effect was observed by comparing calibration curves and no linearity was observed among different dilutions of the fortified aflatoxin M_1 sample, a calibration curve in milk-based buffer is needed to avoid samples cleanup. Being calibration curves obtained by preparing aflatoxin M_1 standards in buffer containing commercial nonfat dried milk (incubation buffer) overlapping with those prepared in centrifuged milk, we used incubation buffer to prepare calibration curves. The use of this milk-based buffer allowed analyzing all kinds of milk samples without interference and with good reproducibility.

Calibration Curves. **Figure 2** shows a mean standard curve in incubation buffer, obtained by averaging 10 standard curves from six different days. The RSD% of individual points ranged

Table 2. Precision and Accuracy Calculated by Analyzing Replicate Samples Spiked with 1, 10, and 100 ppt of Aflatoxin M_1, as Well as Certified Reference Materials[a]

expected concn (ppt)	found concn (ppt)	RSD (%)	recovery (%)
	within assay ($n = 6$)		
1	1.10 ± 0.07	6.3	110 ± 7
10	9.72 ± 0.38	3.9	97.2 ± 3.8
100	99.4 ± 2.5	2.5	99.4 ± 2.5
	between assay ($n = 6$)		
1	1.22 ± 0.10	8.1	122 ± 10
10	9.63 ± 0.54	5.6	96.3 ± 5.4
100	98.5 ± 3.2	3.2	98.5 ± 3.2
	certified reference materials		
760	745 ± 17.2	2.3	98.0 ± 2.3
50	48 ± 1.2	2.5	96.0 ± 2.4

[a] The report data are the mean values ± SD.

from 4 to 10%. The best fitting of the curve was obtained by a nonlinear regression using a three parameters function. The relative standard deviation (RSD%) calculated for the Y_O, b, and c coefficient values was equal to 3.1, 6.5, and 4.1%, respectively, and the R^2 was 0.998 ± 0.002. The results obtained with 10 standard curves generated either in the same day or on different days demonstrated that the developed assay is reproducible and precise.

LOD. The LOD was calculated by interpolation on the aflatoxin M_1 standard curve, as the analyte concentration corresponding to the mean signal of B_0 (obtained by averaging the signal of six replicate sets) minus three times its standard deviation (SD) (according to IUPAC, ISO, and AOAC harmonized guidelines) (*29*), and it was 0.25 ppt.

LOQ. The LOQ, defined as the aflatoxin M_1 concentration producing a signal corresponding to aflatoxin M_1-free milk minus 10 times its SD (*29*), was determined by analyzing six replicate sets of aflatoxin M_1 fortified milk samples ranging from 1000 to 1 ppt. The LOQ of the validated method in milk samples was 1 ppt that is more than 20 times below the legal limit for aflatoxin M_1 in milk. In addition, the LOD and the LOQ of the developed enzyme immunoassay were lower than those of previously developed immunological methods and commercially available kits for aflatoxin M_1 analysis in milk samples (*11*, *14*, *30*).

Within Assay and between Assay Precision and Bias. The precision of the method was determined by analyzing replicates of aflatoxin M_1 fortified milk samples, which contained a nominal aflatoxin M_1concentration of 1 (low level), 10 (medium level), and 100 (high level) ppt. The assays were carried out in six replicates on the same day for the within assay precision evaluation and in six different days for the between assay precision evaluation. The values of the mean, SD, and RSD were calculated at each theoretical concentration level and are summarized in **Table 2**. The RSD (%) and recovery (%) values demonstrate that, even at the lower LOQ, the precision and accuracy of the method are suitable for routine screening of aflatoxin M_1 in a very low milk sample volume.

Stability of the Coated Plates. Coated plates were vacuum-dried and stored at 4 °C under vacuum. Stability of the antigen-coated plates was investigated by comparing six calibration curves obtained using plates stored at either 0, 1, 3, 6, or 8 months. Experimental data show that vacuum storage of the antigen-coated plates at 4 °C for up to 6 months did not significantly alter the performance of the assay in terms of LOQ and LOD.

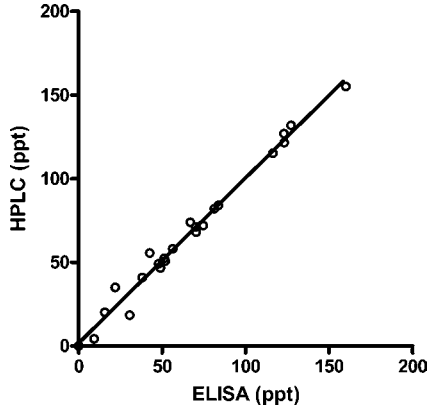

Figure 3. Correlation of results obtained by both CL enzyme immunoassay and reference HPLC method on milk samples**.** The linear regression analysis yielded a good correlation between methods ($y = 0.98x + 1.71$, $r^2 = 0.98$, $n = 24$).

Accuracy. To evaluate the accuracy of the assay, three fortified milk samples, containing a nominal aflatoxin M_1 concentration of 1, 10, and 100 ppt, as well as two certified reference materials, were analyzed. Results are summarized in **Table 2**. Recovery values ranged from 96 to 122%, thus indicating an excellent accuracy of the assay when applied to real samples.

Milk Samples Analysis. A total of 24 milk samples were analyzed with the newly developed immunoassay method. Three fortified milk samples prepared at three aflatoxin M_1 concentration levels (1, 10, and 100 ppt) were used in each microtiter plate as control. For each sample, four determinations were performed on different days and the results were compared with those obtained using the reference HPLC method.

Concordant results were obtained with the two methods: 22 samples were positive for aflatoxin M_1, and only two samples were scored as negative (the aflatoxin M_1 concentrations detected by HPLC were approximately 0.8 and 0.5 ppt). The linear regression analysis (**Figure 3**) yielded a good correlation between methods ($y = 0.98x + 1.71$, $r^2 = 0.98$, $n = 24$), even if a higher scattering of the data was observed for samples with aflatoxin M_1 concentration lower than 50 ppt.

These results prove that the developed enzyme immunoassay method can be applied for the analysis of aflatoxin M_1 in milk samples of different kinds at levels of regulatory relevance, with accuracy and precision comparable to those obtained with the reference method. The developed CL enzyme immunoassay is a robust and simple assay characterized by a high sensitivity and analytical throughput, thanks to the combination of the CL detection of enzymatic activity with the use of a 384 well microtiter format.

In addition, the procedure can be easily automated using conventional instrumentation, easily available in laboratories where a continuous screening for aflatoxin M_1 in milk is performed. In the future, the method will also be validated for the determination of aflatoxin M_1 milk-derived dairy products, such as cheese and yogurt. The continuous growth of the production of organic foods potentially increases the risk of aflatoxins production for uncontrolled mold growth when no chemicals and preservatives are used. Therefore, a sensitive, fast, first level control is highly recommended, and this can be achieved with the present HTS method based on a 384 well format. A HTS screening is useful for a routine quality control of milk from different farms before mixing the different milk bulks, especially when the absence of aflatoxin M_1 above the regulatory limit needs to be documented.

ABBREVIATION USED

BSA, bovine serum albumin; LOQ, limit of quantification; LOD, limit of detection; HPLC, high-performance liquid chromatography; CL, chemiluminescent; HRP, horseradish peroxidase; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FCA, Freud's complete adjuvant; PBS, phosphate-buffered saline; aflatoxin M_{2a}, hemi-acetal aflatoxin M_1 derivative; CR%, cross-reactivity; CV%, coefficient of variation; SD, standard deviation; RSD%, relative standard deviation; HTS, high-throughput screening.

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