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Chapter 15

Lateral Flow Immunoassays for Aflatoxins B and G and for Aflatoxin M_1

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Additional information is available at the end of the chapter

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1. Introduction

Aflatoxins (AFs), secondary metabolites produced by *Aspergillus Flavus* and *Aspergillusparasiticus*, are a numerous group of chemically related compounds characterised by high toxicity. Among these, aflatoxin B_1 (AFB_1) is the most potent known carcinogen for liver and, together with aflatoxins B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) is the most frequently found and the most toxic of the group [1]. Therefore, maximum residue levels (MRLs) for AFB_1 and for the sum of the four AFB_1 + AFB_2 + AFG_1 + AFG_2 (total aflatoxins) in food and feed have been set by the European Union [2-4] and all over the world [5-7].

The occurrence of aflatoxins (AFs) has been widely reported in a variety of crops (including maize, wheat, barley, rice, groundnuts, nuts, pistachios, cottonseed, and spices) which can be infected pre-, during and post-harvest. Moreover, due to the relative stability of AFs to thermal and chemical stresses, they are found on commodities despite the elimination of mould, after long periods of storage, and also after the transformation of raw materials; therefore the presence of AFs has also been ascertained in commodities such as composite feed, flour, bakery products, and roasted peanuts.

In addition, products of the animal metabolism of aflatoxins could retain toxicity, such as in the case of AFB_1, which, once ingested, is rapidly absorbed and transformed into a hydroxylated metabolite. The latter is secreted into the milk and thus has been named aflatoxin M_1 (AFM_1). The hepatotoxicity and carcinogenic effects of AFM_1 have also been demonstrated and IARC have included this toxin in the group I human carcinogens as well as the parent AFB_1[1]. Milk and derived products can consequently also be implicated in the spreading of aflatoxins. Therefore, most countries have also set up MRLs of AFM_1 in milk, which varies

from the 50 ng kg^{-1} established by the EU to the 500 ng kg^{-1} established by US FDA [2, 8]. More restrictive MRLs have been decided by the EU for the presence of AFM_1 in baby food [2].

A part from safety issue, food contamination caused by AFs also strongly affects economic interests; so much effortis devoted to the development of analytical methods for detecting these contaminants. Newly developed methods of analysis are intended both for screening purposes (rapid, economic and simple methods) and for the accurate, reproducible and sensitive quantification by confirmatory methods.

Numerous chromatographic methods to detect AFs in foods have been developed, coupled to fluorescent or mass spectrometric detection [9-11]. Likewise, several methods for aflatoxin M_1 determination in milk based on high-performance liquid chromatography associated to fluorescence or mass spectrometric detection have been developed [12-13]. However, chromatographic techniques are mainly used in confirmatory analyses and are usually not applied to routine controls owing to the necessity to use expensive equipment and extensive clean-up steps.

The first rapid methods of analysis for AFs were based on Thin Layer Chromatography [14]; this technique is still used today even though in a significant lesser extent compared to methods based on the use of antibodies. Immunochemical methods of analysis are widely employed as screening methods for measuring AFs in food and feed [9, 14-18] and also for AFM_1 quantification in milk and dairy products [19-21] thanks to their rapidity, selectivity and sensitivity. Several ELISA kits are commercially available, whose performances are generally adequate to meet legal requirements, and are routinely employed for aflatoxin monitoring. Some of these methods have also been validated [17-18]. However, even immunoassays need to be run in a laboratory, use a minimum of equipment and occasionally require some sample treatments, which may also involve the use of hazardous chemicals. Instead, affordable monitoring of food contaminants requires the highest-through put and more economical methods of detection and, possibly, little or no sample treatment, user-friendliness, employment of non-hazardous chemicals, in situ applicability. Additional requisites in aflatoxin detection would be low detection limits (especially for aflatoxin M_1) and adaptability to very differing commodities (for aflatoxins B and G).

Several innovative strategies have been proposed for the rapid, qualitative, semi-quantitative or quantitative detection of aflatoxins, also based on the use of specific antibodies without constraints of classical immunoassays [22]. For example, an interesting qualitative approach has been described for the detection of AFM_1 in milk [23-24]. The proposed method is based on a flow-through immunoassay with visual detection. Main advantages are represented by the high sensitivity and by the on site applicability of the assay which does not require any equipment for the treatment of the sample, norfor the analysis. In addition, it allowed the possibility of obtaining sample pre-concentration and/or clean-up in the same device used for the analysis [25]. Nevertheless, this method implies several subsequent steps to be carried out, thus limiting simplicity and rapidness of use. Very recently, the same approach has also been demonstrated for the multi-detection of different mycotoxins, thus increasing its potentiality of utilization [26].

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Numerous immunosensors have been described [27] as well, and research is constantly evolving in this area, particularly for the development immunosensors for the selective determination of AFB_1 [28-32] and for AFM_1 detection [33-35].

In parallel, strategies aimed at avoiding the use of antibodies in the development of rapid methods for aflatoxin detection have also been reported, such as those based on the preparation of polymers with molecular recognition properties towards AFB_1as capture systems [36-37] or those based on the exploitation of its natural fluorescence for the detection [38]. A combination of the surface plasmon resonance phenomenon and fluorescence has been exploited in the work of Wang et al and permitteda very sensitive determination of AFM_1, though the proposed assay took almost an hour to be accomplished and couldn't be considered as a truly rapid method [39]. A fancy and cunning approach for the rapid quantification of AFB_1 have been described in the work of Arduini et al, who exploited the inhibiting effect of the toxin towards the enzyme acetylcholinesterase. The measurement of the enzymatic activity was demonstrated to directly allow AFB_1 quantification in 3 minutes and within the 10-60 $\mu g\ l^{-1}$ range [40].

Among the rapid methods for screening of food contaminants, the'lateral flow immunoassay" (LFIA) (also known as immunochromato graphic assayorimmuno-colloid gold immunoassay, ICG) has recently attracted the interest of researchers and industry. This technology has long been known in medical fields for diagnosing blood infections and failure of internal organs, disclosing drug abuse or ascertaining pregnancy and combines a series of benefits, including extreme simplicity, rapidity, and cost effectiveness [41]. These features make it ideally suited for screening large number of samples, for being conducted by non-trained personnel and practically everywhere, thus enabling the effective possibility of food safety assessment at all stages of food and feed production.

2. Lateral flow immunoassays for aflatoxins

Since the early 2000's, scientific papers and commercial devices aimed at measuring mycotoxinsin food and feed have appeared, and recentlya certain amount of literature on this topic has become available, including comprehensive reviews [42-44]. In particular, some LFIAs for the qualitative and semi-quantitative detection of aflatoxins in food and feed have been described and will be discussed below. At the same time, commercial LFDs for the detection of aflatoxinsin various commodities have become available and some of them have also been validated by USDA-GIPSA [45].

2.1. Principle of the method

As aflatoxins are low-molecular-mass compounds, immunoassays in competitive formats should be conceived to measure them. The same principles and reagents as in the microwell-type immunoassays could be applied, except for the fact that,in LFIA, the separation of bound and unbound antibody sites is obtained by means of the lateral flow on a suitable support (the membrane). The liquid flow transports immunoreagents along the membrane

where they encounter their partners in spatially confined zones of the membrane itself where immuno reactions take place.

Besides the porous membrane which assures the flow, lateral flow devices (LFDs) usually include: an absorbent pad (positioned at the top of the membrane to increase the volume of the flowing liquid), a sample pad (to assure contact between the liquid sample and the membrane), and a rigid backing (Figure 1). A release pad can be added, whose role is to adsorb labelled antibodies in such a way that they are included in the device itself.

The membrane is almost exclusively made of nitrocellulose (NC); sample and adsorbent pads are usually made of cellulose, although sample pads could also be made of glass fibre or other materials and sometimes are soaked with proteins and/or surfactants for special applications. Release pads are usually glass fibre pads. Lines are traced on the NC membrane by means of dedicated dispensers which enables the dispensing of small volumes (typically few μl per cm) with high reproducibility.

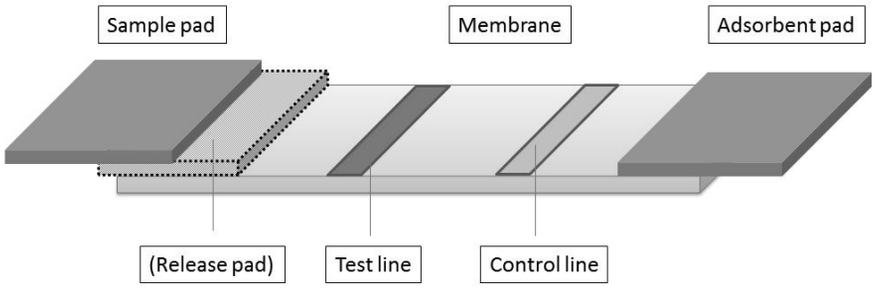

Figure 1. Schematic of a lateral flow device in the dipstick format.

The simplest LFD is a dipstick, which is dipped directly into the sample solution. Labelled antibodies can be added to the sample as a concentrated suspension or provided in a lyophilized form to be re-suspended by the sample itself. Alternatively, the labelled antibody can be pre-adsorbed onto the releasing pad, which partially overlaps the membrane. The liquid sample itself causes the re-suspension of the pre-adsorbed labelled antibodies during the assay. The sample pad is added in such a way that it overlaps the membrane or the releasing pad. Its role is the reduction of matrix interference by filtration alone or combined with some chemical action by means soaked reagents.

Besides the most popular dipstick format, LFDs exist in which the strip is inserted into a rigid plastic cassette provided with a sample well and a reading window. The main advantage of these housings is the guarantee of a reproducible compression of all components in the overlapping zones, which assures faster and more reproducible flows.

With few exceptions, the indirect competitive format, in which the antigen (a protein conjugate of the target toxin) is coated on the membrane and the antibody is labelled, is strongly preferred in the development of LFIA for AFs. Antibody labelling can be obtain by using virtually whatever nanoparticles that have a spectroscopically detectable property, such as,

for example, coloured or fluorescent nanoparticles. However, gold nanoparticles (GNPs) are generally employed, with few exceptions, because of some characteristics which make them particularly suitable for the purpose. First, the conjugation of antibodies with GNPs is very easily obtained by simply mixing the two components at a proper pH (at or above the pI of the antibodies). The preparation and characterisation of stable colloidal solution of GNPs also follows well-established, easy protocols and a wide literature is available on this topic. The surface plasmon resonance of GNPs determines an intense colour of colloidal gold, which varies from orange to pink depending on particle dimensions and on surface overlay, therefore coloured nanoparticles can be prepared and the colour nuance can be use to monitor preparation and conjugation to antibodies.

The principles of the indirect competitive LFIAs which exploit GNP-labelled antibodies have been widely described and are schematized in Figure 2 and 3.

Briefly, the labelled specific antibody is suspended in the liquid sample and flows through the membrane where it first encounters the antigen coated in a zone indicated as "Test line" (T-line). In the absence of the target compound (negative sample, Figure 2), labelled antibodies bind to the coated antigen and are focused on the T-line, so that a visible (detectable) line is formed.

Usually, a second so-called "Control line" (C-line) follows and is constituted by secondary anti-species antibodies which capture any excess of specific antibodies.

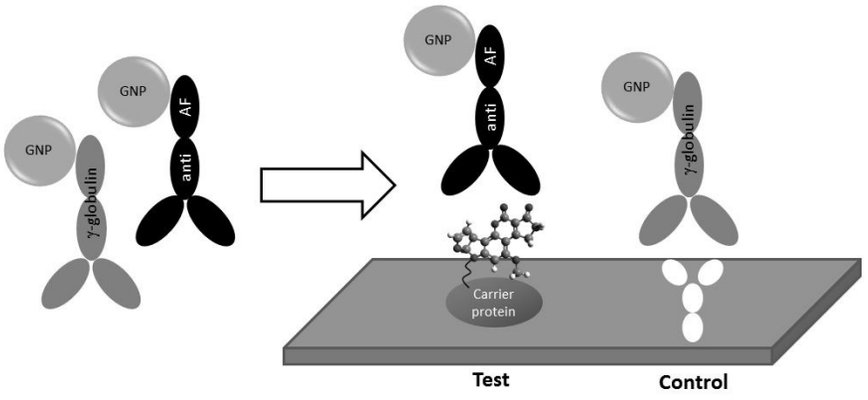

Figure 2. A lateral flow immunoassay in the indirect formatwith GNP-labelled antibodies for a negative sample (no AF is present). The Test line is made by a protein conjugate of the target toxin, while the Control line is constituted of anti-species antibodies. Anti-aflatoxin antibodies mixed together with non-specific γ-globulins (both GNP-labelled) move along the membrane. Anti-AF antibodies bind the antigen coated in the Test zone and are focused, thus forming a visible (detectable) line. Non-specific γ-globulins pass the Test line and are captured by the anti-species antibodies in the Control line where they are focused and form a second visible (detectable) line.

The appearance of a C-line can be regarded simply as the confirmation of the correct development of the assay (reagents and materials integrity) or else can be exploited to calculate the T/C signal ratio with the aim of normalizing strip-to-strip variations [46] or can also be regarded as an internal standard to which the intensity of the T-line is compared to determine positivity/negativity [47-48].

When the target is present above the lower detectable concentration level (positive sample, Figure 3), binding of labelled antibodies to the coated antigen is inhibited, resulting in a non-visible (undetectable) T-line.

Interpretation of assay results depends on the presence and intensity of both Test and Control lines as schematized in Figure 4.

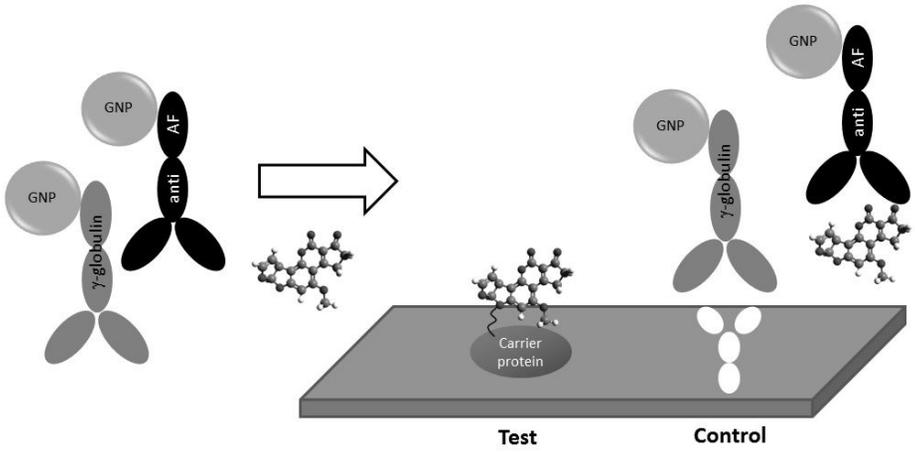

Figure 3. A lateral flow immunoassay in the indirect format for a positive sample (AF above the detectable limit). GNP-labelled anti-aflatoxin antibodies and non-specific γ-globulins move along the membrane. Anti-AF antibodies bind the toxin in the sample and the interaction with coated antigen is thus inhibited. Non-specific γ-globulins pass the T-line and are captured by the anti-species antibodies in the Control line where they are focused. Therefore, a single line (C-line) appears on the membrane.

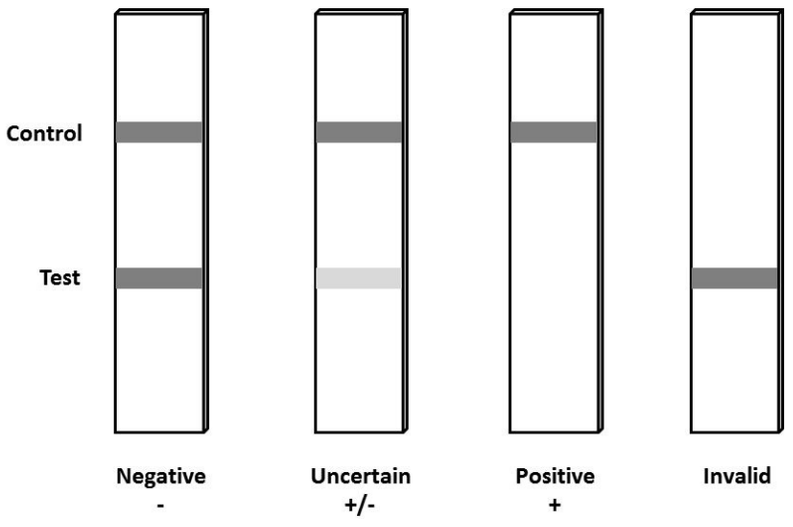

Figure 4. Assay result interpretation. Two intense lines: valid test, negative sample (target toxin below the detection limit of the method); intense C-line and fading T-line: valid test, the amount of the target toxin is near to the detection limit; intense C- line: test valid, positive sample (target toxin above the detection limit); intense or fading T-line: invalid test.

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2.2. LFIAs for aflatoxins B and G

The first LFIA aimed at measuring any one of aflatoxins appeared in the scientific literature ten years ago and was one of the first reported lateral flow assays for food contaminants. The authors described a simplified device formed by aNC membrane on which the T-line had been traced upon by dispensing antibodies towards AFB_1. The signal reporters were liposomes, which were tagged with AFB_1 and encapsulated a visible dye. The tagged liposomes flowed along the membrane where encountered the coated anti-AFB_1 antibodies and were captured, thus determining the appearance of a coloured T-line due to the focalization of the encapsulated dye. If some AFB_1 was present in the sample, the binding of the tagged liposomes to the coated antibodies was inhibited and the colour of the T-line faded. The absolute limit of detection of such a device was 18 ng of AFB_1 and the test could be completed in a total of 12 minutes, including sample preparation [49].

Apart from this early approach, following papers described more usual LFDs based on the use of GNPs as antibody labels. In 2005, Delmulle and co-workers reported the development of a dipstick which allowed authors to detect AFB_1in pig feed. The visual detection limit (VDL) was set at 5 $\mu g\ kg^{-1}$ and the analysis could be completed in 10 minutes [50]. In the same year, the group of Xiulianal so described the preparation of GNP-labelled antibodies towards AFB_1 and their exploitation in a visual LFIA [51]. The application of the developed dipstick to measure AFB_1 in rice, corn, and wheat was reported in a following paper of the same group [52]. The described LFD showed a VDL of 2.5 $\mu g\ l^{-1}$ in buffer, which became 0.05 $\mu g\ l^{-1}$ when the colour intensity of lines was determined by means of a photometric reader. Therefore, a sensitive quantification of the target toxin (limit of detection, LOD, 2 $\mu g\ kg^{-1}$ in food) could be demonstrated; moreover, accuracy of the developed assay was confirmed on 60 samples through comparison with ELISA.

A visual LFIA for detecting AFB_1 was also described by papers of Shim et al [53-54]. The developed LFD was shown to cross-react to some extent to other major aflatoxins (AFB_2, AFG_1, and AFG_2) but not to differing mycotoxins (such as ochratoxin A, citrinine, patulin, zearalenone, and T-2 toxin). Nevertheless, it was applied for selectively measuring the sole AFB_1in rice, barley and feed. VDLs of 5-10 $\mu g\ l^{-1}$(rice, barley) and 10-20 $\mu g\ l^{-1}$(feed) were obtained and the proposed method showed agreeing results towards HPLC analysis on up to 172 food and feed samples. The same group also published results obtained with a multi-analyte device aimed at contemporary measuring AFB_1 and ochratoxin A in feed. The described method allowed the simultaneous detection of the two toxins which could be completed in 15 minutes and showed a VDL of 10 $\mu g\ kg^{-1}$for AFB_1. Method validation by means of ELISA and HPLC confirmatory analyses was also reported [55].

Although regulations prescribe the simultaneous determination of AFB1, AFB_2, AFG_1, and AFG_2 beside AFB_1 quantification, most papers described LFIA selective towards AFB_1.To meet the need of measuring all the four major AFs our group developed a quantitative LFIA for total aflatoxin determination in corn samples. The assay could be completed in 10 minutes, showed a LOD of 10 $\mu g\ l^{-1}$ and was validated through comparison with HPLC on 25 samples. In addition, an aqueous extracting medium was also optimized and proven to allow reliable quantification of total aflatoxin [56]. Except in this case, AFs were always ex-

tracted in methanol/water (typically 70/30 or 80/20 v/v) followed by dilution of the extract before LFIA analysis to reduce the proportion of the organic solvent, which is hardly compatible with materials composing LFDs. However, a methanol amount lower than 15-20% has been demonstrated by most authors to be compatible with LFD materials and further more not to affect immunoassay performance.

Most recent contributes to the topic are due to the group of Zhang and co-workers, who described two LFDs, the first highly selective towards AFB_1 and the second able to measure total aflatoxins [57-58]. Both devices have been applied to visually detect target toxins in peanuts (the highly selective one could also be exploited to detect AFB_1 in pu-erh tea, vegetable oil and feed). Both methods allowed reliable results (agreeing with HPLC determination) to be obtain in 15 minutes. In addition, the LFIA aimed at measuring total AFs was extremely sensitive, with VDL in peanut extracts as low as 0.03, 0.06, 0.12, and 0.25 $\mu g\ l^{-1}$for AFB_1, AFB_2, AFG_1, and AFG_2, respectively.

In addition to papers aimed at describing actually functioning devices for measuring AFs, those targets have often been chosen as system models for the development of original devices which exploited non-traditional signal reporters to label antibodies. Besides the above mentioned approach of Ho and Wauchope, based on the use of dye-encapsulating liposomes, Liao and Li described a visual device which exploited nanoparticles with a silver core and a gold shell as the reporters in the construction of a LFD for AFB_1. The toxin was determined in cereals and nuts and performances were compared to those of a GNP-based LFIA and to results obtained through a classic microwell-based immunoassay. The authors demonstrated that the newly developed LFD was comparable to the GNP-LFD in terms of stability of components and reproducibility of signals. On the other hand, it allowed a great enhancement in sensitivity so that values as low as 0.1 $\mu g\ l^{-1}AFB_1$ could be measured [59].

With the expectation of increasing the useful signal, therefore being able to reduce immunore agents for the benefits of the competition, magnetic nanogold microspheres with a Fe_2O_3 core and a shell of multiple GNPs have also been proposed. The magnetic core of particles allowed authors to simplify separation steps during the labelling of antibodies and their micro- dimensions to enhance colour during the test itself. A three-fold increase in sensitivity was stated for the visual detection of AFB_2 compared to the use of simple gold colloid nanoparticles [60].

2.2.1. Application of LFIA for aflatoxins B and G in food analysis

A major concern in the development of LFDs for aflatoxins is the unpredictable effects due to food components co-extracted from the sample beyond the target and which affect not only the antigen-antibody interaction on which the immunoassay is based, but also the mechanics of the device itself.

Some authors experienced the apparently inexplicable failure of recovery experiments conducted on fortified materials and the incongruity of results attained for artificially and naturally contaminated samples, which necessitate matrix-matched calibrations and recommended the use of naturally contaminated samples blended in varying proportions

with blank samples as calibrators [56, 61-63]. Matrix components not only interfere in defining appropriate standards for calibration but also determine requirement of distinct devices to be developed for individual foods.

Despite the fact that the some authors reported calibration by using standard AFs diluted in buffers (to which methanol is added in limited proportions, as discussed above) and stated no interference from matrix given a limited dilution of sample extracts, the application of LFDs for the effective AF B and G detection in food remains the bottleneck in the development of new LFIAs. This taskis also made particularly complex by the multiplicity andvariety of matrices to be considered in aflatoxin B and G analysis.

2.3. LFIAs for aflatoxin M_1

The development of LFIAs for AFM_1 is one of the most challenging goals in this field of research 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 and did not described any preparation protocols and methods. The ROSA Charm Aflatoxin M_1™ aimed at quantitatively measuring AFM_1 in milk was validated as the result of an inter-laboratory trial, which involved 21 participants, at four levels above and two below the declared LOD of the assay (400 ng l^{-1}) [64]. Less than 5% of false negative (n=83) and no false positive below 300 ng l^{-1} were found. For contaminations between 350 and 450 ng l^{-1} false positivity increased from 21 to 93%.

More recently, Wang et al first described the development of a LFD for the detection of AFM_1 [65]. The cut-off level (0.5-1 μg l^{-1}) is just above the eligible value required by the US regulation [8] and far beyond the more severe limits imposed by the European Union for this contaminant [2]. However, it is an effectively sensitive and rapid assay, provided that the whole analytical procedure can be completed in 10 minutes, as no sample treatment is required.

A visual device has also been developed by Zhang et al which showed a VDL for $AFM_1$of 0.3 μg l^{-1} [66]. Although the sensitivity improvement respect to the work of Wang et al, the obtained VDL remains far away from the detectability demand imposed by EU MRLs for this contaminant.

3. Development of a highly sensitive LFIA for measuring AFM_1 in milk

With the aim of producingasystem sensitive enough to reach the limits imposed by European regulations, we developed a competitive lateral flow immunoassay which exploited rabbit polyclonal antibodies towards AFM_1that had been previously employed in the development of a sensitive ELISA [19]. A classic device, including a NC membrane (onto which the two lines of reagents had been immobilized), cellulose sample and adsorbent

pads, and a glass fibre release pad (on which GNP-labelled antibodies are pre-adsorbed) was conceived.

The method was designed to be a competitive LFIA, in which the Test line comprised an AFM_1 conjugate (competitor) and the Control line was composed of anti-rabbit IgG antibodies. GNP-labelled anti-AFM_1 antibodies were furnished as pre-adsorbed in a release pad. When re-suspended by the sample, flowed across the membrane where first encountered the T-line and bound to the immobilized AFM_1 conjugate. A red colour became visible at the T-line, due to the focusing of nanoparticles. If some AFM_1 was present in the sample, it competed with the immobilized AFM_1-BSA for binding to the GNP-labelled antibodies, resulting in a reduction of the T-line intensity. The anti-rabbit IgG antibodies on the Control line captured any excess GNP-labelled antibodies to produce a C-line as a visible confirmation of particle flow. Signal intensities of the two lines were 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.

Since the methodin development was a competitive immunoassay, its sensitivity was influenced by several well-known factors, such as antibody dilution and competitor concentration, provided that a definite antiserum was used. Additional factors that could be considered were: the chemical structure of the hapten (actually, the use of heterologous competitors had been shown to improve sensitivity [67]), the structure of the antigen used as the competitor in the assay (as far as the nature of the carrier-protein and the degree of conjugation between the hapten and the carrier-protein itself were considered); the specific response of the reporter used to label the antibody; the extent of antibody labelling (moles of reporter per mole of antibody). In effect, the work of Byzovaet al [68] firstly reported the effect of varying some of the described factors on LFIA performances and, in particular, showed that the diminishing of the molar substitution ratio (SR) between the hapten and the carrier-protein in the preparation of the competitor significantly improved as say sensitivity. The same authors also studied the binding capacity of different anti-species antibodies (which were used to trace the C-line) concluding, in this case, that no evident differences could be observed.

The need of developing a very high sensitive assay for determining AFM_1 in milk at levels of regulatory concern according to EU regulation [2], forced us to investigate further in these directions and to question other established practices, such as the assumption that the labelling of antibodies should be conducted in such a way to obtain a complete coating of GNP surfaces.

Therefore, the effects of varying: the competitor (use of homologous or heterologous hapten; nature of the carrier-protein and hapten-to-protein molar ratio) and the reporter (extent of antibody labelling)on sensitivity were studied and optimized.

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3.1. Materials and methods

3.1.1. LFD preparation

Gold colloids with mean diameter of about 40 nm were prepared using the sodium citrate method as previously described [46]. The saturation concentration of the anti-AFM_1 antibody for conjugation with gold nanoparticles was determined according to Horisbergand Rosset [69]. GNP-antibody conjugation was carried out using an amount of antibodies which is the half the saturation concentration and was carried out as follows: 100 μl of a 0.5 mg ml^{-1} anti-AFM_1antibodies in borate buffer 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. The mixture was centrifuged and the pellet was washed twice by re-suspension in borate buffer with 0.1% BSA added. Finally, the pellet was re-suspended in borate buffer supplied with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide and stored at 4°C until use.

Release pads were previously treated with borate buffer supplied with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide. After drying, gold-labelled antibodies were distributed near the lower edge of the pads and left to dry.

Test and Control lines were spotted upon a NC membrane as follows: the AFM_1-protein conjugate (SR 4) at 0.3 mg/ml was the capture reagent, and the goat anti-rabbit IgG antibodies (2 mg/ml) formed the C-line. Then, the membrane was dried. Strips were composed as follows: from the top; the adsorbent pad, the NC membrane, the release pad and the sample pad were pasted, in sequence, with 1-2 mm overlap. Release pad was positioned so that the line of GNP-labelled antibodies was on the opposite site from the edge of the membrane. The prepared membrane was cut into strips of 5 mm, which were inserted into rigid plastic cassettes. Cassettes were stored in plastic bags containing silica at room temperature until use.

3.1.2. Lateral flow immunoassay procedure

Pasteurized milk samples were purchased in large stores, and raw milk samples were obtained from farms. Whole and semi-skimmed milk (1 ml) were centrifuged for 2 min at 6000 rpm. The upper fat layer was discharged, 500 μl of the underlying serum was transferred into a tube and 25 μl of 10% Tween 20 was added. The mixture was immediately used in the lateral flow assay.

The test was carried out by placing 100 μl of sample into the sample well. After 15 minutes of incubation at 37°C, the cassette was placed above a mobile scanner connected to a laptop. The Skannex 3.0 software (SkannexAS,Hoenefoss, Norway) was used to acquire and process images. Calibration curves were obtained by plotting the ratio between the intensity of the test (T) and the control line (C) [46] against the log of AFM_1 concentration. For each experiment, a calibration curve was determined by a nonlinear regression analysis of the data using the four-parameter logistic equation [70]. For the construction of the standard curve and for recovery experiments blank milk samples that did not show any detectable residues of

the target when analysed by a reference ELISA (LOD 5 ng l^{-1}) [19] were fortified with appropriate amounts of an AFM_1 standard solution.

3.2. Optimization of the LFIAs

3.2.1. Effect of varying the hapten, the AFM_1-protein substitution ratio and the carrier protein in the T line

The polyclonal antiserum used in this work had showncertain cross-reactivity towards aflatoxin B_1 (about 35% when measured by means of the ELISA); therefore a competitor synthesized by using a hapten derived from this 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) conjugates which varied in the hapten-to-protein ratio, one conjugate of $AFM_1$with ovalbumin (AFM_1 –OVA) and one conjugate between $AFB_1$and 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 (1µl/cm), however the concentration was varied to obtain an absolute signal of about 20-25 arbitrary units in the T-line when the strip were read by means of the software. 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. Indeed, the reducing of the substitution ratio (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 [68] and with expectations based on the experience with competitive immunoassays in other formats (such as for example in ELISA). In parallel, the absolute signal decreased and forced to increase the amount of antigen to be dispensed. Nevertheless, the advantage of reducing the hapten density strongly predominated over the increase of the absolute antigen concentration.

Conjugate	SR	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

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. SR represents the molar substitution ratio between the toxin and the protein which had been estimated by spectrophotometric measurements.

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On the contrary, the substitution of the bovine serum albumin (which had been used to prepare the immunogen) with ovalbumin as the carrier-protein seemed irrelevant. In fact, antibodies binding the BSA used as the immunogenic carrier-protein are saturated in the preparation of the gold labelled- antibody. This preparation involves the GNP overcoating with exceeding amount of BSA to prevent aggregation; however, the inhibition of further non-specific binding to BSA of antigens could also be attained.

3.2.2. 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. Varying the amount of antibodies is exclusively intended as diluting the colloid 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; a typical protocol prescribes that the saturation amount of antibodies, intended as the amount that prevent GNP aggregation, has to be determined firstly and this stabilizing amount or, more usually, a small excess of antibodies, has to be conjugated to GNPs to prepare the signal reporter. 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 stabilizing amount [47].

Therefore, besides studying the effect of varying GNP-labelled antibody (intended as varying the OD under saturating conditions); we considered that dilution of antibodies to favour competitive conditions would also be achieved by reducing the number of molecules of antibody bound per GNP at a fixed OD value. Consequent risk of GNP aggregation, due to incomplete shielding of the superficial GNP charges, could be efficiently prevented by the further addition of exceeding amount of other proteins, such as for example BSA, which is particularly effective in this purpose.The variation of the amount of GNP-labelled antibodies dispensed at different ODs (3 and 6) under saturating conditions, apparently did not directly influence the sensitivity of the LFIA (data not shown) Nevertheless, the increasing of the OD allows the development of more intense absolute signals, which in turn means that the amount of competitor could be decreased in the T-line therefore improving detectability.

To study antibody dilution intended as the reduction of antibody amount per GNP, different amount of antibodies were reacted with portions of the same GNP colloid as follows: saturation amount (Ab_{SAT}), excess of antibody (Ab/ Ab_{SAT} = 1.5), defect of antibody (Ab/ Ab_{SAT} = 0.7), and half the saturation amount (Ab/ Ab_{SAT} = 0.5). 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 and a concentration of 0.2 mg/ml had been traced upon to form the T-line. AFM1 calibrators prepared in milk were run onto these strips in triplicate. Resulting curves are show in in Figure 4. Besides a significant signal reduction, a certain improvement in sensitivity was observed when the amount of antibody was lowered from saturating conditions (IC50 = 1.71 ± 0.01) to its half (IC50 = 0.99 ± 0.01); however detectability was influenced in a

considerably lesser extent respect than when modifying the nature of the competitor (i.e.: the SR of the conjugate used to obtain the T-line), as discussed above.

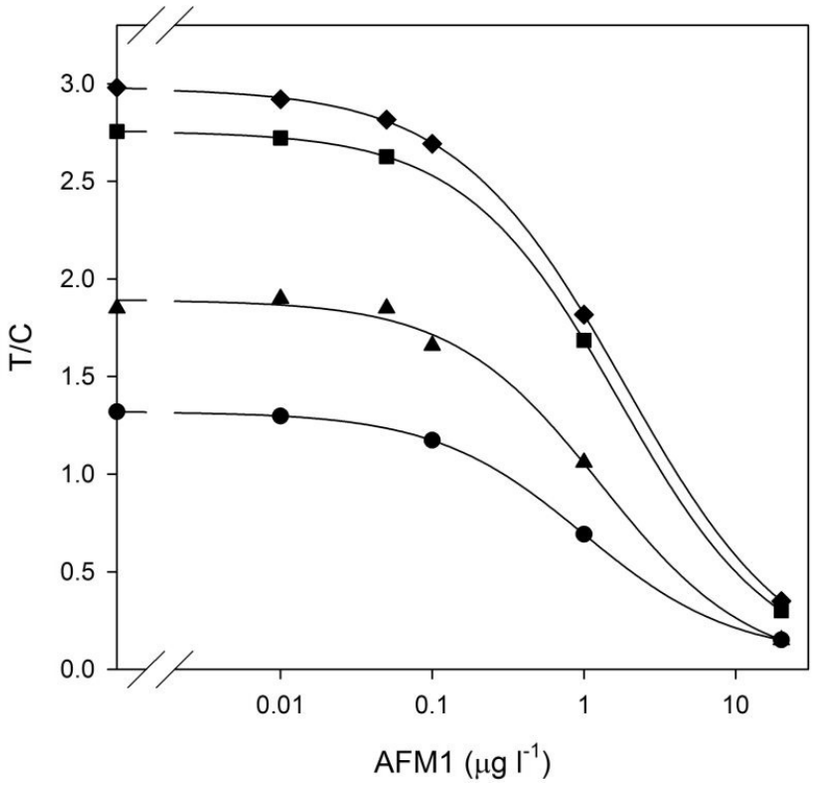

Figure 5. Effect of the amount of antibodies coated onto the GNPs (Ab) compared to the amount needed for saturating GNP surface (Ab_{SAT})for varying Ab/ Ab_{SAT}: 0.5 (▪), 0.7 (▲), 1 (▪), 1.5 (▪).GNP-antibodies dispensed at OD 3; T-line: AFM_1-BSA conjugate (0.2 $mgml^{-1}$, SR=22).

3.3. AFM_1 detection in milk by the developed LFIA

Protein and fat contents of milk may influence test results in various ways: the sample flow can be altered (for example fat content strongly affectsviscosity) and any of the milk components can give specific or non-specific interactions with immunoreagents involved in the assay. In fact, we observed that 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 undergone to different thermal treatments, i.e.: with different levels of protein denaturation (raw, pasteurized, UHT milk) and with variable fat content (whole, semi-skimmed, skimmed milk), samples were standardized by a rapid centrifugation stepto allow the removal of the fat layer and by adding Tween 20 to control protein interferences.

After development (15' at 37°C), strips were scanned. Dedicated software acquires 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

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LFIA for other mycotoxin [56, 61-63], matrix-matched calibration should be carried out to fit experimental results obtained on milk samples. Therefore, a pasteurized whole milk sample in which AFM_1 was found out undetectable when analysed by the reference ELISA kit was used to prepare diluted calibrators. A typical calibration curve is depicted in Figure 5. A LOD (calculated as the average of the blank minus 3 standard deviations from the average) and IC_{50} of 20 ng l^{-1} and 102 ± 19 ng l^{-1} were estimated, respectively.

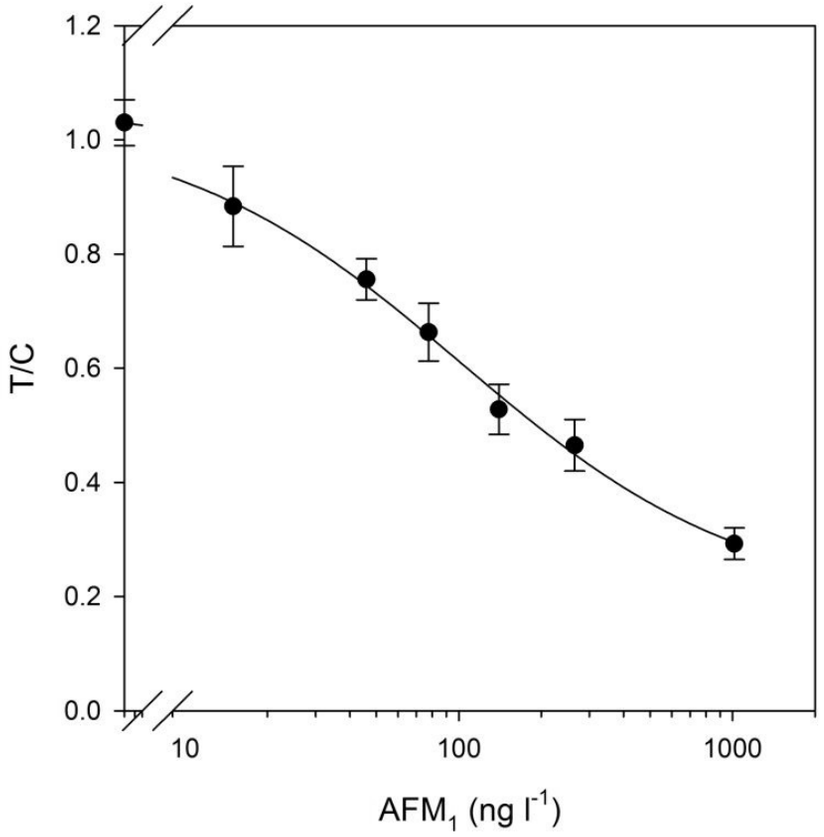

Figure 6. A typical calibration curve for AFM_1 measurement in milk by the developed LFIA. Error bars represent SD of 3 replicates.

Accuracy of the developed LFIA was evaluated on different kind of milk samples (Table 2). Milk samples were purchased on the market and were found undetectable according to the developed LFIA. Therefore, accuracy was evaluated on samples 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.

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%) which makes reliability of quantification questionable.

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	78.4 ± 6.2	121
		100	153.2 ± 14.1	135
whole 1	< LOD	0	<LOD	
		50	40.0 ± 2.0	80
		100	121.5 ± 9.8	122
whole 2	16.0	0	<LOD	
		50	79.0 ± 8.6	126
		100	125.5 ± 11.0	126
skimmed	15.7	0	34.6 ± 1.2	
		50	74.4± 4.0	117
		100	113.0 ± 20.5	97
UHT	<LOD	0	<LOD	
		50	46.8 ± 5.3	94
		100	87.5 ± 10.8	88

Table 2. Recovery of AFM_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

3.4. Intra-laboratory validation of the semi-quantitative LFIA

The objective of analytical methods such as those based on the LFIA technology is the parting between samples surely complying with legislation in force and samples which do not comply. However, a further category of samples should be considered and is represented by those samples in which the toxin content is close to the legal limit which because of measure uncertainty cannot be classified as compliant or noncompliant (Figure 4). These "uncertain samples" should be submitted to further controls before entering the transformation chain. In the case of milk, rejection is more often the fate of such uncertain samples (as for noncompliant samples), because the perishable nature of milk discourages time-consuming investigations. Therefore, the purpose of the work could become the development of a very rapid screening method which allowed the semi-quantitation of AFM_1 in milk in such a way to permit the discrimination between compliant and noncompliant samples. The instrumental quantification of coloured lines and their correlation with a calibration curve, in this context, could be regarded as a way to limit subjectivity in the interpretation of results and to improve detectability [52, 44] rather than going into the direction of factual quantitative measurements.

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To achieve the useful ability to discriminate compliant from noncompliant samples, a proper cut-off value should be established. The eligible EU MRL value (i.e.: 50 ng l^{-1}) would be expected to be at tain able given the high sensitivity of the developed LFIA. Nevertheless, the definition of a cut-off level should consider precision and technical limitations of the method, besides sensitivity. Moreover, the calibration curve being a continuously descending curve characterized by a finite slope, the definition of a single-point cut-off value is less appropriate than the identification of an indicator range of analyte concentrations within which uncertain or "non-attributable" results (neither "compliant" nor "noncompliant") fall [44].

As regard precision, European legislation for screening methods of analysis defines as appropriate a relative uncertainty of 47% of the MRL and as acceptable even 94% for AFM_1 based on the application of Horwitz equation [71]. Accepting the more restrictive criterion, this means that any screening methods should be able to discriminate between AFM_1 content less than 26.5 ng l^{-1} (negative sample) and AFM_1 content over 73.5 ng l^{-1} (positive sample). Samples that have AFM_1 content close to the thres hold limit should thus be defined as uncertain because precision did not allow to reliably attributing them to one or another group.

In spite of this, it should be noted that a "non-attributable" judgement would determine rejection of the sample with a considerable economic damage, as discussed above. Therefore, the minimum number of non-attributable results would be expected for a worth while method and this number obviously depends on the combination of accuracy and precision of the method itself. To indicate the capability of a qualitative/semi-quantitative method to produce the lowest score of non-attributable results, for a defined uncertainty interval, we introduced a new parameter indicated as "efficiency" of the method, defined as the ability of the method itself to detect truly non-attributable as non-attributable. Efficiency was thus calculated as the number of truly non-attributable tests divided by the sum of known non-attributable samples, in strict analogy with "sensitivity" and "selectivity" of qualitative and semiquantitative as says, which are defined as the rate of truly positive e and truly negative test results, respectively [50, 60]. The more efficient the assay, the highest the score of useful results (samples certainly attributed as compliant or noncompliant).

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 (noncompliant) 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}. Since all tested samples were always contaminated below 30 ng l^{-1} as established by the reference ELISA, positive samples were generated through fortification at 50 and 100 ng l^{-1}. Results of this evaluation, together with the definition of sensitivity, selectivity and efficiency, are reported in Table 3.

It can be observed from data that the definition of an indicator range instead of a cut-off level allowed us to avoid occurrence of false compliant and false noncompliant. Incorrect attribution occurred in 15% of samples (6/40), though 3 of them would represent a minor issue being assigned as non-attributable rather than noncompliant, which anyhow mean that samples would be discarded. The efficiency is relatively low, however it could still be considered acceptable.

Parameter	Definition	Calculated as	Value (%)
Sensitivity	truly positive / known positive	tp / (tp + fn + fup)	81.3
Selectivity	truly negative / known negative	tn / (tn + fp + fun)	100.0
Efficiency	truly uncertain / known uncertain	tu / (tu + fun + fup)	62.5
False compliant rate	false negative / known negative	fn / (tn + fn + fun)	0
False noncompliant rate	false positive / known positive	fp / (tp + fp + fup)	0
False non-attributable rate	false uncertain / known uncertain	fu / (tu + fun + fup)	37.5

Table 3. Evaluation of LFIAs performances on 40 milk samples: 16 negatives, 16 positives and 8 uncertain. The AFM_1 reference content was determined by an ELISA kit [19]. 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.

Finally, 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 periods longer than a month.

4. Conclusions

Despite LFIAs still being regarded in some ways as an emerging and incoming technology for food safety monitoring, there are several examples of fully developed devices described in the literature and also available as commercial kits for detecting a variety of natural and xenobiotic contaminants. Annual updates of state-of-the-art techniques underline the growing interest in the field and the increasing relevance of this technology over more established screening techniques. Not with standing the research is conditioned by the attainment of effectively functioning devices, often at the expense of true innovation, except in a few rare cases.

The literature concerning lateral flow immunoassays for aflatoxins is stilllimited, partly because the subject is very recent; indeed, the first published work on this topic dates back to just adecade ago. From this pioneering approach, several papers have been published which describes devices mainly aimed at measuring aflatoxin B_1. The use of LFDs for aflatoxin determination in nuts has also been demonstrated, even if the principal application is represented by their use to monitor aflatoxin contamination in cereals and derived products. This can be explained by the fact that research in this field is strongly driven by industry and by

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the prevalent economic impact of cereals in comparison to other commodities potentially affected by aflatoxin contamination.

The development of reliable devices for AFM_1 detection, conversely, suffers 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 the format of the classic lateral flow assay (such as tailoring the toxin conjugate, used as the competitor in the T-line, and the antibody labelling procedure)a greatdetect ability improvement could be obtained. The estimated LOD of the developed semi-quantitative LFIA was one order of magnitude lower than previously published LFIAs for AFM_1, therefore allowed us to effectively discriminate between compliant and noncompliant samples at a level required by the most severe legislation in force. Matrix-matched calibration was necessary to level 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 easy sample treatment, which involves 2' centrifugation followed by the addition of a small volume of a concentrated solution of a surfactant.

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