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Research Article

Development of an immunochromatographic assay for detection of Aflatoxin B1 in corn and rice

Desarrollo de un ensayo inmunocromatográfico para la detección de aflatoxina B1 en maíz y arroz

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ABSTRACT

Introduction: Aflatoxin B1 is a toxic contaminant, a threat to human health. Its presence in food was common. Rapid and efficient determination of AFB1 in corn and rice is critical for food safety monitoring.

Objectives: To develop a highly sensitive sandwich immunochromatographic assay for the detection of aflatoxin B1, with greater sensitivity than the strip test.

Methods: During sample preparation, aflatoxin B1 bound to bovine serum albumin to form AFB1-BSA conjugates, captured by both the anti-BSA antibody on the conjugate pad and the anti-AFB1 antibody on the test line, forming a sandwich structure. Key parameters were optimized to achieve the highest sensitivity of the test strip. Optimal parameters include: saturation of the antibody coating on magnetic nanoparticles, suitable nitrocellulose membrane, and optimal antibody concentration on the test line.

Results: Test strips were developed for the rapid detection of aflatoxin B1 in maize and rice. Detection limits: 1.0 µg/L for aflatoxin B1 in the assay buffer and 1.6 µg/L for aflatoxin B1 in spiked maize and rice samples. Repeatability: 5/5 for 5 aflatoxin B1 concentrations analyzed; test strip stability was 6 months when stored at 4°C and 25°C.

Conclusion: The immunochromatographic assay provides an efficient and highly sensitive approach for the in situ detection of AFB1 in maize and rice.

Keywords: Aflatoxin B1; immunochromatographic assay; magnetic nanoparticles; sensitivity.

RESUMEN

Introducción: Aflatoxina B1 es un contaminante tóxico; una amenaza para la salud humana. Su presencia en los alimentos era común. La determinación rápida y eficaz de AFB1 en maíz y arroz es fundamental para el control de la inocuidad alimentaria.

Objetivos: Desarrollar un ensayo inmunocromatográfico tipo sándwich, de alta sensibilidad, para detectar aflatoxina B1, con mayor sensibilidad que la prueba de tira.

Métodos: Durante la preparación de la muestra, la aflatoxina B1 se unió a la albúmina sérica bovina, para formar conjugados AFB1-BSA, capturados tanto por el anticuerpo anti-BSA en la almohadilla de conjugado, como por el anti-AFB1 en la línea de prueba, para formar una estructura tipo sándwich. Se

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optimizaron los parámetros clave para lograr mayor sensibilidad de la tira de prueba. Los parámetros óptimos incluyen: saturación del recubrimiento de anticuerpos sobre nanopartículas magnéticas, membrana de nitrocelulosa adecuada y concentración óptima de anticuerpos en la línea de prueba.

Resultados: Se desarrollaron tiras de prueba para la detección rápida de aflatoxina B1 en maíz y arroz. Límites de detección: 1,0 µg/L para la aflatoxina B1 en el tampón de análisis y de 1,6 µg/L para la aflatoxina B1 en muestras de maíz y arroz enriquecidas. Repetibilidad: 5/5 para 5 concentraciones analizadas de aflatoxina B1; la estabilidad de la tira de prueba fue de 6 meses cuando se almacenó a 4 ^{0}C y 25 ^{0}C.

Conclusión: El ensayo inmunocromatográfico proporciona un enfoque eficaz y altamente sensible para la detección *in situ* de AFB1 en maíz y arroz.

Palabras clave: Aflatoxina B1; ensayo inmunocromatográfico; nanopartículas magnéticas; sensibilidad.

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INTRODUCTION

Aflatoxin B1 (AFB1) is a toxic secondary metabolite produced by several strains of fungi, like *Aspergillus flavus* and *A. parasiticus*.[1] Exposure to AFB1 is associated with a risk of hepatocellular carcinoma. It is categorized as a group 1 human carcinogen by the International Agency for Research on Cancer.[2] AFB1 is widely found as contaminants in a great variety of unprocessed foods, such as cereals, nuts, coffee, etc. Contaminated AFB1 food products can pose serious hazard to human health. For protecting the health of consumers, detection of food products contaminated with AFB1 is necessary.

Several analytical methods, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and enzyme-linked immunosorbent assay (ELISA), have been developed to analyze AFB1 in food.[3] However, these methods are time-consuming as well as require expensive equipment and skilled

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technicians. Lateral flow immunochromatography assay (LFIA), which is another perspectively more efficient method has been studied and developed.

Magnetic nanoparticles (MNPs), which are relatively new and inexpensive, are recently employed to develop LFIAs with both qualitative (optical signal) and quantitative (magnetic signal) capabilities. However, MNPs can easily be oxidized in air, and have poor solubility, dispersibility and biocompatibility. Gold coated MNPs is an option to reduce these limitations. Therefore, Fe_3O_4@Au magnetic nanoparticles (MNPs) has been used as color indicators in the present study.

The objective of this resarch was to develop a high-sensitivity sandwich-based immunochromatographic assay for the detection of aflatoxin B1 in corn and rice samples, with greater sensitivity than the strip test.

METHODS

Chemicals and Materials

Anti-AFB1 monoclonal antibodies (OAEF00325; Biotrend), Anti- bovine serum albumin monoclonal antibodies (abx179053; Abbexa), were used. Anti-Mouse IgG antibodies (M5899), bovine serum albumin (BSA), Aflatoxin B1, 1-ethyl-3-(3-dimethyllaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS), were purchased from Sigma, Germany. The following membranes were used: nitrocelullose membranes (HF075MC100, HF090MC100, HF120MC100, Merck; Germany); conjugate pad (Standard 17 whatman; USA).

Principle of the method

The LFIA has developed using Fe_3O_4@Au (MNPs) as color indicator, and based on antigen-antibody reactions to form a sandwich structure. As illustrated in figure 1, anti-AFB1 is immobilized on the detection line (the test line). When sample solution flows into the conjugate pad, the anti-BSA antibody coating on MNPs bind to BSA, which has previously been attached to the target antigen AFB1 during sample processing. The formed complexes (MNPs-antibody-antigen) migrate to the nitrocellulose membrane and are captured by anti-AFB1 antibodies present in the test line. The remaining complexes

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then continue to migrate to the control line, where they are captured by the goat anti-mouse antibody. If the target antigen AFB1 is present in the test sample, the signal must be observed both the control and test lines. Besides, the intensity of the signal from the test line is proportional to the concentration of AFB1 in the test sample.

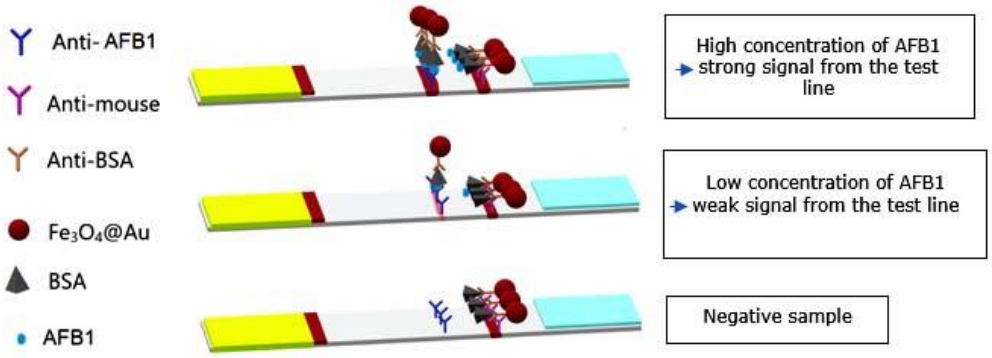

Fig. 1 - Scheme of the sandwich-based immunochromatography assay designed in this study for rapid detection of AFB1.

Optimization of the immunochromatographic strip

Synthesis of the Fe_3O_4@Au magnetic nanoparticles

Firstly, Fe_3O_4@PEI with a diameter of approximately 100 nm was directly synthesized.[4] 0.68 g of $FeCl_3.6H_2O$ was dissolved in EG under magnetic stirring at 60^0C for 10 min. After that, sodium acetate and 1 g of PEI were dissolved in 10 mL EG. Subsequently, these two mixtures were vigorously stirred at 60^0C for 20 min, and then transferred to a Teflon thermos flask to react at 220^0C for 2 h.

Next, Fe_3O_4@Au (MNPs) was synthesized. First, 2 mg Fe_3O_4@PEI was dispersed in 37.5 mL water and then 12.5 mL $HAuCl_4$ solution (6 mM) was added to mixture. The solution was heated at 80^0C for 10 min under vigorous mechanical stirring. After that, sodium citrate was rapidly added to the flask and stirred for another 20 min. As a result, Fe_3O_4@Au MNPs were formed. The morphology and size of synthesized MNPs were observed by transmission electron microscopy (TEM).

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Saturation of the antibody coating on Fe_3O_4@Au MNPs and determination of the detection limit of the test strip

The process of antibodies binding to Fe_3O_4@Au MNPs included two steps: activating the carboxyl group on the MNPs' surface and covalently binding to the antibody. Firstly, Fe_3O_4@Au-COOH was activated with EDC and NHS. The anti-BSA antibody was then added and incubated at 25 ^{0}C for 60 min. After the washing steps, the MNPs-antibody was blocked in Borat pH 7.2, containing 2% glycine.

Different antibody concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, 150 µg/mL, and 175 µg/mL) were added to 1 mg of Fe_3O_4@Au MNPs to determine optimal concentration.

The amount of antibody coating on Fe_3O_4@Au MNPs was determined by UV-Vis spectroscopy. First, the adsorption peaks for MNPs-antibody and the free MNPs were compared to confirm that MNPs were successfully coated with the antibody. A standard curve was next constructed using different concentrations of the protein standard. By interpolation from the constructed standard curve, the amount of antibody coating on Fe_3O_4@Au MNPs was determined indirectly through the amount of residual antibody in the solution.

Dispersity, hydrodynamic size and zeta potential of MNPs before and after antibody coating were measured using a Litesizer DLS 100. The optimal antibody concentration was the sample with the strongest color signal intensity on the test line when tested on a positive sample with an antigen concentration of 2 µg/L.

The determined optimal antibody concentration was subsequently used to prepare LFIA for figuring out the detection limit. Concentrations of AFB1 standard solution, including 0 µg/L, 0.5 µg/L, 1.0 µg/L, 10 µg/L, 100 µg/L and 200 µg/L, were tested.

Selection of nitrocellulose membrane

HF075MC100, HF090MC100, HF120MC100 membranes were compared to find out which membrane is the best to develop LFIA for detection of AFB1. The test strips then were prepared and tested with AFB1 samples at a concentration of 2.0 µg/L. These nitrocellulose membranes were evaluated to identify the optimal membrane for AFB1 detection. The most suitable membrane was defined as having a high sensitivity and no false positives.

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Optimization of the antibody concentration on the test line

The following antibody concentrations on the test line: 1.2 mg/mL, 1.0 mg/mL, 0.8 mg/mL, and 0.6 mg/mL. Samples with AFB1 at a concentration of 2.0 µg/L were used to determine which antibody concentration on the test line gives the strongest color signal. 60 µL of a running buffer (15 mM pH 7.4 PBS comprising 0.6% glycine, and 1% Tween 20) was used for each test.

Analytical performance evaluation of the LFIA

The rapid detection efficiency of the LFIA was determined by parameters: limit of detection (LOD), stability, and repeatability.

Determination of detection limit on experimental samples containing AFB1

Preparation of AFB1-spiked food

The negative samples were confirmed by HPLC method. Blank corn and rice samples were spiked with AFB1 standard solution at different concentrations of 1.0 µg/L, 1.6 µg/L, 10 µg/L, 100 µg/L, or 200 µg/L. The samples were then tested on the LFIA test strip. The LOD is the lowest AFB1 concentration that can be detected by the LFIA test strip. The LOD results in PBS buffer solution were used to compare with the sensitivity in experimental samples spiked with AFB1 standard solution.

Determine the stability of the LFIA

The developed LFIA were sealed in bags with silica gel and stored under different temperature conditions (4^0C, 25^0C, and 37^0C). After 1 month, 3 months, and 6 months of storage, the LFIA was used for AFB1 detection in positive samples, and its stability was determined by the change of color intensity of the test line. To check for stability and false negatives, we ran each parameter five times with samples of rice and maize having AFB1 antigen at a concentration of 2 µg/L. To check for false positives, we ran each parameter five times with blank samples.

Repeatability of the LFIA

The repeatability of the test strip was determined by the results of AFB1 detection in corn and rice samples, each of which was spiked with AFB1 at a concentration of 1.0, 1.6, 10, and 100 µg/L.

Samples preparation

One gram of crushed corn or rice samples was mixed with 5 mL of methanol-PBS (7:3; v/v). Then centrifuged at 1600 rpm for 5 min.

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Conjugation of AFB1 to BSA

AFB1-BSA conjugate was synthesized using the oxime active ester (OAE).[5] AFB1 in the food sample and 12 mg of CMO (0.06 mM) were dissolved in 5 mL of pyridine and incubated at 37 ^{0}C for 1 hour in the dark under stirring. Then, AFB1 oxime was dissolved in 1.0 mL of DMF, followed by the addition of 10 mM EDC and 10 mM NHS. The activated solution was slowly added to the BSA (20 mg/mL) solution under stirring for 1 hour. The resulting mixture was used for LFIA.

RESULTS

Description of Fe_3O_4@Au (MNPs)

The diameter, dispersion, gold nanoparticles layer, and stability of MNPs affect the sensitivity, and reaction time of LFIA, so was observed the synthesized MNPs under transmission electron microscopy (TEM). The formed monodisperse Fe_3O_4@Au MNPs had a spherical shape with an average diameter of about 100 nm (Fig. 2 A). The MNPs dispersed well, and gold nanoparticles formed a layer that covered the MNPs completely. These TEM images show that Fe_3O_4@Au MNPs were successfully synthesized.

Saturation of the detection antibody coating on Fe_3O_4@Au (MNPs) and detection limit of the test strip

The hydrodynamic size of the MNPs before antibody coating was 210.5 nm and increased to 244.3 nm after conjugation with detection antibody (Fig. 2 B). Furthermore, the zeta potential of the MNPs to explore the surface electric charge before and after antibody coating, which were −32.7 mV and −21.8 mV, respectively (Fig. 2 C). The hydrodynamic size and the zeta potential increased demostrated that the antibody was successfully attached to the nanoparticle surface.

Fig. 2 D shows the amount of antibody molecules binding to MNPs increased as the amount of antibody added increased as long as it was lower than 100 µg/mL. When the amount of antibody added was higher than 100 µg/mL, the amount of antibody molecules binding to MNPs did not increase significantly ($p > 0.05$). This indicated that saturation of anti-BSA antibody coating on MNPs was achieved. Therefore,

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the optimal antibody concentration we selected as 100 µg/mL for 1 mg of gold-coated magnetic beads and the amount of conjugated antibody was approximately 62 µg.

Furthermore, was compared the adsorption peaks for MNPs-antibody and the free MNPs. The results showed that there was no adsorption peak in the wavelength range of 270 – 280 nm with the free MNPs, while an adsorption peak for MNPs-antibody was observed at the wavelength of 270 nm (Fig. 2E). This confirms that the antibody has conjugated to MNPs'surface.

The MNPs-antibodies, which were obtained after adding anti-BSA antibody with concentrations of 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, were used to prepare the test strips for AFB1 detection in the sample with 2 µg/L AFB1. The test results showed that increasing the concentration of added antibodies resulted in an increase in the color intensity on the test and control lines (Fig. 3 A). This suggested that high density of antibody covering MNPs'surface increased the efficiency of the antigen-antibody interaction. The LOD of LFIA in our study was 1.0 µg/L (Fig. 3 B). Since the allowable standard of AFB1 in the samples is 5 µg/Lfor rice products, 2 µg/L processed rice products,[6] this LFIA can be a useful tool for the detection of AFB1 in corn and rice samples.

Selection of nitrocellulose membrane

As shown in figure 3C, all three test strips gave positive results, but the color signal on the test line of the test strip using HF075MC100 membrane was the weakest. On HF075MC100 membrane the time MNPs-antibody-antigen flow through the test line was not enough for efficient antigen-antibody interaction. In contrast, on HF120MC100 membrane with high capillary flow times can lead to a higher sensitivity, but false positive results may occur due to non-specific adsorption. Therefore, HF090MC100 membrane was the best choice for LFIA.

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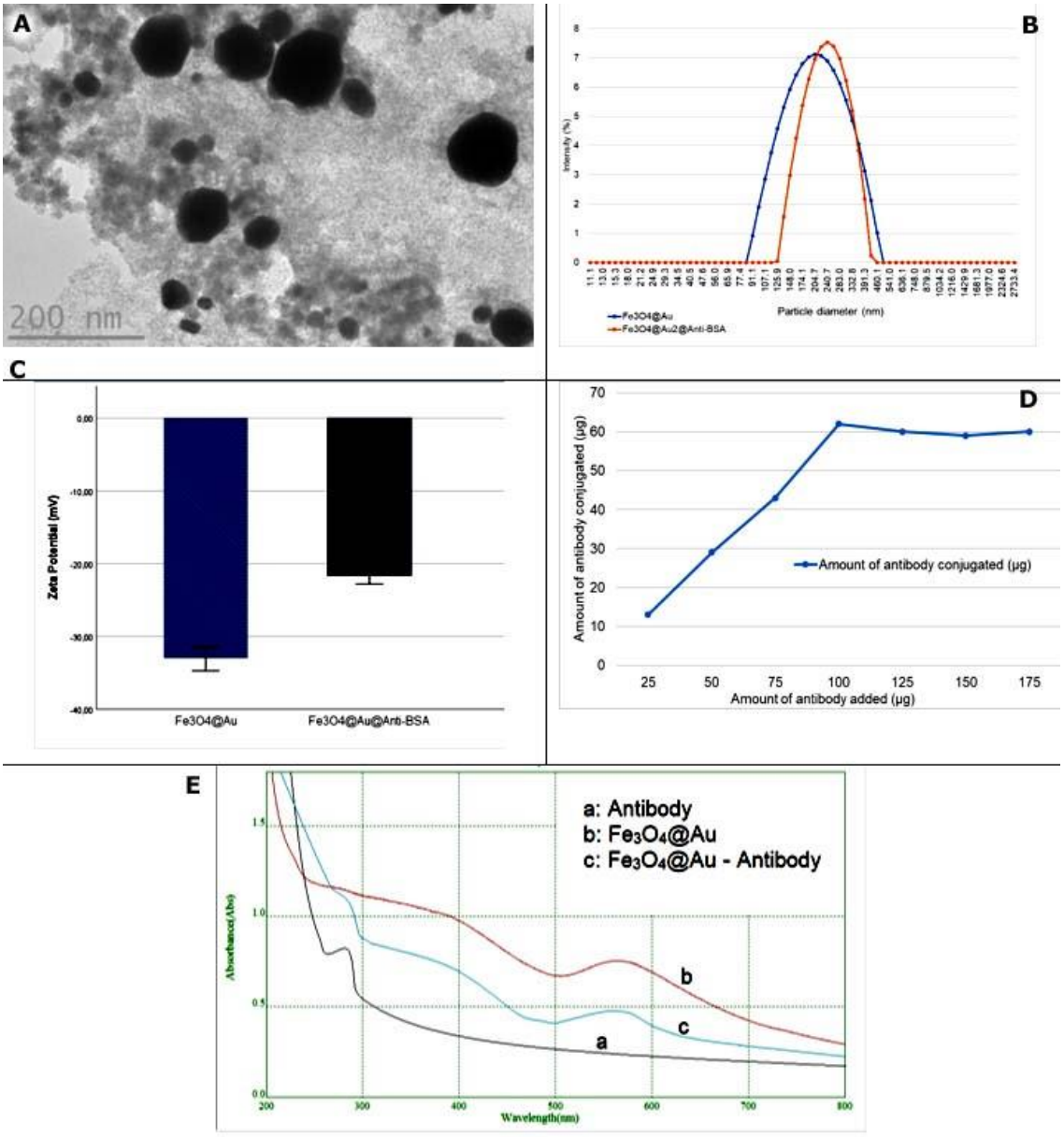

Fig. 2 - A: TEM images of Fe_3O_4@Au (MNPs); B: Hydrodynamic size of MNPs before and after antibody coating; C: Zeta potential of MNPs before and after antibody coating; D: Relationship between the amount of antibody conjugated to MNPs and the antibody concentration added; E: UV-Vis absorption peaks of monoclonal antibodies, Fe_3O_4@Au and Fe_3O_4@Au-Antibody.

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Selection of antibody concentration on the test line

As shown in figure 3D, the color intensity of the test line increased with increasing antibody concentration and reached a peak value at antibody concentration of 1 mg/mL and then decreased significantly at concentration of 1.2 mg/mL. Therefore, the antibody concentration of 1 mg/mL was chosen.

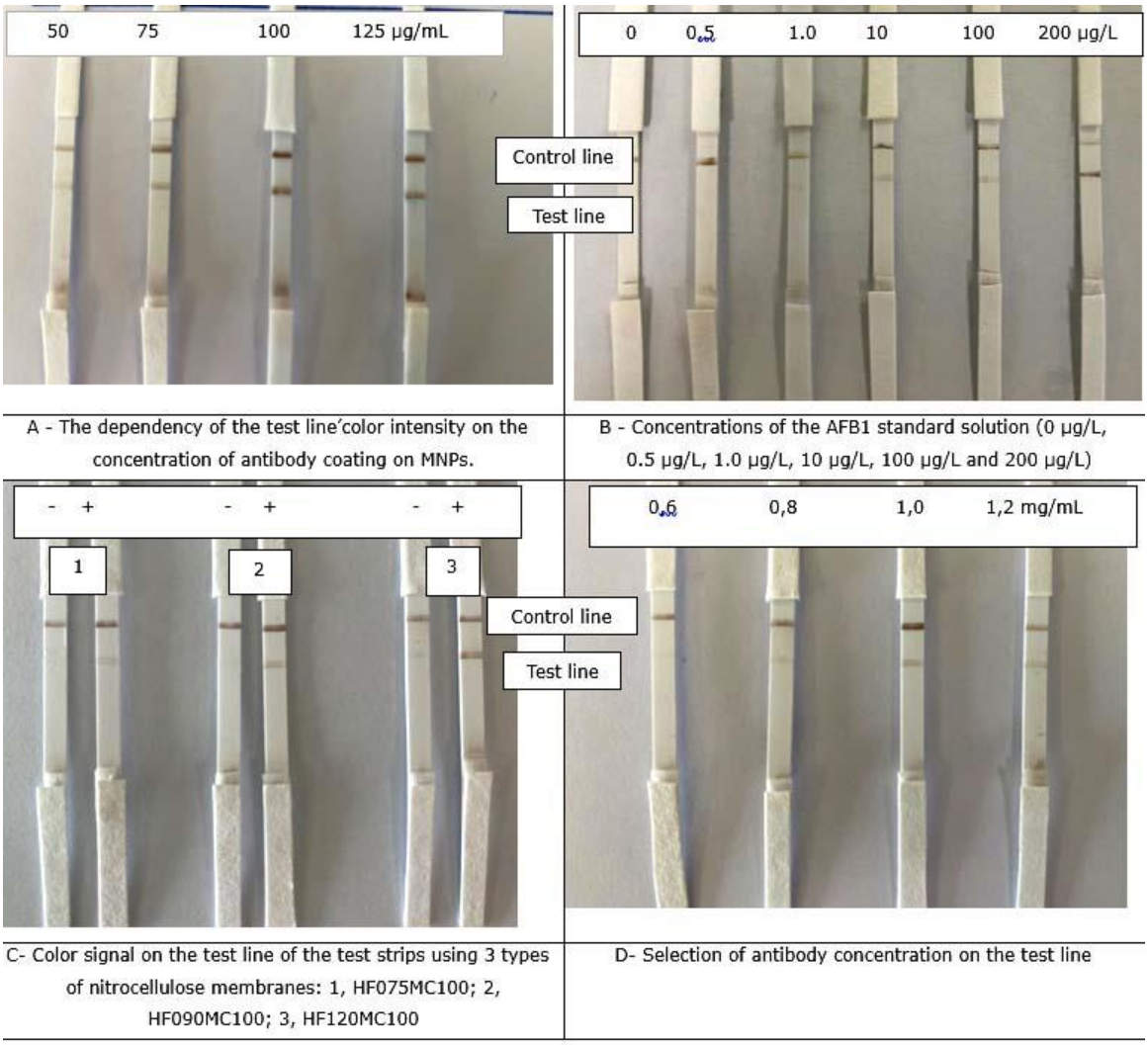

Fig. 3 – A: The results of the LFIA with the different concentrations of antibody coating on MNPs included 50 µg/mL, 75 µg/mL, 100 µg/mL, and 125 µg/mL; B: the LOD of the LFIA; C: Results of 3 types of nitrocellulose; D: Antibody concentration on the test line.

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Analytical performance evaluation of the LFIA

Results of LOD of the LFIA on corn and rice samples

AFB1 concentrations of 0 µg/L, 0.5 µg/L, 1.0 µg/L, 10 µg/L, 100 µg/L and 200 µg/L were added to the PBS buffer. The resulting solutions were then tested to determine LOD of the LFIA (Fig. 4). The analysis was completed within 12 minutes. AFB1 concentrations of 0 µg/L was used in order to check for the presence of non-specific binding. The LOD of the LFIA was 1.0 µg/L in the PBS buffer (Fig. 4 A).

Blank corn, and rice samples spiked with AFB1 concentrations of 0 µg/L, 1.0 µg/L, 1.6 µg/L, 10 µg/L, 100 µg/L and 200 µg/L were tested by the LFIA (Fig. 4 B). The LOD of the LFIA was 1.6 µg/L in the spiked samples (Fig. 4 A), which was higher than AFB1 in the PBS buffer. The intensity of the signal from the test line was proportional to the concentration of AFB1 in the test sample. However, in comparison with AFB1 in the PBS buffer at the same concentration, corn and rice samples gave lower test line's color intensity.

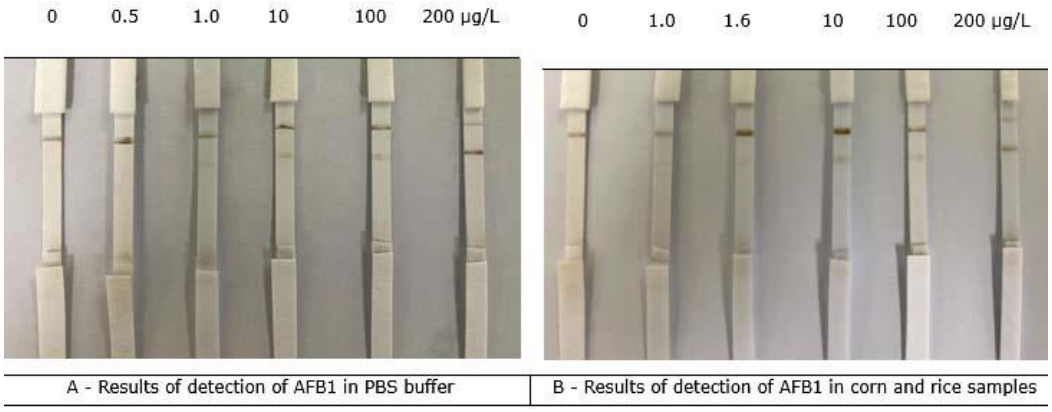

Fig. 4 - LODs of the LFIA in the PBS buffer (A) and the spiked food samples (B).

Stability of the LFIA

The LFIA still gave clearly positive results with an AFB1 concentration of 2 µg/L without the test line's color intensity decreasing after 6 months of storage at 4 ^{0}C and 25 ^{0}C. There were not false positive and false negative results. After 3 months of storage at 37 ^{0}C, the LFIA gave faded positive results with an

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AFB1 concentration of 2 µg/L, and the color intensity continuously decreased after 6 months of storage. However, false positive and false negative results weren't observed (table 1).

Table 1 - Stability of the LFIA stored at different temperatures

Storage time (month)		1	3	6
25 ^{0}C	False negative	0/5	0/5	0/5
	False positive	0/5	0/5	0/5
	Number of samples passed	10/10	10/10	10/10
	Color intensity of the test line	++	++	++
4 ^{0}C	False negative	0/5	0/5	0/5
	False positive	0/5	0/5	0/5
	Number of samples passed	10/10	10/10	10/10
	Color intensity of the test line	++	++	++
37 ^{0}C	False negative	0/5	0/5	0/5
	False positive	0/5	0/5	0/5
	Number of samples passed	10/10	10/10	10/10
	Color intensity of the test line	++	+	+

Notes: "++": strong color intensity. "+": light color intensity.

Repeatability of the LFIA

Five lots of the LFIA were used to assess their repeatability in food samples spiked with AFB1 concentrations of 1.0, 1.6, 10, and 100 µg/L.

DISCUSSION

Saturation of the detection antibody coating on Fe3O4@Au (MNPs) and detection limit of the test strip

The amount of antibody covering the MNPs plays an important role in determining the LOD and specificity of the LFIA. The concentration of anti-BSA conjugated to MNPs must be as high as possible to increase the efficiency of antigen binding, lower LOD, and avoid false positives. In current study, the antibody concentration was 100 µg/mL, the color signal on the test line was the most intense. Too high

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an antibody concentration on the surface of MNPs reduced the ability to bind to the antigen and recorded false positive results.

When testing LFIA with increasing antigen concentrations of 0 µg/L, 0.5 µg/L, 1.0 µg/L, 10 µg/L, 100 µg/L and 200 µg/L, the intensity of the color signal in the test line increased. For negative samples, no false positive was found. The smallest antigen concentration that produces a positive signal was 1.0 µg/kg, which was the LOD of the test strip. The test strip had good repeatability.

The LOD in this study was better than that in the previous studies on AFB1 toxin detection.[7,8] The reason for this difference might be the use of different methods. The previous studies developed competitive LFIA for AFB1 toxin detection;[7,8] while the sandwich LFIA, which was known as more sensitive than competitive mechanism, was used in current study.

The amount of MNPs-antibody sprayed on the conjugate pad to achieve the best sensitivity was optimized. If there was too little MNPs-antibody on the conjugate membrane, the amount of antibody was not enough to react completely with the target antigen. The amount of MNPs-antibody sprayed on the conjugate pad must be just enough to react completely with the antigen in the sample and create a clear color signal on the control line in both negative and positive samples. On the contrary, if too many MNPs – antibody were on the conjugated pad, a background signal may be generated on the nitrocellulose membrane, lead to increased nonspecific absorption.

Analytical performance evaluation of the LFIA

Limit of detection of the LFIA

Both the LOD of the LFIA in the PBS buffer and food samples were lower than the allowable standard of AFB1 in cereals[7] (Fig. 4). Current results indicated that the sensitivity of LFIA for AFB1 detection in PBS buffer solution was better than that in corn and rice samples. In addition, the intensity of the color signal of the LFIA tested in the food-spiked samples was slightly weaker than that in the PBS solution. These results demonstrated that the LFIA could be applied for AFB1 detection analysis of corn and rice samples.

Stability and repeatability of the LFIA

These results indicated that the LFIA can be stored at 4 ^{0}C and 25 ^{0}C for at least 6 months without losing their efficacy.

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To evaluate the repeatability of the LFIA, five lots of the LFIA were used to assess their repeatability in corn and rice samples spiked with AFB1 antigen.

LFIAs employing Fe_3O_4@Au MNPs were used for qualitative analysis. Additionally, the magnetic can be used to enrich the antigen in the samples. Therefore, gold-coated MNPs with core-shell structure for the development of LFIAs was choosen.

A high-sensitivity sandwich-based immunochromatographic assay for rapid detection of AFB1 in corn, rice spiked samples have successfully developed. The LOD of the LFIA in the PBS buffer and food spiked samples were 1,0 µg/L and 1,6 µg/L, respectively. The stability of the test strips was 6 months if they were stored at 4 ^{0}C and 25 ^{0}C. Current results have shown that optimization of some experimental parameters plays an important role in the development of the LFIA test strips.

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Conflict of interest

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Data Availability Statement

All data underlying the results are available as part of the article.

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