Optimization of a lateral flow immunoassay for the ultrasensitive detection of aflatoxin M1 in milk

UNIVERSITÀ DEGLI STUDI DI TORINO

Optimization of a lateral flow immunoassay for the ultrasensitive detection of aflatoxin M_1 in milk

Anfossi Laura*, Baggiani Claudio, Giovannoli Cristina, Biagioli Flavia, D'Arco Gilda, Giraudi Gianfranco

Department of Chemistry, University of Turin.

Via Giuria, 5 – I-10125 Turin, Italy

*to whom correspondence should be addressed. Tel: +390116705219, fax: +390116705242. E-mail: laura.anfossi@unito.it

Abstract

A high sensitive immunoassay-based lateral flow device for semi-quantitatively determine aflatoxin M_1 (AFM_1) in milk was developed. Investigation and optimisation of the competitor design and of the gold-labelling strategy allowed the attainment of the ultra-sensitive assessment of AFM_1 contamination at nanograms per litre level (LOD 20 ng l^{-1}, IC_{50} 99 ng l^{-1}), as requested by European regulations. A one order of magnitude detectability enhancement in comparison to previously reported gold colloid immunochromatographic assays for this toxin was obtained.

Direct detection of the target toxin in milk could be obtained by acquiring images of the strips and correlating intensities of the coloured lines with analyte concentrations. The one-step assay can be completed in 17 minutes, including a very simple and rapid sample preparation, which allowed the application of the assay to milk samples which differ in fat and protein contents. Although imprecise (mean RSD about 30%), the method proved to be accurate and sensitive enough to allow the correct attribution of sample as compliant or non-compliant according to EU legislation in force. Agreeing results to those of a reference ELISA were obtained on 40 milk samples by matrix-matched calibration in pasteurized milk.

Keywords Immunochromatographic assay; gold colloid; mycotoxins; food analysis

Introduction

Aflatoxins are highly toxic and carcinogenic metabolites produced by some fungi. The most toxic and diffuse is the aflatoxin B_1 (AFB_1), which has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) [1]. AFB_1 contamination can affect a variety of crops, including cereals used as feed for dairy cattle. Once ingested, it is rapidly absorbed and transformed into a hydroxylated metabolite named aflatoxin M_1 (AFM_1), which is secreted into the milk [2]. The hepatotoxicity and carcinogenic effects of aflatoxin M_1 have also been demonstrated, therefore it has been included in group I human carcinogens, as well [1]. Most countries have regulated the levels of aflatoxin M_1 in milk, which varies from the 50 ng l^{-1} established by the EU to the 500 ng l^{-1} established by US FDA [3-5]. In addition, the European Union have set up maximum permissible levels of 25 ng l^{-1} for baby food [3].

Several analytical methods are currently available for aflatoxin M_1 determination, including high-performance liquid chromatography associated to fluorescence or mass spectrometric detection [6-10]. Enzyme-linked immunosorbent (ELISA)-methods have also been described [11-15] and are widely employed as screening methods in routine analysis, mainly because of their simplicity and rapidity. Among rapid methods for the first level screening of food contaminants lateral flow immunoassay (LFIA) technology has recently attracted a growing interest particularly for mycotoxin determination [16-18], mainly because of allowing very rapid, simple, *in situ* analyses to be carried out. Nevertheless, the development of LFIAs for AFM_1 is challenging because of the extreme sensitivity required by legislation for this contaminant (particularly in the European Union). The first paper dealing with the subject reported a validation study on a commercial device which was conceived for meet US regulations. The ROSA Charm Aflatoxin M_1™ was evaluated in an inter-laboratory trial at four levels above and two below the declared LOD of the assay (400 ng l^{-1}) [19]. Less than 5% of false negative (n=83) and no false positive below 300 ng l^{-1} were found. Nevertheless, for contaminations from 350 and 450 ng l^{-1}, false positivity increased from 21 to 93%. More

recently, Wang et al first described the development of a rapid LFIA for the detection of AFM_1 [20]. The whole analytical procedure could be completed in 10 minutes, as no sample treatment was required and the visual detection limit (VLD, 0.5-1 μg l^{-1}) was just above the eligible value required by the US regulation. A visual device has also been developed by Zhang et al which showed a VDL for AFM_1 of 0.3 μg l^{-1} [21]. Although the slight sensitivity improvement, the VDL remains far away from the detectability demand imposed by EU regulations for this contaminant.

With the aim of producing a system sensitive enough to reach the levels required by European regulations, we developed a competitive lateral flow immunoassay which exploited rabbit polyclonal antibodies towards AFM_1 that had been previously employed in the development of a sensitive ELISA [12]. The method was designed to be a competitive LFIA, in which the Test line comprised an AFM_1 conjugate and the Control line was composed of anti-rabbit IgG antibodies. Anti-AFM_1 antibodies labelled with gold nanoparticles (GNP-Ab) were furnished as pre-adsorbed in a release pad to be re-suspended by sample flow Colour intensities of the Test and Control lines, which appear as the result of strip development, were determined by means of a portable reader, which also interpolates values on a memorized standard curve and returns the concentration of the analyte in the sample.

Sensitivity of competitive immunoassay is influenced by several well-known factors, such as antibody dilution, competitor concentration, and, above all, by antibody affinity and avidity. Nevertheless, some other factors have been demonstrated to play a role in determining the sensitivity of LFIA for haptens, such as: the use of heterologous competitors, which had been shown to improve sensitivity in the development of an immunochromatographic assay to measure parathion [22]; the structural modification of the antigen used as the competitor in the assay [23-24]; the specific response of the reporter used to label the antibody; and the extent of antibody labelling (moles of reporter per mole of antibody [25]). In particular, Byzova and co-workers [23] demonstrated that the diminishing of the molar substitution ratio (SR) between the hapten and the carrier protein in the preparation of the competitor strongly improved sensitivity in a lateral flow for detecting chloramphenicol in milk. In addition, Laycock et al [25] showed great dependence of detectability on the extent of antibody labelling in the development of their gold colloidal-based

immunochromatographic assay for measuring saxitoxins. Therefore, with the aim of setting up a ultrasensitive assay for determining AFM_1 in milk at levels of regulatory concern according to EU regulation, the influence of the competitor features and of the signal reporter on sensitivity were studied and optimized. Investigated factors included, for the competitor: (i) use of homologous or heterologous hapten, (ii) substitution of the carrier protein, (iii) modulation of the hapten-to-protein molar ratio; and, for the signal reporter: (iv) the extent of antibody labelling.

Materials and methods

Preparation of colloidal gold and colloidal gold-labelled polyclonal antibodies

Gold colloids were prepared using the sodium citrate method as previously described [26].

The saturation concentration of the anti-AFM_1 antibody for conjugation with gold nanoparticles was determined according to Horisberg [27], as follows: increasing amounts of the anti-AFM_1 antibody were added to GNPs and incubated for 30 min at room temperature. Then sodium chloride was added and the amount of antibody needed to stabilize GNPs (saturation amount, SAT) was visually established as the minimum quantity that not determine colour shift from red to purple-blue of the gold colloidal solution.

GNP-antibody conjugation was carried out in borate buffer (BB, 20 mM, pH 8.0) by using an amount of anti-AFM_1 antibodies which was the half the saturation amount . After 30 min incubation at room temperature, GNPs-Ab were overcoated with BSA (10 min at 37°C)and , washed twice withBB that contains 0.1% BSA. Finally, the pellet was re-suspended in BB supplemented with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide and stored at 4°C until use.

Preparation of the test strips

: The AFM_1-protein conjugate (SR 4) at 0.3 mg/ml, and the goat anti-rabbit IgG antibodies (2 mg/ml) both diluted in PBS were applied onto nitrocellulose membranes to trace the Test and Control lines, respectively. Gold-labelled antibodies (OD 3) were distributed at a flow rate of 5 µl/cm near the lower edge of release pads, which had been previously treated with BB supplemented with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide. .

Strips of 5 mm were composed as follows: from the top; the adsorbent pad, the nitrocellulose membrane, the conjugate pad and the sample pad,and were inserted into rigid plastic cassettes. Cassettes were stored in plastic bags containing silica at room temperature until use.

Lateral Flow Immuno Assay procedure

The test was carried out by placing 100 μl of sample into the sample well. After 15 minutes of incubation at 37°C, colour density was measured by a mobile scanner connected to a laptop. The Skannex 3.0 software (Skannex AS, Hoenefoss, Norway) was used to acquire and process images.

The calibration curve was obtained by plotting the ratio between the intensity of the test (T) and the control line (C) [26] against the log of AFM_1 concentration and was determined by a nonlinear regression analysis of the data using the four-parameter logistic equation [28]. For the construction of the standard curve and for recovery experiments blank samples that did not show any detectable residues of the target when analysed by the reference ELISA (LOD 5 ng l^{-1}) [12] were fortified with appropriate amounts of the standard AFM_1 solution. The limit of detection was calculated as the average of 8 blank milk samples (according to ELISA measurements) minus 3 standard deviations from the average.

Samples and sample preparation

Pasteurized milk samples were purchased in large stores, and raw milk samples were obtained from farms. Whole and semi-skimmed milk (1 ml) were spun down for 2 min by a mini centrifuge (Spectrafuge Mini, Labnet, NY, USA). The upper fat layer was discharged, 500 μl of the underlying serum was transferred into a tube and 25 μl of a Tween 20 solution in water (10% v/v) was added. The mixture was immediately used in the lateral-flow assay. Skimmed milk could be analysed without centrifugation.

Results and discussion

Effect of varying the hapten, the AFM_1-protein molar ratio and the carrier protein in the Test line

The polyclonal antiserum used in this work had shown cross-reactivity towards AFB_1 (about 35% when measured by means of the ELISA); therefore a competitor synthesized by using a hapten derived from the parent toxin was considered as a "heterologous" competitor respect to AFM_1 protein conjugates (which were homologous to the immunogen). Therefore, three conjugates of AFM_1 with Bovine Serum Albumin (AFM_1-BSA), which varied in the hapten-to-protein rate (SR), one conjugate of AFM_1 with ovalbumin (AFM_1 –OVA) and one conjugate of AFB_1 with BSA (AFB_1-BSA) were evaluated as potential competitors to be immobilized in the Test line (Table 1). Each conjugate was dispensed on the membrane at the same rate and volume, however the concentration was varied to obtain a similar absolute T-line colour . AFM_1 standard solutions (0-10-100-1000 ng l^{-1}) prepared in a blank pasteurized whole milk were measured in triplicate and IC_{50} values were compared (Table 1). The AFB_1 conjugate qualitatively behaved as the AFM_1 conjugate with a similar SR, except for the absolute signal, which was less intense at the same concentration of dispensing. Interestingly, the decrease of the amount of AFM_1 per mole of protein strongly influenced the sensitivity of the assay. Reducing SR from about 22 to about 4 allowed an improvement of nearly 10-folds in the IC_{50} to be obtained. This result is in good agreement with the observation of Byzova and co-workers [23] and with expectations based on the experience with competitive immunoassays in other formats (such as for example in ELISA). Despite the need of increasing the competitor amount to maintain readable signals, the advantage due to reduction of the hapten density predominated. On the contrary, the substitution of BSA (which was the carrier protein also employed to raise antibodies) with ovalbumin seemed irrelevant. Presumably, the antibody fraction directed towards the carrier protein, if present, would be saturated in the preparation of the gold labelled- antibody, during GNP-Abs over-coating with BSA to prevent aggregation.

Table 1 Effect of varying the competitor to be used in the Test line of the LFD. Protein conjugates were dispensed onto the membrane at different concentrations to reach an absolute signal comprises between 20 and 25 arbitrary units on the T-line.

Conjugate	SR[a]	Dispensing concentration (mg ml^{-1})	IC_{50} (µg l^{-1})
AFM_1-BSA	4	0.8	0.2
AFM_1-BSA	15	0.4	1.1
AFM_1-BSA	22	0.2	1.7
AFM_1-OVA	10	0.8	0.6
AFB_1-BSA	24	0.4	1.6

[a] molar ratio between AFM1 and protein as estimated by spectrophotometric measurements

Labelling of antibodies with gold nanoparticles

Optimization of LFIA usually involves checkerboard titrations where the amounts of antibodies and of the competitor are varied to achieve the lower limit of detection and the maximum slope of the calibration curve. The modulation of antibody amount is almost exclusively obtained by diluting the solution of GNPs coated with antibodies themselves. The parameter used to measure this dilution is the optical density (OD) of the gold colloid, assuming that GNPs surface had been saturated with antibodies. Nevertheless, contrarily to this generally accepted assumption, Laycock et al reported a huge increase in sensitivity due to the reduction of antibodies coated onto GNPs in comparison to the saturation amount [25]. Moreover, the comparison of calibration curves carried out by the same antibody-saturated GNPs dispensed at two ODs (3 and 6) apparently did not influence the sensitivity of the LFIA directly (data not shown).

Therefore, we evaluated the effect of diminishing of the number of antibody molecules bound per gold nanoparticle at a fixed OD value. The colloidal gold obtained as described in the Experimental section showed absorption maximum at 525 nm, from which the mean diameter of 30.5 nm could be calculated according to Khlebtsov [29]. Under the approximations of having homogeneous spherical nanoparticles and

a complete conversion of tetrachloroauric acid into metallic gold, the concentration and the superficial area of GNPs could be estimated as 1.75 x 10^{+11} number of GNP/ml and 2902 nm^2, respectively. Roughly estimating that each antibody molecule occupies 45 nm^2 [30-31], about 64.5 antibody molecule could accommodate on each nanoparticle and thus the expected amount of antibody needed to saturate the colloidal gold preparation would be approximately 2.8 μg of IgG per millilitre of GNPs. The experimental value, visually established as the amount of antibodies which prevented GNP flocculation when 0.5M sodium chloride was added [27], was determined to be 3.5 μg/ml, in an acceptable agreement with the approximate calculation. Thereafter, variable amounts of antibodies were reacted with portions of the GNP colloid as summarised in Table 2.

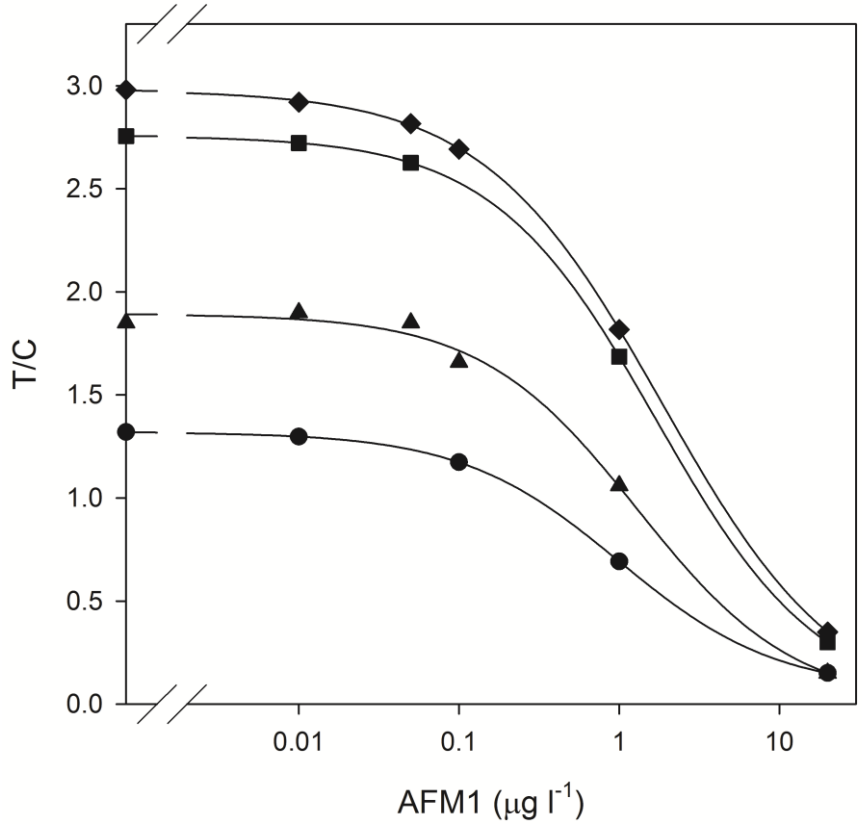
Table 2 Antibody amount used to coat GNPs at variable levels of saturation. The saturation amount was experimentally determined by the method of Horisberg [31] (SAT_{Exp}) and approximately calculated from the concentration of gold nanoparticles, their superficial area and the hypothetical area occupied by an IgG molecule (SAT_{Calc})

Antibody amount (Ab), μg/ml	Ab / SAT_{Exp}	Ab / SAT_{Calc}
1.5	0.4	0.5
2.5	0.7	0.9
3.5	1.0	1.25
5.0	1.4	1.8

The four GNP-antibody preparations were dispensed onto release pads at OD 3 and applied to strips where the AFM1-BSA with SR of 22 had been traced upon to form the T-line. AFM_1 calibrators prepared in milk were run onto these strips in triplicate. Resulting curves are show in in Figure 1. Besides a marked signal reduction, a slight improvement in sensitivity was also observed when the amount of antibody was lowered from saturating conditions (IC_{50} = 0.17 ± 0.01 μg l^{-1}) to its half (IC_{50} = 0.09 ± 0.01 μg l^{-1}); however

detectability was influenced in a lesser extent in comparison to what observed for the modulation of the competitor, as discussed above.

Figure 1 Inhibition curves obtained from signal reporters with variable amount of antibodies coated onto the GNPs. Antibody amount compared to the experimentally determined saturation: 0.4 (●), 0.7 (▲), 1 (■), 1.5 (◆). Labelled antibodies: OD 3 ; T-line: AFM_1-BSA conjugate (0.2 mg/ml, SR=22).



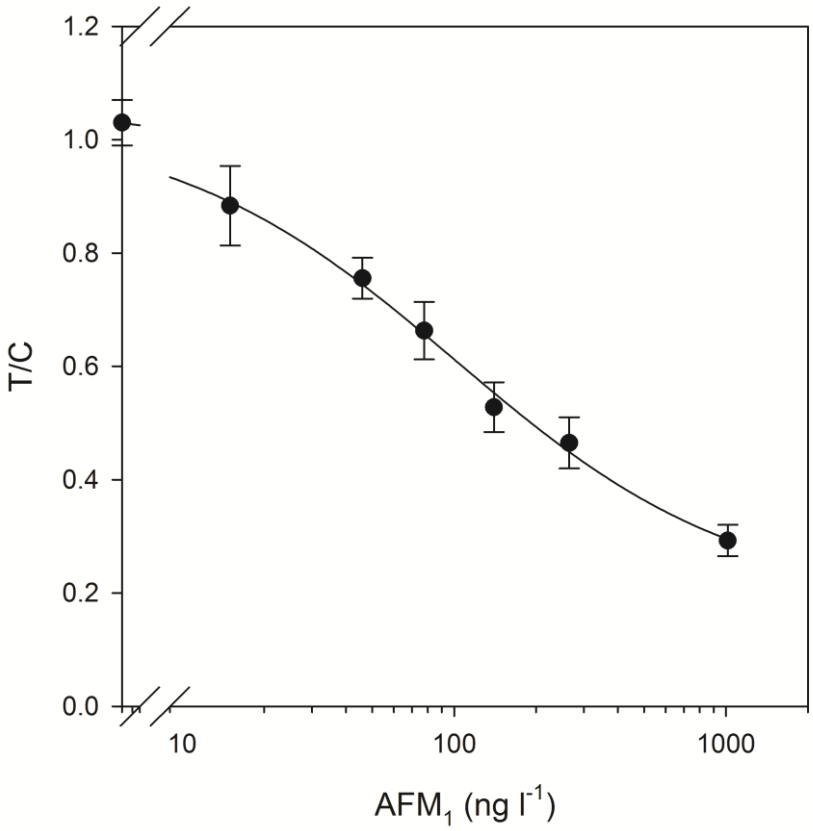
AFM_1 detection in milk by the ultrasensitive LFIA

Protein and fat of milk strongly influenced the test; in particular, fats altered sample flow through augmenting sample viscosity and native casein determined a strong signal depression of both the Test and Control lines. With the aim of developing a unique system that could be used on milk samples with variable fat content (whole, semi-skimmed, skimmed milk) and which were undergone to different thermal treatments, that is with different levels of protein denaturation (raw, pasteurized, UHT milk), samples were

standardized by: (i) a rapid centrifugation step that allowed the removal of the fat layer, and (ii) by adding a small amount of Tween 20 to overthrow protein interference.

After 15 min development at 37°C, strips were scanned. Dedicated software acquired and processed images and the signal, intended as the T/C ratio, was plotted against the logarithm of AFM_1 concentration to carry out calibration. As previously observed in the development of LFIA for other mycotoxin [32-35], matrix-matched calibration should be carried out to fit experimental results obtained on milk. Therefore, pasteurized whole milk samples in which AFM_1 was found out undetectable when analysed by the reference ELISA kit were used to prepare diluted calibrators. A typical calibration curve, obtained by combining the AFM_1-BSA with SR =4 as the competitor and GNPs covered with half the saturation of antibodies as the signal reporter, is depicted in Figure 2. In these optimal conditions, a limit of detection (calculated as the average of the blank minus 3 standard deviations from the average) and IC_{50} of 20 ng l^{-1} and 99 ± 19 ng l^{-1} were estimated, respectively.

Figure 2 A typical inhibition curve, obtained under optimized conditions, for the developed lateral flow immunoassay



Accuracy of the developed LFIA was evaluated on different kind of milk samples (Table 3) purchased on the market. All samples were found undetectable according to the developed LFIA. Therefore, accuracy was evaluated on milk fortified at two levels (50 and 100 ng l^{-1}). Acceptable results were obtained, although a slight overestimation or underestimation were observed for the raw and the UHT samples, respectively, which can be attributed to the fact that calibration was carried out in pasteurized milk.

Table 3 Recovery of Aflatoxin M_1 determination from artificially contaminated milk samples undergone to various thermal treatments and with different fat content as determined by the developed LFIA. Recovery was calculated as follows: (estimated AFM_1 for the fortified sample – estimated AFM_1 for the non fortified sample) / fortification level *100. Semi-quantitative attribution was done according to following criteria: negative sample (-), samples with AFM_1 content lower than 30 ng l^{-1}; positive sample (+) , samples with

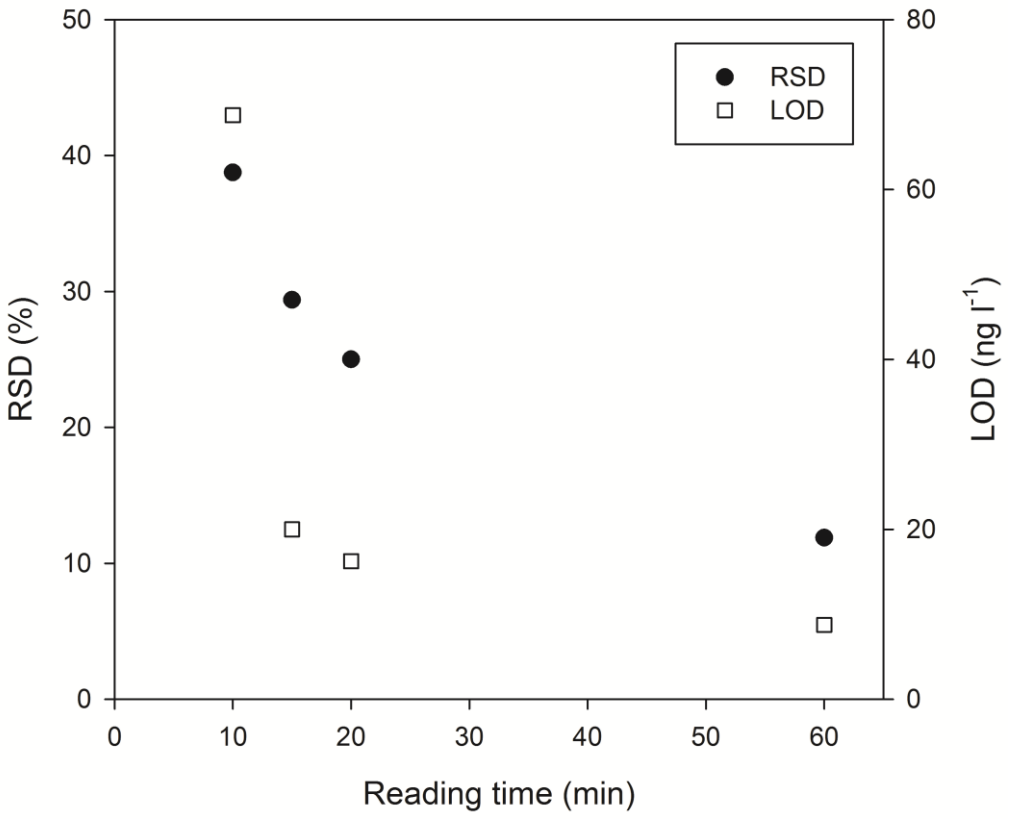
AFM_1 content higher than 70 ng l^{-1}; uncertain sample (±), AFM_1 content comprised between 30 and 70 ng l^{-1}.

Milk sample	AFM_1 measured by ELISA (ng l^{-1})	Fortification level (ng l^{-1})	Estimated AFM_1 ± SD (ng l^{-1})	Recovery (%)
raw	17.8	0	<LOD	
		50	78 ± 6	120
		100	153 ± 14	135
whole 1	< LOD	0	<LOD	
		50	40 ± 2	80
		100	122 ± 10	122
whole 2	16.0	0	<LOD	
		50	79 ± 9	126
		100	126 ± 11	110
skimmed	15.7	0	35 ± 1.2	
		50	74± 4	117
		100	113 ± 20	97
UHT	<LOD	0	<LOD	
		50	47 ± 5	94

	100	88 ± 11	88

The intra- and inter-day precision was evaluated at 3 levels of fortification (0-50-100 ng l^{-1}). RSD values were generally high (above 30%) and depended on reading time (Figure 3). 15 min of strip development before reading was chosen as the best compromise between precision and assay rapidity.

Figure 3 Precision of AFM1 quantification and LOD (calculated from the mean of the blank minus three standard deviation of the mean) as a function of the time elapsed before line intensity reading



Intra-laboratory evaluation of the semi-quantitative LFIA

The purpose of screening methods for AFM_1 monitoring in milk is to be very rapid and to allow the semi-quantitation of AFM_1 in such a way to permit the discrimination between samples surely complying with legislation in force and samples which do not comply. , The discrimination requires that assay cut-off value could be established at the EU maximum permissible value. The value established for milk (50 ng l^{-1}) would be expected to be attainable by the developed LFIA given its high sensitivity,while the lower limit set for baby food, was too near to the detection limit of the method to allow reliable discrimination of samples. In addition, samples in which the toxin content was close to the legal limit would be unreliably attributed as "compliant" or "non-compliant", because of measure imprecision and the limited slope of the calibration curve. Therefore, rather than defining of a single-point cut-off value,the identification of an indicator range of analyte concentrations (above and below 50 ng l^{-1}) within which uncertain or "non-attributable" results fall, was preferred [18, 36-37]. This indicator range had also been defined "unreliability region", and upper and lower limits of the unreliability region have been used to discriminate positive and negative results, respectively [38]. Limits for the unreliability region were established according to European legislation for screening methods for detecting AFM_1, which defines a relative standard deviation (RSD %) of 47% of the maximum permissible value as appropriate and 94% as acceptable based on the application of Horwitz equation [39]. According to the most restrictive criterion, it achieves that a method is appropriate if able to discriminate between AFM_1 content below 26.5 ng l^{-1} (compliant sample) and AFM_1 content above 73.5 ng l^{-1} (non-compliant sample). Samples that have AFM_1 content close to the threshold limit should be defined as uncertain (non-attributable)and should be submitted to further controls before entering the transformation chain. In the case of milk, rejection is often the fate of such uncertain samples (as for noncompliant samples), because the perishable nature of milk discourages time-consuming investigations. Therefore, the minimum number of uncertain results would be expected for a worthwhile method, as the "non-attributable" judgement would determine a considerable economic damage

The ability of the developed LFIA to correctly attribute to each of the groups milk samples found on the market was thus assessed; in particular, negative (compliant) samples were defined as those in which AFM_1

content was below 30 ng l^{-1}, positive (non-compliant) samples those in which AFM_1 content was above 70 ng l^{-1} and uncertain (non-attributable) those having an AFM_1 content between 30 and 70 ng l^{-1}. The instrumental quantification of coloured lines and their correlation with a calibration curve, in this context, was regarded as a way to limit subjectivity in the interpretation of results and to improve detectability [18] rather than going into the direction of true quantitative measurements. All obtained samples resulted to be contaminated below 30 ng l^{-1}, as established by the reference ELISA; therefore positive samples were generated through fortification at 50 and 100 ng l^{-1}. Results of the evaluation on a total of forty samples (which included 16 negatives, 16 positives and 8 uncertain) together with the definition of sensitivity and selectivity are reported in Table 4. No false negatives, summing truly positive and truly uncertain samples (0/24), occurred. However, three non-attributable samples were assigned as non-compliant and three truly positive samples were incorrectly attributed as uncertain (3/24). Additional five samples were not attributable and, although their classification was correct (truly uncertain samples) this meant that the width of the unreliability region chosen (30-70 ng l^{-1}) determined 20% samples that would have needed further investigation before making a decision on them. To indicate the capability of the method to produce the highest score of useful responses Ellison and Fearn introduced a parameter, which they called efficiency [40] and defined as the proportion of right responses on the total. The same concept, aimed at evaluating the fit for purpose of qualitative analytical methods, has also been proposed by several other authors, although under different names [41-42]. Efficiency was thus calculated as the number of truly positive and truly negative samples divided by the sum of tested samples (Table 4). The lowest the number of non-attributable samples, the more efficient the assay.

Table 4. Evaluation of LFIAs performances on 40 milk samples. The AFM_1 reference content was determined by an ELISA kit [12]. According to the definition of the indicator range: 16 milk samples were tested negative, 16 positive and 8 uncertain. Abbreviations used: tp, truly positive (AFM_1 below 30 ng l^{-1}); tn, truly negative (AFM_1 above 70 ng l^{-1}); tu, truly uncertain (AFM_1 between 30 and 70 ng l^{-1}); fn, false negative; fp, false positive; fun, false uncertain and known to be negative; fup, false uncertain and known

to be positive. According to the definition of a single point cut-off value: 19 milk samples were tested negative and 21 positive by the reference ELISA.

		Indicator range (30-70 ng l^{-1})		Cut-off value (50 ng l^{-1})	
Parameter	Definition	Calculated as	Value (%)	Calculated as	Value (%)
Sensitivity	truly positive / known positive	tp / (tp + fn + fup)	100	tp / (tp + fn)	86
Selectivity	truly negative / known negative	tn / (tn + fp + fun)	100	tn / (tn + fp)	100
Efficiency	truly positive + truly negative / total sample	tp+tn / (tp+tn+fp+fn+tu+fun+fup)	72.5	na[a]	
False compliant rate	false negative / known negative	fn / (tn + fn + fun)	0	fn / (tn + fn)	14
False non-compliant rate	false positive / known positive	fp / (tp + fp + fup)	0	fp / (tp + fp)	0
False non-attributable rate	false uncertain / known uncertain	fup + fun / (tu + fn + fp)	37.5	na	

[a] na, not applicable

Sensitivity and selectivity of the developed assay at a single cut-off value (50 ng l^{-1}) were also calculated as 86% and 100%, respectively (Table 4). By applying the single cut-off value, three truly positive samples were incorrectly assigned as negatives, producing an excessive false compliant rate (14%). The definition of an indicator range instead of a single cut-off value allowed us to avoid the occurrence of false compliant, at the expenses of having some non-attributable assignments.

Besides, the stability of the overall device at room temperature was evaluated as the possibility of correctly measuring samples contaminated at low (<30 ng l^{-1}) and high levels (> 70 ng l^{-1}) and by using calibration curves carried out with freshly prepared strips; nevertheless, it could not be confirmed for longer than a month.

Conclusions

The development of reliable LFIAs for detecting AFM_1suffers the extreme sensitivity required to analytical methods aimed at measuring such a contaminant. Very few papers have been published which describe LFIAs for AFM_1 and none actually meet those requirements, despite the high interest in obtaining adequate systems for the rapid and on site monitoring of this toxin.

In this paper, we demonstrated that modifying of the format of the classic lateral flow assay (such as tailoring the toxin conjugate used as the competitor in the Test line and the antibody labelling procedure) a great detectability improvement could be obtained. The estimated LOD of the developed semi-quantitative LFIA was more than one order of magnitude lower than previously published LFIAs for AFM_1. Therefore, despite imprecision, the proposed method demonstrated to be effective in discriminating between compliant and non-compliant samples at a level required by the EU legislation in force for milk, while method sensitivity did not allowed the discrimination at the level set for baby food. Matrix-matched calibration was necessary to resemble results obtained on milk samples, however, various matrices (undergone to different thermal treatment and with differing fat contents) could be analysed after a very rapid and simple treatment, which involves 2 min centrifugation followed by the addition of a small volume

of a surfactant. The proposed sample treatment and the instrumental reading of test results pose some limitations to the use of the developed assay onsite as require a minimum of equipment. However, all employed tools are portable and designed to fit the need of onsite applications.

References

1. International Agency for Research on Cancer (IARC). Evaluation of carcinogenic risks in humans, IARC vol. 82. Lyon, 2002, pp 171–274
2. H.P. Van Egmond, in: H.P. Van Egmond (Ed) Mycotoxins in Dairy products. Elsevier Applied Science Publisher, London, 1989, pp 11-59
3. European Commision (EC), Off. J. Eur. Comm. L364 (2006) 5-24
4. U.S. Food and Drug Administration, Available at: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm077969.htm (last accessed 13 June 2012)
5. European Mycotoxins Awareness Network (EMAN), Available at: http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=79 (last accessed 25 June 2012)
6. A.C. Manetta, L. Di Giuseppe, M. Giammarco, I. Fusaro, A. Simonella, A. Gramenzi, A. Formigoni, J. Chromatogr. A 1083 (2005) 219-22
7. M. Muscarella, S. Lo Magro, C. Palermo,D. Centonze, Anal. Chim. Acta 594 (2007) 257-264
8. H. Wang, X.J. Zhou, Y.Q. Liu, H.M. Yang, Q.L. Guo, Food Addit. Contam. A 27 (2010) 1261-1265.
9. W.L. Chen, T.F. Hsu, C.Y. Chen, J. AOAC Int. 94 (2011) 872-877
10. C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Laganà, J. Chromatogr. A 1101 (2006) 69–78
11. M. Magliulo, M. Mirasoli, P. Simoni, R. Lelli, O. Portanti, A. Roda, J. Agric. Food Chem. 53 (2005) 3300-3305
12. L. Anfossi, M. Calderara, C. Baggiani, C. Giovannoli, E. Arletti, G. Giraudi, J. Agric. Food Chem. 56 (2008) 1852-1857
13. D. Guan, P. Li, Y. Cui, Q. Zhang, W. Zhang, Anal. Chim. Acta 703 (2011) 64-69
14. L. Kanungo, S. Pal, S. Bhand, Biosens. Bioelectron. 26 (2011) 2601–2606
15. A. Radoi, M. Targa, B Prieto-Simon, J.L. Marty, Talanta 77 (2008) 138-143
16. R. Krska, A. Molinelli, Anal. Bioanal. Chem. 393 (2009) 67–71
17. B. Ngom, Y. Guo, X. Wang, D. Bi, Anal. Bioanal. Chem. 397 (2010) 1113–1135

18. L. Anfossi, C. Baggiani, C. Giovannoli , G. D'Arco, G. Giraudi, Anal. Bioanal. Chem. 2012: 1-14 DOI 10.1007/s00216-012-6033-4
19. R. Salter , D. Douglas , Tess M, Markovsky B, Saul SJ, J. AOAC Int. 89 (2006) 1327-1334.
20. J.J. Wang, B.H. Liu, Y.T. Hsu, F.Y. Yu, Food Control 22 (2011) 964-969
21. D. Zhang, P. Li, Q. Zhang, Y. Yang, W. Zhang, D. Guan, X. Ding, Anal. Methods 4 (2012) 3307-3313
22. Y.H. Liu, R. Xie, Y.R. Guo, G.N. Zhu, F.B. Tang, J. Environm. Sci. Health B 47 (2012) 475-483.
23. N.A. Byzova, E.A. Zvereva, A.V. Zherdev, S.A. Eremin, P.G. Sveshnikov, B.B. Dzantiev, Anal. Chim. Acta 701 (2011) 209-217
24. B.H. Liu, Z.J. Tsao, J.J. Wang, F.Y. Yu, Anal. Chem. 80 (2008) 7029–7035
25. M.V. Laycock, M.A. Donovan, D.J. Easy, Toxicon 55 (2010) 597–605
26. L. Anfossi, M. Calderara, C. Baggiani, C. Giovanoli, E. Arletti, G. Giraudi, Anal. Chim. Acta 682 (2010) 104–109
27. M. Horsiberger, J. Rosset, J. Histochem. Cytochem. 25 (1977) 295-305
28. J.W. Findlay, W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, R.R. Bowsher, J Pharmaceut. Biomed. Anal. 21 (2000) 1249-1273
29. N.G. Khlebtsov, Anal. Chem. 80 *(*2008) 6620–6625
30. http://www.nanoprobes.com/tech_help/TechCG.html (last accessed 10 October 2012)
31. E.S. Grabbe, Langmuir, 9 (1993) 1574-1581
32. A. Molinelli, K. Grossalber, M. Führer, S. Baumgartner, M. Sulyok, R. Krska, J. Agric. Food Chem. 56 (2008) 2589-2594
33. A. Molinelli, K. Grossalber, R. Krska, Anal. Bioanal. Chem. 395 (2009) 1309-1316
34. L. Anfossi, G. D'Arco, M. Calderara, C. Baggiani, C. Giovannoli, G. Giraudi, Food Addit. Contam. A 28 (2011) 226-234
35. L. Anfossi, G. D'Arco, C. Baggiani, C. Giovannoli, G. Giraudi, Food Control 22 (2011) 1965-1970
36. A.Y. Kolosova, L. Sibanda, F. Dumoulin, J. Lewis, E. Duveiller, C. Van Peteghem, S. De Saeger, Anal. Chim. Acta 616 (2008), 235–244

37. R.C. Wong, H.Y. Tse, in: R.C. Wong, H.Y. Tse (Eds), Lateral flow Immunoassay, Humana Press c/o Springer Science +Business Media, New York, USA, 2009, pp 188-189
38. E. Trullols, I. Ruisanchez, F. X. Rius Trends Anal. Chem. 23 (2004) 137-14
39. European Commission. Off. J. European Comm. L070 (2006) 12-34
40. S.L.R. Ellison, T. Fearn, Trends Anal. Chem. 24 (2005) 469-476
41. S. Cardenas, M. Valcarcel, Trends Anal. Chem. 24 (2005) 477-487
42. AOAC Int availaible from http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-foods-gen/documents/document/ucm088702.pdf (last accessed 10/12/2012
43. D. Tang, Z. Zhong, R. Niessner, D. Knopp, Analyst 134 (2009) 1554-1560

TABLES

Table 1 Effect of varying the competitor to be used in the Test line of the LFD. Protein conjugates were dispensed onto the membrane at different concentrations to reach an absolute signal comprises between 20 and 25 arbitrary units on the T-line.

Conjugate	SR[a]	Dispensing concentration (mg ml^{-1})	IC_{50} (µg l^{-1})
AFM_1-BSA	4	0.8	0.2
AFM_1-BSA	15	0.4	1.1
AFM_1-BSA	22	0.2	1.7
AFM_1-OVA	10	0.8	0.6
AFB_1-BSA	24	0.4	1.6

[a] molar ratio between AFM1 and protein as estimated by spectrophotometric measurements

Table 2 Antibody amount used to coat GNPs at variable levels of saturation. The saturation amount was experimentally determined by the method of Horisberg [27] (SAT_{Exp}) and approximately calculated from the concentration of gold nanoparticles, their superficial area and the hypothetical area occupied by an IgG molecule (SAT_{Calc})

Antibody amount (Ab), µg/ml	Ab / SAT_{Exp}	Ab / SAT_{Calc}
1.5	0.4	0.5

2.5	0.7	0.9
3.5	1.0	1.25
5.0	1.4	1.8

Table 3 Recovery of Aflatoxin M_1 determination from artificially contaminated milk samples undergone to various thermal treatments and with different fat content as determined by the developed LFIA. Recovery was calculated as follows: (estimated AFM_1 for the fortified sample – estimated AFM_1 for the non fortified sample) / fortification level *100. Semi-quantitative attribution was done according to following criteria: negative sample (-), samples with AFM_1 content lower than 30 ng l^{-1}; positive sample (+) , samples with AFM_1 content higher than 70 ng l^{-1}; uncertain sample (±), AFM_1 content comprised between 30 and 70 ng l^{-1}.

Milk sample	AFM_1 measured by ELISA (ng l^{-1})	Fortification level (ng l^{-1})	Estimated $AFM_1 \pm$ SD (ng l^{-1})	Recovery (%)
raw	17.8	0	<LOD	
		50	78 ± 6	120
		100	153 ± 14	135
whole 1	< LOD	0	<LOD	
		50	40 ± 2	80
		100	122 ± 10	122

whole 2	16.0	0	<LOD	
		50	79 ± 9	126
		100	126 ± 11	110
skimmed	15.7	0	35 ± 1.2	
		50	74 ± 4	117
		100	113 ± 20	97
UHT	<LOD	0	<LOD	
		50	47 ± 5	94
		100	88 ± 11	88

Table 1. Evaluation of LFIAs performances on 40 milk samples. The AFM_1 reference content was determined by an ELISA kit [12]. According to the definition of the indicator range: 16 milk samples were tested negative, 16 positive and 8 uncertain. Abbreviations used: tp, truly positive (AFM_1 below 30 ng l^{-1}); tn, truly negative (AFM_1 above 70 ng l^{-1}); tu, truly uncertain (AFM_1 between 30 and 70 ng l^{-1}); fn, false negative; fp, false positive; fun, false uncertain and known to be negative; fup, false uncertain and known to be positive. According to the definition of a single point cut-off value: 19 milk samples were tested negative and 21 positive by the reference ELISA.

		Indicator range (30-70 ng l^{-1})		Cut-off value (50 ng l^{-1})	
Parameter	Definition	Calculated as	Value (%)	Calculated as	Value (%)
Sensitivity	truly positive /	tp /	100	tp /	86

	known positive	(tp + fn + fup)		(tp + fn)	
Selectivity	truly negative / known negative	tn / (tn + fp + fun)	100	tn / (tn + fp)	100
Efficiency	truly uncertain / known uncertain	tu / (tu + fun + fup)	62.5	na[a]	
False compliant rate	false negative / known negative	fn / (tn + fn + fun)	0	fn / (tn + fn)	14
False non-compliant rate	false positive / known positive	fp / (tp + fp + fup)	0	fp / (tp + fp)	0
False non-attributable rate	false uncertain / known uncertain	fup + fun / (tu + fn + fp)	37.5	na	

[a] na, not applicable

FIGURES

Figure 1 Inhibition curves obtained from signal reporters with variable amount of antibodies coated onto the GNPs. Antibody amount compared to the experimentally determined saturation: 0.4 (●), 0.7 (▲), 1 (■), 1.5 (◆). Labelled antibodies: OD 3 ; T-line: AFM_1-BSA conjugate (0.2 mg/ml, SR=22).

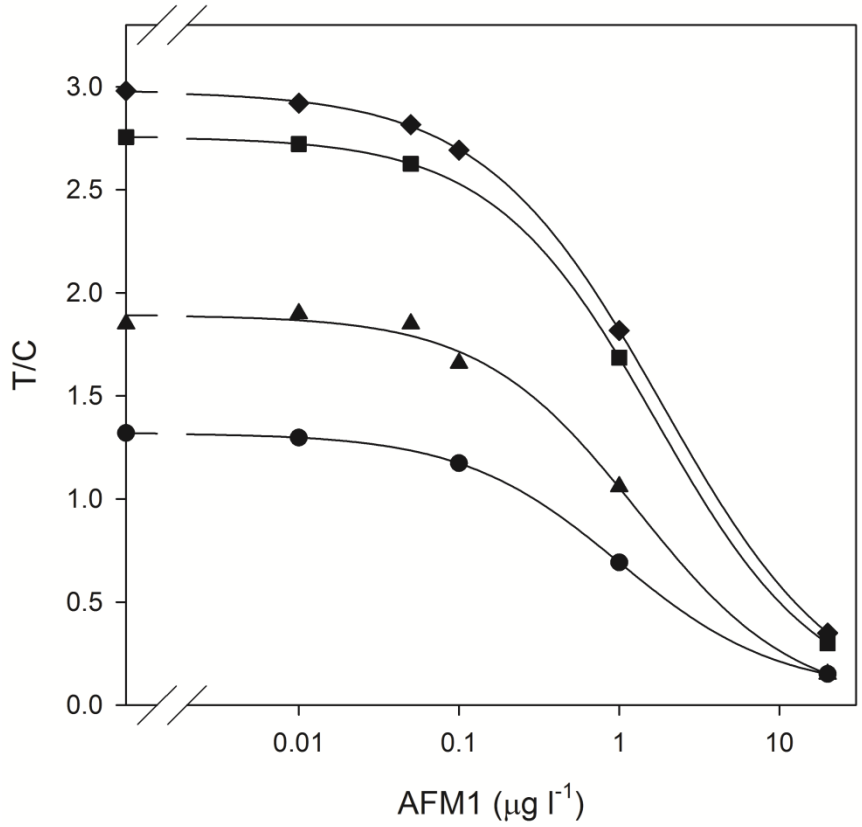


Figure 2 A typical inhibition curve, obtained under optimized conditions, for the developed lateral flow immunoassay

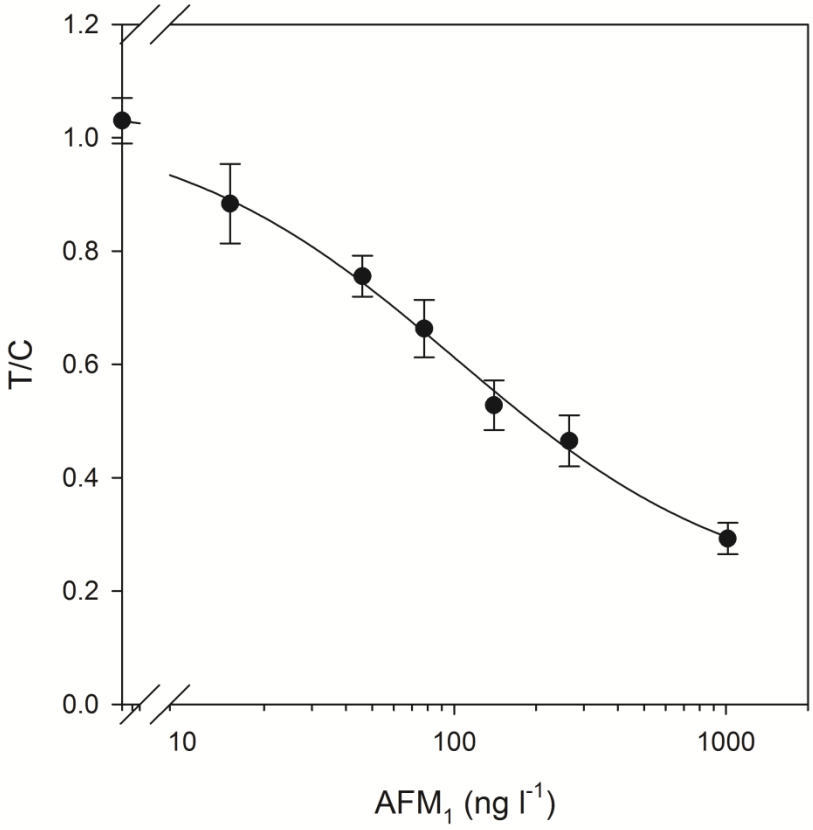
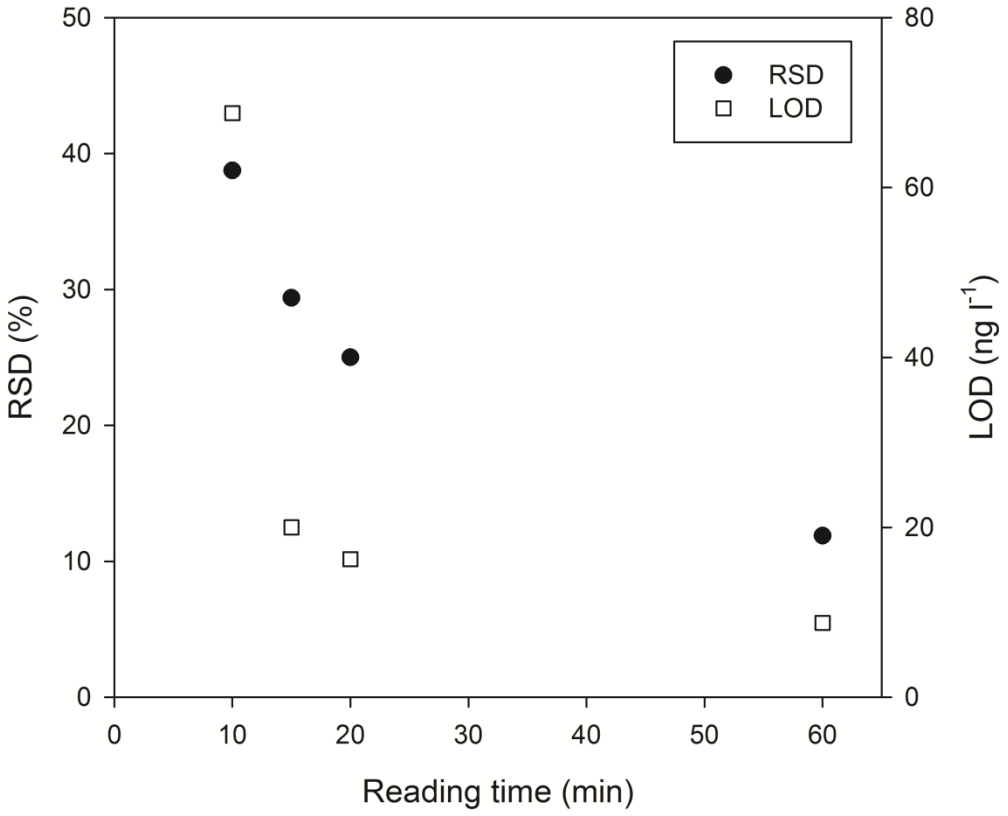
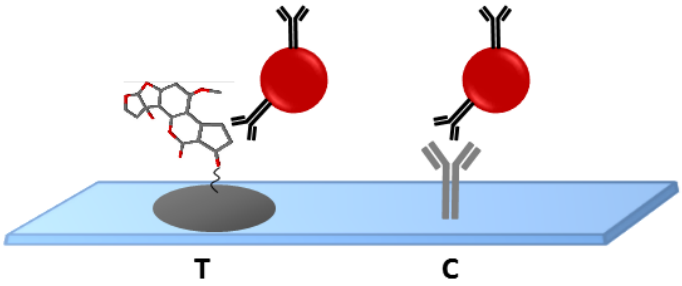
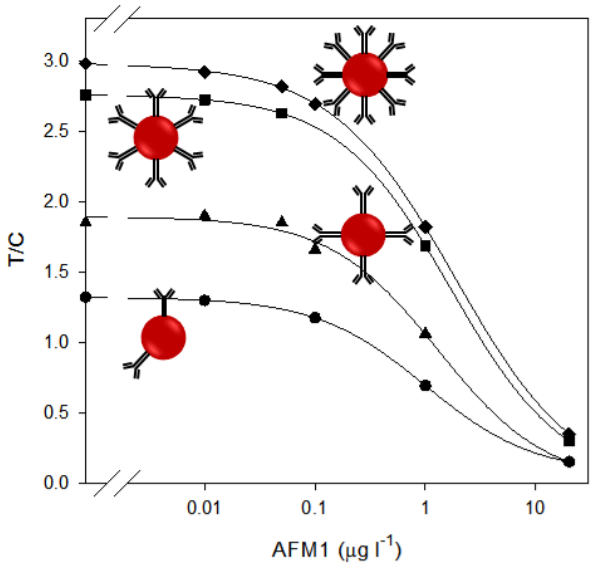


Figure 3 Precision of AFM1 quantification and LOD (calculated from the mean of the blank minus three standard deviation of the mean) as a function of the time elapsed before line intensity reading

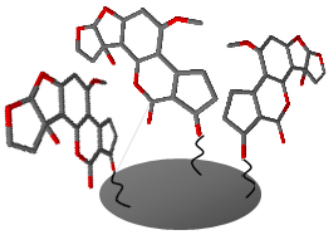


Graphical Abstract A significant improvement in the sensitivity of a gold colloid immunochromatographic assay could be obtained by reducing the amount of antibody coated onto gold nanoparticles and, mainly, by decreasing the hapten-to-protein rate in the preparation of the competitor used to trace the Test line. In optimal conditions, the developed assay is sensitive enough to allow aflatoxin M_1 detection at a ng/l level, which means a detectability increase of more than an order of magnitude in comparison to previously reported immunochromatographic methods for this toxin and, in general, for haptens.





Conjugate	Hapten/Protein	IC_{50} (µg l^{-1})
AFM_1-BSA	4	0.2
AFM_1-BSA	15	1.1
AFM_1-BSA	22	1.7
AFM_1-OVA	10	0.6
AFB_1-BSA	24	1.6



SUPPLEMENTARY MATERIALS

Materials

Gold (III) chloride trihydrate (ACS reagent), bovine serum albumin (BSA), goat anti-rabbit immunoglobulin antibodies, aflatoxin M_1 (Oekanal certified standard solution), aflatoxin M_1-BSA conjugate (substitution ratio 4:1) were purchase from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies (ammonium sulphate precipitated IgG fraction) directed towards AFM_1, AFB_1-BSA, AFM_1-BSA (substitution ratios 15:1 and 22:1), AFM_1-OVA (substitution ratio 12:1) and the ELISA kit (Mycotoxins Competitor IPS ELISA - Aflatoxin M1Quantitative 96X Kit) were kindly provided by Generon srl.

Sample and adsorbent pads were cellulose fibre (CFSP20300, 20x300 mm), release pads were glass fibre (GFCP103000, 10x300 mm), membranes were nitrocellulose (Hi-Flow Plus 180 membrane cards, 60x300 mm); all these materials were purchased from Millipore (Billerica, MA, USA).

Release pads and the membranes had spots traced upon them by means of an XYZ3050 platform (BioDot, Irvine, CA, USA), equipped with two BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing. Membranes were cut into strips, each one of 5 mm in width, by a CM4000 guillotine (BioDot, Irvine, CA, USA).

Preparation of colloidal gold and colloidal gold-labelled polyclonal antibodies

Gold colloids were prepared using the sodium citrate method as previously described [26]. Briefly, 1 ml of a sodium citrate solution (1 % w/v) were added to 100 ml of a boiling solution of $HAuCl_4$ (0.01% w/v) under vigorous stirring. The solution was left to stand under stirring and warming until the colour turned to a clear red and for the following 10 minutes. Then, it was cooled and the absorption maximum in the visible region (400-600 nm) was measured. The pH of the gold colloids was adjusted to about 8-8.5 before conjugation with antibodies by means of a carbonate buffer (50 mM, pH 9.6).

The saturation concentration of the anti-AFM_1 antibody for conjugation with gold nanoparticles was determined according to Horisberg [27], as follows: increasing amount of the anti-AFM_1 antibody (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 μg) were added to 1 ml of pH-adjusted gold colloid and incubated for

30 min at room temperature. Then 100 µl of sodium chloride (0.5M) was added and the colour of the colloidal solution was visually evaluated. The amount of antibody needed to stabilize GNPs (saturation amount, SAT) was established as the minimum quantity that not determine colour shift from red to purple-blue.

GNP-antibody conjugation was carried out using an amount of antibodies which is the half the saturation amount and was carried out as follows: 100 µl of a 0.15 mg ml^{-1} anti-AFM_1 antibody solution in borate buffer (BB, 20 mM, pH 8.0) was added to 10 mL of pH-adjusted colloidal gold solution. After 30 min incubation at room temperature, 1 ml of BB containing 1% of BSA was added and incubated for additional 10 min. The mixture was centrifuged at 10000 rpm at 25°C for 30 min, and the pellet was washed twice by re-suspension in BB which contains 0.1% BSA. Finally, the pellet was re-suspended in BB, supplemented with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide as a preservative and stored at 4°C until use. The absorption maximum shift was checked and was considered acceptable if lower than 10 nm.

Preparation of the test strips

Nitrocellulose membranes, pasted onto an adhesive polyester layer of 5 cm x 30 cm, had spots traced upon them with Test and Control lines at a distance of 4 mm from each other: the AFM_1-protein conjugate (SR 4) at 0.3 mg/ml, used as a capture reagent, and the goat anti-rabbit IgG antibodies (2 mg/ml) both diluted in PBS were applied onto the membranes at 1 µl/cm. Then, the membranes were dried at 37°C under vacuum for 60 min.

Release pads were previously treated with BB supplemented with 1% (w/v) BSA, 0.25% (v/v) Tween 20, 2% (w/v) sucrose, and 0.02% (w/v) sodium azide. After 60 minutes drying at 65°C, gold-labelled antibodies were distributed at a flow rate of 5 µl/cm near the lower edge of pads, and these were dried again at room temperature for 2 hours.

Strips were composed as follows: from the top; the adsorbent pad, the nitrocellulose membrane, the conjugate pad and the sample pad were pasted, in sequence, with 1-2 mm overlap. The prepared

membrane was cut into strips of 5 mm, which were inserted into rigid plastic cassettes (D.M. Varese, Italy). Cassettes were stored in plastic bags containing silica at room temperature until use.