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A LATERAL FLOW IMMUNOASSAY FOR MEASURING OCHRATOXIN A: DEVELOPMENT OF A SINGLE SYSTEM FOR MAIZE, WHEAT AND DURUM WHEAT

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Abstract

A quantitative lateral flow immunoassay for the detection of ochratoxin A (OTA) in cereals has been developed. The ready-to-use device includes a nitrocellulose membrane on which capturing reagents are immobilized in spatially confined zones and a conjugate pad on which the antibody labelled with gold nanoparticles is pre-adsorbed. Stabilization of gold labelled antibodies and blocking of nonspecific interactions of the nitrocellulose membrane were obtained by using 1% ovalbumin. This, combined with the use of PEG in the extraction of OTA from cereal samples, allows us to level the matrix effects caused by various cereals (maize, wheat and durum wheat). The immunochromatographic assay developed includes a rapid and very simple treatment of samples, which, in addition, does not involve the use of organic solvents and allows the quantitative determination of OTA with limits of detection as low as 1.5 μg kg^{-1}. The coefficients of inter–assay variations are below 20%, with recoveries which ranged from 87 to 119%. Fifteen maize samples, four wheat and six durum wheat samples were extracted with the aqueous medium and analysed by the developed assay. A good correlation was observed (($y = 0.99\ x + 0.18$, $r^2 = 0.982$, n=25) when data was compared with that obtained through a reference method.

Keywords: Lateral flow, maize, wheat, ochratoxin A, quantification

Introduction

Penicillium verrucosum, *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* produce a secondary metabolite, named "ochratoxin A" (OTA) with such nephrotoxic properties as to be classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC, 2002). OTA contamination could occur in various crops: cereals, coffee, grapes, and spices, not only as basic products but also in their derivates due to its chemical stability to heat treatments and hydrolysis during food processing (Bullerman et al., 2007). Therefore several countries have set guidelines and recommendations for maximum residue levels (MRL) acceptable in crops for this compound; in particular, maximum limits for OTA in cereals have been proposed by the World Health Organization (5 µg kg^{-1}), China (10 µg kg^{-1}) (Lai et al., 2009), and the European Union (5.0 µg kg^{-1} for raw cereals 3.0 µg kg^{-1} for cereal derivates)(Reg. CE 1881/2006).

Official methods for detecting OTA involve extraction with organic solvents (typically aqueous methanol or acetonitrile), a clean-up and pre-concentration step by means of solid-phase extraction or immunoaffinity extraction, and determination by reverse-phase HPLC with fluorescence detection (MacDonald et al., 1999) . Alternative methods have been proposed as capillary electrophoresis with laser-induced fluorescence (Corneli et al., 1998) or GC/MS methods (Monaci et al., 2004); however instrumental techniques are expensive, time-consuming and require skilled personnel. Alternatively, immunochemical assays have been developed for a rapid detection of toxins in foods during routine analysis, becoming more and more reliable and sensitive. The enzyme-linked immunosorbent assay (ELISA) and the fluorescence polarization immunoassay (FPIA) are widely used in this field as screening method thanks to their rapidity and simplicity, but their costs, and the errors caused by overestimation or false positives or negatives have slowed their diffusion on a large scale (Krska et al., 2005).

Another assay based on the reaction between an antibody and its antigen is the immunochromatographic (ICG) assay on a nitrocellulose membrane, also called "lateral-flow immunoassay" (LFIA), widely employed for clinical controls and, more recently, for food analysis.

The increase in sales of test kits based on this technique is due to several advantages such as easy storage, user-friendly format and easy result reading. Also, the expense of staff training and analysis times are highly reduced compared to other screening techniques.

Until now, a quantitative rapid LF test for OTA detection in foods has not existedWang et al. (2007) made membranes which were able to detect OTA in cereals, beer and coffee with a visual detection limit of 1 µg kg^{-1}, but sample extraction required the use of methanol or chloroform, an ultrasonic system and a cooled centrifuge, with a total extraction time of over 30 minutes. Shim et al. (2009) prepared strips for the multi-analysis of OTA and Zearalenone with a visual cut-off of 5 µg kg^{-1} of

OTA in spiked corn samples, after the use of methanol as an extracting solvent, and centrifugation. The use of hazardous chemicals and laboratory equipment strongly frustrates the main advantages of the immunochromatographic assay's being point-of-use test, i.e. applicable outside the laboratory and virtually everywhere.

The aim of the present work was to develop a sensitive lateral flow assay for the quantification of OTA in cereals of major economical relevance (maize and wheat) which required limited sample manipulation, short analysis times and avoid using organic solvent as the extracting medium to allow the real application of the assay outside the laboratory. The developed LFIA is based on a competitive format: the analyte competes with an analogue coated onto the membrane, in a zone named "Test line" (T-line) for the binding sites of an antibody labelled with gold nanoparticles; the more the analyte there is, the lower the binding of the labelled antibodies to the T-line. The binding of labelled antibodies to the T-line is attested by the appearance of a red colour, due to the focalisation of the gold nanoparticles. Therefore, the development of a red colour in the T-line indicates the absence of the analyte and the fading of the T-line colour means that the sample does contain the analyte. Two of the key steps in the preparation of immunochromatographic devices to be applied in food control are the stabilization of gold nanoparticles conjugated to the antibodies and the pre-absorption of various additives in the membrane and/or in the sample, adsorbent and conjugate pads aimed at eliminating or reducing or managing matrix effects. In fact, a crucial aspect in the monitoring of food contaminants such as ochratoxin A is that a wide variety of raw and treated matrices should be analysed and it should be advisable to develop devices that can run on classes of food and not dedicated devices for very specific applications. Several authors reported the use of bovine serum albumin (BSA) both for the stabilization of gold colloids conjugated to antibodies (Molinelli et al., 2009; Shim et al., 2009; Xu et al., 2010;) and as a helpful additive to avoid food matrix interference (Molinelli et al., 2008, Molinelli et al., 2009, Anfossi et al., 2010, 2011, Tang et al., 2009) Unfortunately, BSA shows a remarkable binding of ochratoxin A with binding constants estimable at levels of 10^5-10^6 M^{-1} (Chu, 1971). The use of such a protein in the immunochromatographic assay would dramatically affect detectability, because of the competition for the binding of ochratoxin A between the antibody and the protein, the latter being in greater amount compared to the first. Therefore, a further object of this work has been the evaluation of alternatives (other proteins, surfactants, polymers, organic cations) as agents for the stabilization of the gold nanoparticles in the labelled antibody solution and as a surface modifier of the nitrocellulose membrane aimed at avoiding interference from food matrices.

Experimental

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Materials

Gold (III) chloride trihydrate (ACS reagent), ochratoxin A (Oekanal standard solution), bovine serum albumin (BSA), ovalbumin (OVA), thyroglobulin from bovine thyroid, pork skin gelatine, hexadecyltrimethylammonium chloride (CTAB), polyethylene glycol (PEG, average mw 10 kDa), and barbital buffer were purchase from Sigma-Aldrich (St. Louis, MO, USA). 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, 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).

The anti-OTA antibody (the immunoglobulin fraction of a polyclonal rabbit antiserum) and the goat anti-rabbit antibody were kindly provided by Generon srl (Modena, Italy).

Preparation of gold nanoparticles (AuNP) and gold-labelled antibodies (AuNP-Ab)

Gold colloids with an adsorption maximum of 525 nm were prepared using the sodium citrate method as previously described (Anfossi et al., 2010). An excess of the polyclonal antibody (pAb) towards ochratoxin A was used for the conjugation to gold nanoparticles. Briefly, 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' incubation at room temperature, 1 ml of borate buffer containing 1% of BSA was added and incubated for 10 min at 37°C. The mixture was then centrifuged at 10000 rpm for 30 min, and the pellet was washed twice by re-suspension in borate buffer which contains 0.1% OVA. Finally, the pellet was re-suspended in BB, with 1% OVA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide as a preservative and stored at 4°C until use. The OD of the obtained labelled antibody was measured by diluting it in water (1:10) and the absorption maximum shift was checked and was considered acceptable if lower than 10 nm.

Preparation of the test strips

Nitrocellulose membranes (NC 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 OTA-BSA conjugate, used as a capture reagent, was dispensed at 0.3 mg/ml, the goat anti-rabbit IgG antibodies were distributed at 2 mg/ml, both diluted in PBS and applied onto the membranes at

1 μl/cm. After drying at 37°C for 60 min, the membranes were blocked with PBS containing 1% (w/v) OVA at room temperature for 5 min and washed twice with PBS containing 0.1% 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) OVA, 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 dispensed at 5 μl/cm on pads and these 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 of overlap.

The prepared membrane was cut into strips of 5 mm, 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. Cassettes were stored in plastic bags containing silica at room temperature until use.

The stability of strips stored with silica gel at room temperature was assessed by measuring fortified and naturally contaminated samples after 1, 2 and 6 months.

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 analysed without fortification.

Ground samples (5 g) were weighed and extracted with 50 ml of PBS containing 2% PEG (w/v) by manual shaking for 2 min. After 10 min of decantation, the clear supernatant was used in the lateral-flow assay.

For the construction of the standard curve appropriate amounts of the reference solution of ochratoxin A were diluted in the cleared extract of a blank maize sample (certified material).

Lateral Flow ImmunoAssay procedure

The test was carried out by adding 150 μl of extract into the sample well. After 8 minutes 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 and the control line (T/C) against the log of ochratoxin concentration. Linearization of the calibration curve was carried out by the logit-log transformation, by plotting the logit of the ratio between the T/C at each analyte concentration level and the T/C in the absence of analyte against the log of analyte concentration. The best data fit was obtained by linear regression of the standard points. The unknown ochratoxin concentration in cereal samples was 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 reference method (Zinedine et al., 2006), with slight modifications. Ground samples (25g) were extracted using 100 ml of water/acetonitrile 60/40. The extract was filtered through Whatman filter paper n.4 and diluted at 1:12 with phosphate buffered saline. 48 ml of the diluted solution was passed through an Ochraprep™ column (R-Biopharm AG, Darmstadt, Germany). After column washing, ochratoxin A was eluted by applying 1.5 ml of methanol. The eluate was diluted with 1.5 ml of water and injected into a HPLC (LaChrom Elite, VWR International, Milan, Italy) equipped with a fluorescence detector (λ_{ex}=333 nm, λ_{em}=460 nm). The analytical column was an Alltima C18 (250 x 3.2, particle size 5 μm, Alltech, Grace, IL, USA); the mobile phase consisted of water/acetonitrile (50/50) acidified by 2% acetic acid and was fluxed at a flow rate of 0.5 ml/min. Quantification was obtained by interpolation on an external calibration curve. The limit of detection of the HPLC-FLD method is 0.01 μg/kg.

Results and Discussion

Gold nanoparticle-labelled antibodies

Colloidal gold has been widely employed as a label for the preparation of immunochromatographic devices. Despite some limitations (i.e. in the development of multi-analyte detection), it is far preferable to coloured latex particles for its simplicity of binding with antibodies and for the small and easily tunable dimension of nanoparticles. However, gold colloids are unstable and must be stabilized by modifying the charge state of the gold nanoparticle surface with proteins to prevent aggregation. Coating with a sufficient number of antibodies should allow the stabilization of the gold colloid, nevertheless most authors suggest the further addition of an excess of some proteins. In most cases BSA is effectively used for this purpose, however BSA strongly interacts with OTA (Chu, 1971), thus an excess of such a protein would compete with the antibody for the binding of

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the toxin. However, stable colloidal gold-antibody conjugates could not be prepared if no protein were added during washing steps. Similarly, gold-antibody conjugates which were re-suspended without any added proteins did not flow through the nitrocellulose membrane. Therefore, other proteins such as ovalbumin, bovine thyroglobulin and casein (Table 1) were tested as stabilizing agents to prevent aggregation of AuNPs . All the considered proteins were effective in controlling AuNP aggregation during the preparation of the gold-antibody conjugate. However, their behaviour differed during the immunochromatographic assay. In particular, despite that the use of casein has been previously reported (Xu et al., 2010), this protein strongly interferes with the binding of the gold labelled antibody with the T-line, resulting in signals indistinguishable from the membrane background for all the considered amounts of casein. Thyroglobulin (bTG)and ovalbumin (OVA) behave very similarly, with a certain degree of precipitation of the gold-antibody conjugate onto the NC membrane, in correspondence with the start of the run. However, the overall flow, signal intensities of the two lines and the reproducibility of results could be considered acceptable. Therefore, a 1% (w/v) OVA added to the borate buffer was established as the modifier of the gold nanoparticle surface after antibody conjugation and to pre-treat the glass fibre pad on which it was deposited, to satisfy economic criteria.

Blocking of NC membranes

Nitrocellulose membranes are the most commonly employed support for lateral flow immunoassays and usually only the porosity of the nitrocellulose is varied to improve assay performances. Based on the previous experience, (Anfossi et al., 2010, 2011), the HiFlow180 membrane was chosen as the best compromise between thinner pore size, which corresponds to the high sensitivity of the assay, and an acceptable flow rate, which means an acceptable time required to complete the experiment.

The optimal amount of the OTA-BSA conjugate to be dispensed in the T-line and the optimal gold-labelled antibody amount were optimized in checkerboard titrations, using Ochratoxin A diluted in the extraction buffer as the reference analyte. An OTA-BSA solution at 0.3 mg ml^{-1} as the coating antigen for the T-line and a gold-labelled antibody solution showing an OD of 4 were selected as the most suitable on the basis of the IC$_{50}$ value that was the lowest. The amount of goat anti-rabbit IgG antibodies to be dispensed on the C-line was established to have a T to C intensity ratio between 1 and 1.5 when the analyte was absent.

As previously described by several authors (Molinelli et al., 2008; Molinelli et al., 2009), a large flow variability is observed when extracts from cereals are applied to the strips. In particular, the difference depends not only on the sample types (corn, wheat, barley,...) but also on the treatment undergone by the sample (raw or processed material) and even only on the grain size of the sample.

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Variations of the capillary flow determine different intensities of the lines and, therefore, the incorrect assignment of the contamination value for the sample. Strategies for the management of matrix effects on strip performance in the analysis of cereals have been reported (Molinelli et al., 2009; Anfossi et al., 2010, Anfossi et al., 2011) and are mainly focused on the modification of the surface properties of the nitrocellulose membrane typically by adsorption of proteins or polymers. Previous works which reported lateral flow strips for the detection of mycotoxin in cereal samples used blocking solution that contained BSA, sometimes followed by washing steps with buffers containing Tween 20 (Wang et al. 2007, Anfossi et al., 2010, Tang et al., 2009). Nevertheless, as discussed above, the presence of large amounts of BSA should be avoided, therefore, other additives aimed at modifying the nitrocellulose were tested: casein, pork skin gelatine, OVA, PEG, CTAB. All additives were dissolved in a phosphate buffered solution (PB). In addition, the PB alone, sodium barbital and TRIS buffers were tested (Table 2). Gelatine inhibited all bindings of the antibody and casein discoloured the T line, as described above; the addition of PEG, CTAB and sodium barbital to the blocking buffer did not lead to positive effects on signal development. TRIS slowed the flow rate thus intensifying the signals, however was ineffective in reducing the difference between samples of different kinds. Best results in terms of capillary flow and reduction of matrix interference were obtained using PB containing OVA at 1% as a modifier of the nitrocellulose membrane. The washing with Tween 20 and Triton X-100 of the membrane to remove the excess of the protein and to influence the capillary flow rate were also considered (Table 2).. High concentrations of surfactants interfered with the formation of immunocomplexes on the membrane. In addition, Triton worsened the uniformity of the results, caused the precipitation of gold nanoparticles when these come into contact with the membrane,and made the flow so fast that it partially prevented antibody binding to the T-line. The best washing solution, which cleans the membrane pores efficiently and maximizes line intensities, was established as being PB containing Tween 20 (0.05% v/v).

Extraction of OTA from maize and wheat

The extraction method represents a critical phase for the assay user-friendliness.. Aqueous organic solvents are often used to extract mycotoxins from food and feed samples, despite the interference, due to organic solvents, in the antigen-antibody binding. Besides, methanol, ethanol or acetonitrile solutions can cause undesirable co-extraction of substances which may interfere with the analysis, often causing false positives (Krska et al., 2009; Shim et al., 2009; Burmistrova et al., 2009). Kulisek et al. (2000) showed that a phosphate buffer was suitable to extract fumonisin from cereals. More recently, the efficiency of an aqueous extraction of aflatoxin B1, B2, G1, G2 from cheese and from maize respectively have been demonstrated (Anfossi et al., , 2011] The same buffered solution

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(phosphate buffer 20 mM pH 7.4) was tested for the extraction of OTA from cereals, considering that OTA solubility in water is just below 1 mg l^{-1} (Mortensen et al., 2006) and that it has to be measured at contamination levels which lie in the μg l^{-1} range. In the meantime, additives aimed at increasing the viscosity of sample extracts to slow down capillary flow and to minimize flow rate variations associated to different cereals(Anfossi et al., 2011) were tested: pork skin gelatine (1%, w/v), dextran (2%, w/v), arabic gum (1-1.5%, w/v) and PEG (2%, w/v). In the first two cases signal intensities decreased, in the third, a rise in the absolute signals was observed however flow rate diminished excessively. The addition of PEG to the extraction buffer produced intensively coloured lines and made flow rate similar for several different kinds of samples (raw maize, maize meal, wheat, durum wheat). Besides, various dilution factors and decantation times were attempted to level results showed by maize and wheat samples;the following conditions were established for the extraction of both maize and wheat samples (raw material and flour): PB containing 2% (w/v) PEG as the extraction medium, a 1:10 ratio between the solid sample and the extracting solution, a decantation time of 10 minutes. In these conditions, extracts could be directly analysed. Calibration curves carried out by fortifying a blank maize sample and a blank wheat sample were overlapping within the experimental error (Figure 1). Despite the modification of the membrane surface and the addition of PEG allowed the equalization of the capillary flow of different cereal extracts, line intensities observed for buffered standard solution of OTA remained significantly different respect to those observed for sample extracts. This behaviour has been previously observed (Molinelli et al., 2008) and is due to the substantial difference in viscosity between aqueous extract of cereals and buffered solutions. The use of a matrix calibration, in such conditions as to equalise matrices among them, partially solves this drawback (Anfossi et al., 2011). Nevertheless, it remains a limitation of the applicability of the developed assay to food matrices strongly differing from those considered.

Cereal samples analysis

Quantification of OTA in cereals was obtained by means of an appropriate calibration curve, which could be stored and used to measure OTA contamination in different days and under varying experimental conditions (temperature, operator, and laboratory). Calibrators were prepared by fortifying the solution belonging to the extraction of a blank maize sample at various levels. The calibration curve was obtained by plotting the ratio T/C to compensate for the variability between different strips (Anfossiet al., 2010) against OTA concentration. Linearization of the calibration curve was performed by the logit-log transformation (Findlay et al., 2000) as requested by the software of the reader.

The limit of detection of the method, estimated as the signal (T/C) of a blank sample minus three standard deviations of the blank, was 0.15 µg kg^{-1}, with an IC$_{50}$ of 3 µg kg^{-1}, which correspond to 1.5 µg kg^{-1} and 30 µg kg^{-1} in the sample, respectively, due to dilution during extraction.

Reproducibility was evaluated by testing a blank and three naturally contaminated samples (5.3 – 12.8 – 23 µg kg^{-1}) on six different days: coefficients of variation were in all cases lower than 20% (Table 3).

Finally, naturally contaminated samples (15 samples of maize, 4 of wheat and 6 of durum wheat) were analysed by the newly developed LFIA and by a reference HPLC-FLD method. The comparison between results obtained via the two methods showed a good correlation (Figure 2), which confirms that the developed LFIA method can be proposed as a screening method for the quantitative determination of OTA in maize and wheat samples. Ochratoxin A was detected in 15 of the 25 tested samples. Among these, 3 samples resulted in OTA levels below the EU MRL, 5 near the EU MRL, and 7 were contaminated at levels above the EU MRL. In particular, two maize samples and one durum wheat sample were highly contaminated (OTA > 10 µg kg^{-1}).

Conclusion

Existing LFIAs for measuring ochratoxin A have been used as qualitative or semi-quantitative assays and samples were declared positive or negative on the basis of a cut-off level. In this paper a LFIA able to quantify the presence of OTA in maize and wheat samples has been developed and the optimization of the preparation of the strips aimed at substituting BSA (which is widely employed as a stabilizing agent in the preparation of gold-labelled antibodies and as a modifier of the nitrocellulose membrane) has been described. The use of appropriate software and the reproducibility of data allow for the quantification of OTA by means of the developed LFIA dipstick without the use of expensive equipment or skilled personnel, and require little sample manipulation. The total time for processing a sample (extraction and analysis) is twenty minutes. In addition, organic solvents were not used, thus respecting economic and environmental criteria. The method is applicable to different matrices (maize and wheat) and allows for the measurement of OTA at contamination levels complying with international regulations.

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FIGURE CAPTIONS

Figure 1: Examples of standard calibration curves for ochratoxin A. Calibrators were obtained by diluting a standard OTA solution in blank sample extracts from maize ($\bigcirc$), and wheat ($\triangle$).

Figure 2: Correlation of results obtained by both LFIA and reference LC-FLD method for the ochratoxin A detection on maize and wheat samples. The linear regression analysis yielded a good correlation between methods ($y = 0.99\,x + 0.18$, $r^2 = 0.982$, n=25)

TABLES

Table 1 Effectiveness of different proteins in the stabilization of gold nanoparticles conjugated to the antibody (AuNP-Ab).

	OVA		bTG		Casein	
	0.1%	1%	0.1%	1%	0.1%	1%
T and C line intensity	++	+++	++	+++	+/-	-
AuNP-Ab	Ppt on the NC membrane		Ppt on the NC membrane		Flow over (till the adsorbent pad)	

Table 2 Various investigated parameters aimed at inhibiting non-specific interaction of AuNp-Ab with the NC membrane and at levelling results obtained with different cereals samples (wheat, durum wheat, and maize)

NC membrane blocking solution							
Buffer	PB (pH 7.4)					Tris (pH 8.5)	Barbital (pH 8.5)
Conc (mM)	20					50-200	50
Additive	/	OVA	Casein	Gelatin	PEG	CTAB	/	/
Conc (w/v)		0.1-1%	0.1-1%	0.1%	0.1%	0.02%		
NC membrane washing solution							
Surfactant	Tween 20			Triton X-100			
Conc (v/v)	0.05%	0.1%	0.5%	0.02%	0.05%		
Extracting solution							
Thickener	Gelatin	Dextran	Arabic gum		PEG		
Conc (w/v)	1%	2%	1%	1.5%	2%		

Table 3 Inter-day precision and accuracy of the developed LFIA for three maize samples naturally contaminated at low (5.3 μg kg^{-1}), medium (12.8 μg kg^{-1}) and high (23.0 μg kg^{-1}) levels. Quantification of ochratoxin A was obtained on six different days by the same stored calibration curve.

OTA concentration [a] (μg kg^{-1})	average measured concentration (μg kg^{-1})	% RSD (n=6)	% Accuracy
5.3	6.3	14	119
12.8	14.7	13	115
23.0	20.0	18	87

[a] as determined by the LC-FLD reference method

FIGURES

Figure 1

Figure 2