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Development of a quantitative lateral flow immunoassay for the detection of aflatoxins in maize

Laura Anfossi*, Gilda D'Arco, Marianna Calderara, Claudio Baggiani, Cristina Giovannoli and Gianfranco Giraudi

Department of Analytical Chemistry, University of Turin, Via Giuria 5, Torino 10125, Italy

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An immunoassay-based lateral flow device for the quantitative determination of four major aflatoxins in maize has been developed. The one-step assay has performance comparably with that of other screening methods, as confirmed by the intra- and the inter-day precision of the data (RSD 10–22%), and can be completed in 10 min. Quantification was obtained by acquiring images of the strip and correlating intensities of the coloured lines with analyte concentration by means of a stored calibration curve carried out by diluting aflatoxins in the extract from a blank maize sample. Limit of detection ($1\,\mu g\,kg^{-1}$) and dynamic range ($2–40\,\mu g\,kg^{-1}$) allows the direct assessment of aflatoxin contamination in maize at all levels of regulatory relevance. All reagents are immobilized on the lateral flow device. In addition, very simple sample preparation, using an aqueous buffered solution, has been demonstrated to allow the quantitative extraction of aflatoxins. Twenty-five maize samples were extracted with the aqueous medium and analyzed by the developed assay. A good correlation was observed ($y = 0.97x + 0.07$, $r^2 = 0.980$) when data was compared with that obtained through an official method. The developed method is reliable, rapid and allows for application outside the laboratory as a point-of-use test for screening purposes.

Keywords: immunoassays; screening, immunoassays; aflatoxins; cereals

Introduction

Aspergillus flavus and *A. parasiticus* are two fungal species that attack crops in mainly tropical and subtropical countries, due to high humidity, during growth and storage. These moulds, besides damaging the harvests, also produce toxic secondary metabolites, known as aflatoxins (AFs). There are more than 18 compounds belonging to this group, each one with a different level of toxicity. Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are the most common and are classified as carcinogenic substances of group I (IARC 2002). Maximum residue levels (MRL) for the sum of the all four AFs (total aflatoxins) have been set by the European Union (2010) and other agencies (US FDA, 2010; CFIA 2010). In particular, EU MRLs for total aflatoxins in cereals stand at $10\,\mu g\,kg^{-1}$ for corn to be submitted for any type of treatment before human consumption (without any treatment) and at $4\,\mu g\,kg^{-1}$ for other cereals and corn for direct human consumption. In the liver of dairy cattle, AFB1 is hydroxylated to form aflatoxin M1, which is transferred into milk; therefore maximum admissible levels have also been set up for aflatoxin contamination in feed (European Commission 2003). The occurrence of aflatoxins has been widely reported in a variety of crops, including maize, wheat, barley, rice, groundnuts, nuts, pistachios, cottonseed, and spices.

The official method of analysis for aflatoxins consists of the extraction with an organic solvent, immunoaffinity clean-up, chromatography separation, and derivatization and UV detection (AOAC 2005). To date, numerous techniques to detect AFs in foods have been developed (Sforza et al., 2006; Shephard 2009); however, instrumental techniques are mainly used in confirmatory analyses and are usually not applied to routine controls owing to equipment costs and extensive clean-up steps. Immunochemical methods of analysis are widely employed as screening methods for measuring food contaminants, thanks to their rapidity, selectivity and sensitivity (Krska et al., 2008). Nevertheless, immunoassays still need to be realized in the laboratory, use equipment and occasionally require sample pre-treatment. Several immunoassays for detecting aflatoxins in a variety of commodities have been described (Kolosova et al., 2006; Li et al., 2009; Peiwu et al., 2009; Lupo et al., 2010).

Immunochromatographic assays carried out on strips, or lateral flow immunoassays (LFIAs), are well known in the medical field for diagnosing blood infections or contamination, drug use or for ascertaining pregnancy (Posthuma-Trumpie et al., 2009).

Devices based on lateral flow immunoassays have a number benefits, including extreme simplicity, rapidity and cost effectiveness. These make them ideally suited for screening large number of samples at all stages of food and feed production and for use by those who are close to the site of contamination. Recently, the LFIA format has been applied to develop portable kits for on-site monitoring of mycotoxins (Kolosova et al., 2007, 2008; Krska and Molinelli 2009; Molinelli et al., 2008, 2009). In particular, some LFIAs for the qualitative and semi-quantitative detection of aflatoxins in food and feed have been described (Delmulle et al., 2005; Sun et al., 2006; Shim et al., 2007; Tang et al., 2009). Nevertheless, quantification would be of great interest to reduce uncertainty due to the subjective interpretation of visual results and to meet regulatory requirements. Indeed, some studies on quantitative lateral flow immunoassays for mycotoxins are beginning to appear as patents or in the literature (Ho and Wauchope 2002; Sun et al., 2007; Saul and Tess, 2008; Anfossi et al., 2010).

In the present work, a LFIA for the quantification of aflatoxins in maize was developed. The method is based on the use of lateral flow strips. A sample extract is directly applied to the strip, where all reagents have been pre-adsorbed. The lateral flow allows gold-labelled antibodies to cross the strip and to focus in two lines corresponding to immobilized immunoreagents. The lines become red coloured due to gold nanoparticle-focusing and the intensity of the red colour is correlated to the amount of analyte in the sample extract. Strips are read by a portable scanner connected to a laptop and processed by dedicated software, which acquires images, determines colour intensity, interpolates values on a memorized standard curve and returns the concentration of the analyte in the sample. Optimization of strip design, adaptation of flow characteristics and evaluation of the system's stability and reproducibility allowed us to develop a portable device for the rapid on-site quantification of total aflatoxins in maize samples.

Another aspect evaluated in this study has been a protocol for extracting aflatoxins from cereals that is simple, fast, does not require the use of hazardous or toxic substances and permits application of the assay outside the laboratory. Therefore, several aqueous extraction solutions were examined as extracting media to determine whether the use of an organic solvent was mandatory for the purpose.

The developed aqueous extraction protocol and lateral flow immunoassay were validated together through comparison with a reference LC–FLD method (AOAC 2008) for measuring aflatoxins in cereals. Results prove the method is capable of the quantitative determination of total aflatoxins in maize of different sizes and subjected to different processing at levels of regulatory relevance.

Materials and methods

Materials

Gold(III) chloride trihydrate (ACS reagent), bovine serum albumin (BSA), mixture of aflatoxin B_1, B_2, G_1 and G_2 (0.5 µg ml^{-1} for B_2 and G_2 and 2 µg ml^{-1} for B_1 and G_1 in acetonitrile; Oekanal standard solution), polyethylene glycol (PEG, average mw 10 kDa) were purchase from Sigma-Aldrich (St. Louis, MO, USA). Certified reference maize samples were obtained from AIA (Rome, Italy). The ELISA kit for measuring total aflatoxins in cereals was purchased from Generon (Modena, Italy). AFB1–BSA conjugate, the anti-aflatoxin antibody (rabbit polyclonal antiserum, IgG fraction by ammonium sulfate precipitation) and the goat anti-rabbit immunoglobulin antibody were kindly supplied by Generon srl (Modena, Italy). Triton X-100, Tween 20 and all other chemicals were obtained from VWR International (Milan, Italy).

Sample and adsorbent pads were cellulose fibre, release pads were glass fibre, membranes were nitrocellulose (Hi-Flow Plus 180 membrane cards, capillary flow 180 s per 4 cm, 60 × 300 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^{TM} 3000 line dispensers for non-contact dispensing. Membranes were cut into strips, each 5.2 mm in width, by a CM4000 guillotine (BioDot, Irvine, CA, USA).

Preparation of colloidal gold and colloidal gold-labelled polyclonal antibodies

Gold colloids with an adsorption maximum of 525 nm and mean diameter of 40 nm were prepared using the sodium citrate method, as previously described (Arai et al., 2001; Du et al., 2008). Briefly, 1 ml of sodium citrate (1% p/v) were added to 100 ml of a 0.01% solution of tetrachloroauric acid under vigorous stirring and warming (100°C). The colloidal gold solution was adjusted to pH 8.5 with a 50-mM carbonate buffer (pH 9.6) for coating with the antiserum. The optimum concentration of the polyclonal antibody towards aflatoxin B1 (pAb) for conjugation with gold nanoparticles was determined prior to conjugation, according to Horisberg and Rosset (1977). Briefly, increasing amounts of a 0.1 mg ml^{-1} antiserum solution (0–100 µl) were added to 1 ml of the gold nanoparticle colloid. After 30 min of incubation at room temperature, 100 µl of a NaCl solution (10% w/v) were added and the colour of the obtained solutions was observed after 10 min. High salt concentrations induce gold nanoparticle aggregation when an insufficient amount of antibodies have been adsorbed on the surface of the nanoparticles themselves, and the aggregation can be

Figure 1. Image of lateral flow devices used for measuring aflatoxins in three maize samples, naturally contaminated at three levels. Concentrations of aflatoxins (obtained by the LC–FLD method) from left to right: not detectable (negative sample), 5.4 μg kg^{-1} (low contamination level, close to EU MRL (European Commission 2003), and 17.1 μg kg^{-1} (high contamination level).

visually detected because the red colour of the gold colloid turn to blue-grey. An amount of antiserum allowing the red colour to be preserved should be used for conjugation to obtain stable gold-labelled antibody preparations. Excess antibody was used for conjugation as follows: 100 μl of a 1 mg ml^{-1} pAb 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 borate buffer containing 1% of BSA was added. The mixture was centrifuged at 10,000 rpm at 25°C for 30 min, and the pellet was washed twice by re-suspension in borate buffer containing 0.1% BSA. Finally, the pellet was resuspended in BB, with 1% BSA, 0.25% Tween 20, 2% sucrose and 0.02% sodium azide as 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 × 30 cm, had spots traced upon them with test and control lines at a distance of 4 mm from each other. The AFB1–BSA conjugate, used as a capture reagent, was dispensed at 0.2 mg ml^{-1}, the goat anti-rabbit IgG antibodies were distributed at 1 mg ml^{-1}, both diluted in PB (disodium hydrogen phosphate/sodium dihydrogen phosphate 20 mM, pH 7.4) and applied onto the membranes at 1 μl cm^{-1}. After drying at 37°C for 60 min, the membranes were blocked with PB containing 1% (w/v) BSA at room temperature for 5 min and washed twice with PB containing 0.05% Tween 20. Then, the membranes were dried at 37°C under vacuum for 60 min.

Release pads were previously treated with BB containing 1% (w/v) BSA, 0.25% (v/v) Tween 20, 2% (w/v) sucrose and 0.02% (w/v) sodium azide. After 60 min drying at 65°C, gold-labelled antibodies were dispensed at 10 μl cm^{-1} on pads and these dried again at room temperature for 2 h.

Strips were composed as follows: from the top, the adsorbent pad, nitrocellulose membrane, conjugate pad and the sample pad were pasted, in sequence, with a 1–2 mm of overlap. The prepared membrane was cut into 5-mm strips, which were inserted into rigid plastic cassettes (Dima Diagnostics, Goettingen, Germany), each one with a sample well, a reading window and a barcode for strip identification (Figure 1). Cassettes were stored in plastic bags containing silica at room temperature until use.

Samples and sample preparation

Maize samples were obtained directly from producers or mills. Grain samples were ground and homogenized, flour was directly extracted. All samples used in comparative studies were analyzed without fortification.

Ground samples (5 g) were weighed and extracted with 50 ml of PB containing 2% PEG (w/v) by manual shaking for 2 min. After 5 min of decantation, the clear

Table 1. Recovery experiments for the extraction of aflatoxins from certified reference maize samples using an aqueous medium (PB).

Sample	Certified concentration of AFs ± SD ($\mu g\, kg^{-1}$)	Estimated concentration of AFs ± SD ($\mu g\, kg^{-1}$; $n = 8$)
A	<1	0.5 ± 0.2
B	6.1 ± 1.8	7.1 ± 0.5
C	13.3 ± 3.0	11.6 ± 0.9
D	4.1 ± 1.1	6.5 ± 0.5

supernatant was immediately used in the lateral-flow assay.

For the construction of the standard curve a blank maize sample (certified material) was fortified with appropriate amounts of the reference solution of total aflatoxins. Solvent was then evaporated and contaminated samples were homogenized and kept at 4°C overnight before extraction and analysis.

Development of an aqueous extraction of aflatoxins from cereals

Evaluation of the extraction protocol was conducted on four certified reference materials of maize flour (Table 1). Firstly, the sample named A, which was considered as a blank sample, was fortified by adding total aflatoxins at a final concentration of $10\,\mu g\, l^{-1}$. Sub-samples of 1 g were weighed from the pool and extracted with 10 ml of different solvents: water, aqueous NaCl (200 mM), phosphate buffer pH 7.4 (20 mM), and phosphate/citrate buffer pH 5.0 (20 mM). After a 5-min shaking, the mixture was allowed to stand for 15 min to separate the supernatant. The clear supernatant was diluted 1:1 (v/v) with the extracting solution and filtered through a syringe filter (0.22 µm pore size nylon membrane) before the analysis. Each sub-sample was extracted in duplicate and analyzed in quadruplicate.

The following recovery experiments were conducted on the three positive reference materials. Samples were thoroughly homogenized and divided into 5-g sub-samples. Two sub-samples of the same material were separately extracted with 50 ml of PB by the above described procedure and each extract solution was analyzed in quadruplicate.

A commercial ELISA kit was used to quantify aflatoxins, in which calibrators were substituted with aflatoxin standards prepared in each solution used in the extraction experiments.

Lateral flow immunoassay procedure

The test was carried out by adding 150 µl of extract into the sample well. After 10 min of incubation at room temperature, the cassette was placed above a mobile scanner (OpticSlim 500, Plustek Technology GmbH, Norderstedt, Germany) connected to a laptop. The Scannex 3.0 software (Skannex AS, Hoenefoss, Norway) was used to acquire and process images. The program recognizes a barcode printed on the cassette containing the strip and converts the ratio between line intensities into a concentration value according to a calibration curve, which has been stored in the barcode itself.

The calibration curve was obtained by plotting the ratio between the intensity of the test (T) and the control line (C) against the log of aflatoxin concentration. Linearization of the calibration curve was carried out by the logit–log transformation, by plotting the logit of the ratio (as a percentage) between the absorbance at each analyte concentration level (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. Total aflatoxins in samples were determined by interpolation on the linear calibration curve.

LC–FLD analysis

Preparation, extraction and clean-up of samples for validation purposes were performed by an AOAC (2008) reference method, with slight modifications. Ground samples were extracted using water/methanol (20:80, v/v) and diluted 1:6 with phosphate-buffered saline (pH 7.2). The diluted solution was passed through an Aflaprep™ column (R-Biopharm AG, Darmstadt, Germany). After column washing, aflatoxins were eluted with acetonitrile. The solvent was evaporated to dryness and extracts were reconstituted with the mobile phase. Aflatoxin separation was carried out by a HPLC (LaChrom Elite, VWR International, Milan, Italy) equipped with a fluorescence detector ($\lambda_{ex} = 362$ nm, $\lambda_{em} = 440$ nm) and a Kobra cell™ photochemical reactor (R-Biopharm AG, Darmstadt, Germany). The analytical column was an Alltima C_{18} (250×3.2, particle size 5 µm; Alltech, Grace, IL, USA), the mobile phase consisted of water/methanol (60:40, v/v) fluxed at a flow rate of $0.65\, ml\, min^{-1}$. Quantification was obtained by interpolation on an external calibration curve.

Results and discussion

Development of an extraction protocol using an aqueous solution

The presence of organic solvent strongly affects LFIA performance, as far as both the flow properties and the immunochemistry are concerned. Therefore, most authors recommend dilution of sample extracts

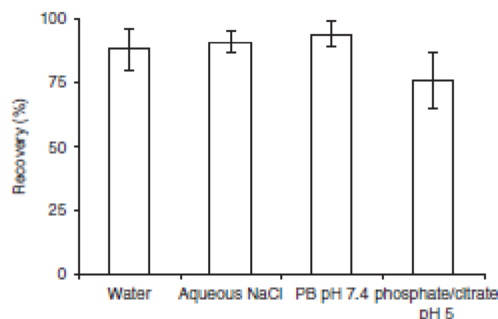

Figure 2. Recovery values for the extraction of aflatoxins from a fortified maize sample (blank reference material) using different aqueous solution as the extracting solvent.

(Delmulle et al., 2005, Sun et al., 2006; Shim et al., 2007). However, as previously observed (Sun et al., 2006; Molinelli et al., 2009) and confirmed by our experience, the use of organic solvent, typically aqueous methanol, determines the co-extraction of fatty materials which may interfere in the assay. On the other hand, good results from extraction procedures, which avoid the use of organic solvents, have been demonstrated for some mycotoxins (Kulisek and Hazebroek, 2000; Anfossi et al., 2008). Therefore, a preliminary evaluation of the possibility of using an aqueous solution to extract aflatoxins from cereals was performed. A commercial ELISA kit for measuring total aflatoxins in cereals was used to assess recovery rates obtained with various aqueous extracting solutions: water, aqueous NaCl (200 mM), phosphate buffer pH 7.4 and phosphate/citrate buffer pH 5.0. A reference blank sample of maize flour fortified with total aflatoxin at 10 µg l^{-1} was used as a model system to investigate extraction recoveries. All investigated aqueous media gave acceptable recovery values (comprising between 76 and 94%; Figure 2) and good reproducibility. In particular, the maintenance of neutral pH seems to favour aflatoxin extraction and is especially preferable for the following immunoassay, thus we opted for the use of the phosphate buffered solution (PB) as the extracting solution. This was used for extracting aflatoxins in certified reference materials. The accuracy and precision, determined as the mean and standard deviation of two recovery experiments (each measured in quadruplicate) on each of the certified reference maize flour samples, are shown in Table 1 and firstly proved the method to be accurate and reproducible enough to be tested together with the developing lateral flow immunoassay.

Development and optimization of the strip

Competitive immunoassays can be carried out on lateral flow dipsticks, which are composed of three main elements: a release pad, a membrane and an adsorbent pad. In addition to those, a sample pad aimed at filtrate samples can be used. The release pad has the function of trapping the gold conjugate of the antibody directed towards aflatoxins, in such a way that the conjugate is released when it comes into contact with a liquid sample and flows through the membrane together with the sample itself. Two lines of reagents are immobilized onto the membrane: the test line comprises an AFB1–BSA conjugate and the control line is a line of anti-species antibody. When the gold-antibody conjugate flows across the membrane it first encounters the test line and binds to the immobilized AFB1–BSA; thus, a red line becomes visible due to the focusing of nanoparticles. If some AFB1 is present in the sample, it competes with the immobilized AFB1–BSA for binding to the gold–antibody conjugate, resulting in a reduction of test line intensity. The anti-species antibody on the control line captures any excess gold particles, bound or unbound, to produce a control line as a visible confirmation of particle flow.

The pore size of the membrane directly affects thickness of the lines and the flow rate. Both these parameters influence the slope of the dose–response curves. Indeed, membranes with small pore sizes give narrower lines, which allow us to reduce the amount of AFB1–BSA conjugate and gold-labelled antibody needed to produce a visible test line. On the other hand, the time necessary to develop the colour on the strip augments. A HiFlow180 membrane was chosen as the best compromise between lower LOD and the time required to complete the experiment.

The sensitivity of the method, being a competitive assay, depends on the amount of AFB1–BSA conjugate and gold-labelled antibodies used. These parameters were optimized by comparing dose–response curves obtained using different combinations of AFB1–BSA (concentrations: 0.1, 0.2, 0.5, and 1 mg ml^{-1}; dispensation rate: 0.5–1–2 µl cm^{-1}) and gold labelled-antibody (OD: 1–2–5–10; dispensation rate: 2–5–10 µlcm^{-1}). The lowest LOD (limit of detection) and the highest sensitivity (slope of the curve) were obtained by using a 0.2 mg ml^{-1} of AFB1–BSA solution (dispensed at a flow rate of 1 µlcm^{-1}) as the coating antigen and a gold-labelled antibody (OD = 2) dispensed at 10 µl cm^{-1}.

The gold-labelled antibodies are dispensed onto glass fibre, which is used as the release pad. As previously observed (Ho and Wauchope, 2002), this procedure introduces considerable non-reproducibility because some drops of the suspension can cross the fibre and disperse on the supporting surface or, in general, can diffuse in an irregular manner along and across the fibre. In addition, when the line of gold-labelled antibodies widens, due to diffusion, the sensitivity of the assay is also affected. To reduce

diffusion phenomena, release pads were treated before the deposition of the gold conjugate by a pre-washing of the pad in a buffer containing sucrose and BSA (Anfossi et al., 2010). The nitrocellulose membrane was also blocked with a solution which contains BSA to reduce the variability of the response due to the difference between the matrices (Molinelli et al., 2008; Anfossi et al., 2010).

Cereals sample analysis

As previously described, the flow rate observed with sample extracts and with aqueous standards of aflatoxins was quite different (Anfossi et al., 2010) and results strongly depended on the type of sample analyzed, as also emphasized by Molinelli et al. (2009). These phenomena were only partially reduced by blocking membranes after line deposition by means of a buffer which contains BSA (Molinelli et al., 2008). A significant improvement was achieved by adding a cellulose sample pad to the bottom of the strip, which contributes to level sample extracts through a filtering action, and by adding polyethylene glycol to the buffer used for preparing aflatoxin standards and for extracting samples (Anfossi et al., 2010). Indeed, PEG determines the viscosity of solutions, which allowed us to level results obtained from different matrices and from sample extracts and aqueous standards. In addition, the flow slows down so that line intensities increase and the amount of AFB1–BSA conjugate in the test line and gold-labelled antibody could be diminished, which improved the sensitivity of the assay. However, both the use of a sample pad and the addition of PEG increase the total time needed to complete the assay. The PEG amount added was thus optimized by considering completion of colour development within 10 min as being acceptable and was established at 2% (w/v).

Despite these attempts to match results obtained for aflatoxin standards and for cereal samples, which allowed us to level samples amongst these, some matrix effects remained uncompensated for (Molinelli et al., 2008). Figure 3 highlights that calibration curves obtained by measuring standard solutions and extracts from fortified samples diverge. In particular, highly-contaminated samples interpolated on the curve, obtained by using diluted standards, would be dramatically over-estimated. Therefore, calibration was carried out by fortifying a certified blank sample of maize at various aflatoxin levels and by submitting each sub-sample to extraction. The described conditions and by using a 1:10 sample to buffer ratio for aflatoxin extraction, compensated for most matrix interference and variability.

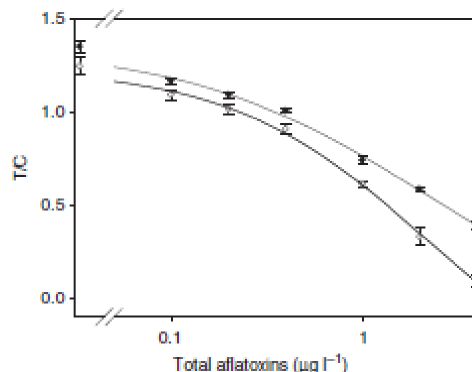

Figure 3. Matrix interference: inhibition curves obtained with aflatoxin standard solutions diluted in PB with 2% (w/v) PEG added (•) and extracts from fortified maize samples (O).

Quantitative measurements and optimization of a stored calibration curve

A developed strip shows two coloured lines: the test and the control line. The first, due to the binding of gold-labelled anti-AFB1 antibodies to the immobilized AFB1, is the analytical signal which varies as a function of the analyte concentration. However, there is certain variability from strip to strip mainly due to the amount of the gold–antibody conjugate retained by the release pad. Standardization of the amount of gold-labelled antibodies could be obtained by mixing a measured volume of gold conjugate with the sample before carrying out the test (Molinelli et al., 2008). However, one of the objectives of this work was to minimize handling; therefore gold-labelled antibodies were pre-adsorbed, in order to provide a fully ready-to-use device. Strip-to-strip variation due to the variability of the amount of gold conjugate dispensed and of any other phenomena affecting the assay (bad flow, different temperatures, etc.) was overcome by the normalization of data, which uses the C line intensity as the normalizing factor (Anfossi et al., 2010). Indeed, using a polyclonal antiserum implies that the intensity of the control line mainly depends on non-target antibodies and it is minimally involved by changes in concentrations of the target. Therefore, this intensity can be used as a corrective factor aimed at reducing strip-to-strip variations and for compensating for any differences in experimental conditions should occur. Results were hence expressed as the test line intensity (T) divided by the control line intensity (C). Accordingly, the calibration curve was obtained by plotting the measured T/C ratio versus aflatoxin concentration.

Table 2. Intra- and inter-day precision for three maize samples naturally contaminated at three levels. Quantification of total aflatoxins was obtained on different days by the same stored calibration curve.

AF theoretical concentration ($\mu g\,kg^{-1}$)	Intra-day			Inter-day		
	Average measured concentration	RSD ($n=5$)	Accuracy %	Average measured concentration	RSD ($n=5$)	Accuracy %
2.7	1.9	22	70	3.5	22	130
5.4	6.4	10	118	5.0	19	93
17.1	16.7	12	98	17.1	11	90

Antibodies used in the present work were raised against aflatoxin B1; nevertheless, the developing lateral flow immunoassay was intended to measure total aflatoxin contamination, namely the sum of AFB1 + AFB2 + AFG1 + AFG2, as statutory (European Commission 2003; European Union 2010; US FDA 2010; CFIA 2010). Data about cross-reactivity supplied by the antibody supplier (relative cross-reactivity towards AFB2 12%, AFG1 27%, and AFG2 2%) demonstrates that the antibody is substantially selective towards AFB1, though it is able to recognize AFG1 in some extent and partially recognizes AFB2.

Therefore, we use a certified standard solution of total aflatoxins as the calibrator, where the four aflatoxins are mixed together. The proportion of the four aflatoxins in the calibrator (B1:G1:B2:G2, 2:2:0.5:0.5) reflects natural occurrence of these contaminants (Resnik et al., 1996). In this way, the calibration curve itself takes into account the response of the antibody to the presence of the different aflatoxins.

The developed competitive lateral flow immunoassay has a dynamic range of 0.2–4 $\mu g\,l^{-1}$, with an IC_{50} of 2.4 $\mu g\,l^{-1}$. The LOD, calculated as the concentration corresponding to the T/C of the blank minus three standard deviations of the blank (Findlay et al., 2000), was 0.10 $\mu g\,l^{-1}$. Considering dilution due to extraction, the dynamic range of the developed LFIA becomes 2–40 $\mu g\,kg^{-1}$, which allows direct measurement of samples at all levels prescribed by EU legislation European Union, 2010; European Commission 2003).

Since lateral flow immunoassays are intended to be point-of-use tests, applicable outside the laboratory for very rapid screenings, the ultimate goal should be supplying a system to read strips and obtain quantitative results without any additional operations. Some fully automated devices aimed at this have been patented and are commercially available. In the current work, a mobile scanner connected to a laptop was used to read strips, coupled with dedicated software (Skannex 3.0, Skannex 2010), which aims to acquire the image and process it (i.e. to find lines on a specified window and determine their intensity). The same software interpolates the measured intensity by means of a stored calibration curve, returning the concentration of aflatoxins in the sample as the ultimate output. To confirm that the stored calibration curve could be used to interpolate data obtained in different days and possibly in different experimental conditions (in particular at different temperatures), intra-day and inter-day precision and accuracy were measured using naturally contaminated maize samples at three levels of contamination: low (2.7 $\mu g\,kg^{-1}$), medium (5.4 $\mu g\,kg^{-1}$), and high (17.1 $\mu g\,kg^{-1}$) as controls. Each of them was quantified in five replicates in the same day (intra-day) and on five different days for the inter-day experiment. Results are summarized in Table 2. The measured accuracy and precision prove that results are sufficiently reproducible to allow the quantitative measurement of aflatoxins in maize samples through the developed LFIA. In detail, the intra and inter-day precision vary in the range 10–22%; relatively high RSD (above 20%) were observed at low contamination levels; however they were considered acceptable for a screening method of analysis (Findlay et al., 2000; Wiswanathan et al., 2007). On the other hand, significant variations in strips response from batch to batch were observed. Each batch was formed using different membrane cards and pads, but the same gold-labelled antibody and AFB1–BSA preparation. Different batches needed different calibration curves; however, the use of a proper calibration curve allowed us to normalise differences. Within the batch, the same calibration curve was used to calculate aflatoxin concentrations in samples.

Comparison of LFIA with aqueous extraction and LC–FLD determination

A cross-validation of the whole developed method (aqueous extraction and lateral flow immunoassay) with the AOAC LC–FLD method (AOAC 2008) has been carried out. The reference method has a limit of detection of 0.05 $\mu g\,kg^{-1}$ for each of the four aflatoxins and a mean RSD% of 25%. The concentration of total aflatoxins was measured in 25 naturally contaminated

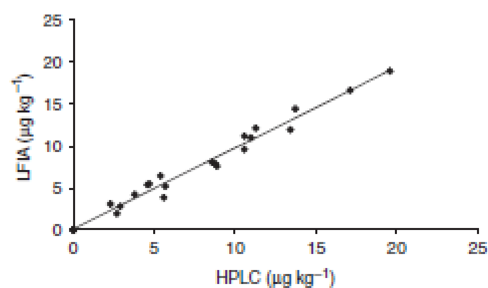

Figure 4. Correlation of results obtained by both LFIA and reference LC–FLD method for the aflatoxin detection on raw corn grain, corn feed, maize starch and cornmeal. The linear regression analysis yielded a good correlation between methods ($y = 0.97x + 0.07$, $r^2 = 0.980$, $n = 25$).

samples, which included raw corn grain, corn feed, maize starch and cornmeal. Results were compared with those obtained using the LC–FLD method and agreeing results were obtained via the two methods: the linear regression analysis (Figure 4) yielded a good correlation between the methods ($y = 0.97x + 0.07$, $r^2 = 0.980$, $n = 25$). These results proved that the developed lateral flow immunoassay can be applied for the quantitative measurement of total aflatoxins (AFB1 + AFB2 + AFG1 + AFG2) in maize samples at levels of regulatory relevance, with accuracy comparable with that obtained through the reference method.

In addition, even though it was beyond the main objective of the work, this is the first time that the possibility of extracting aflatoxins from maize using an aqueous buffer has been demonstrated.

In conclusion, the newly developed LFIA allows the rapid and accurate determination of the target analytes after a simple extraction. Minimal operations (dilution, addition or reagents, incubations) are needed. Strips were read by means of a scanner connected to a PC and the assay could be carried out without temperature control (in the range between 22 and 30°C). In addition, the extraction does not involve the use of organic solvents or other hazardous chemicals, thus further simplifying the feasibility of the assay outside the laboratory. Therefore, the developed quantitative LFIA method proved to be applicable as a point-of-use test for first level screening of aflatoxins in various maize products.

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