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Influence of the Compounds Used in the Immunochromatographic Test-System on its Analytical Characteristics: Multi-Factor Optimization of the System for Aflatoxin B1

Alexandr E. Urusov, Anatoly V. Zherdev and Boris B. Dzantiev

A.N. Bach Institute of Biochemistry of the Russian Academy of Sciences,
Leninsky prospect 33, 119071 Moscow, Russia

Abstract: Analytical parameters of immunochromatographic test-systems, such as the detection limit and working range, depend on many factors. However, the process of choosing the concentration and composition of reagents applied to test strip membranes and choosing the membranes themselves has not been systematically described for commercially produced tests or for scientific studies. Here, we present the results of a study to determine how the properties of the test system components, using mycotoxin aflatoxin B1 as an example, affect its analytical performance. A comparison of different membranes used for immunochromatography was performed as well as reagent concentrations and composition of intermolecular conjugates. Optimization of the immunochromatographic system indicated a limit of aflatoxin B1 detection equal to 0.2 ng/mL. The effectiveness of the system in testing plant extracts with minimal (twice) dilution prior to analysis was determined and the final content of methanol in the course of immunoassay was 35%.

Key words: Immunochromatography · Aflatoxin B1 · Analysis · Detection Limit

INTRODUCTION

Immunochromatography is of great practical interest as a rapid method of analysis, since all the components of the immunochromatographic test systems are previously deposited on the membrane test strip and the contact of this strip with the tested sample initiates all further processes, leading to the result (staining certain zones of the test strip or absence of the staining) for 5-15 minutes. To date, immunochromatographic tests are successfully commercialized and sold by a number of companies and are also described in a large number of scientific articles [1-6].

Previous studies noted that analytical characteristics of the immunochromatographic test system, especially the threshold for the staining appearance, as well as the working range for measuring the intensity of colour (by quantitative photometric analysis) were largely dependent on the affinity of immunochemical interactions (i.e. the choice of antibodies), as well as all other parameters that reflect the process of test strip manufacturing, i.e. intermolecular composition of conjugates (such as hapten-carrier proteins and antibody-colloidal gold), the amount of immunoreagent applied to the membrane, the properties of the membranes themselves and the reaction medium used in the analysis. However, the influence of these factors is described only in general terms, without demonstrating the extent to which they change the characteristics of the analysis [7]. However, previous studies have reported a detailed analysis of individual factors, such as the composition of antibody-colloidal gold conjugates [6] and the size of nano carriers required for immobilization of antibodies [8].

The absence of a detailed consideration of all the factors involved in the immunochromatographic test system has resulted in complications when developing new analytical systems. This lack of knowledge does not allow the analysis of accumulated data to make general recommendations for the immunochromatographic detection of various compounds.

Corresponding Author: Boris B. Dzantiev, A.N. Bach Institute of Biochemistry of the Russian Academy of Sciences, Leninsky prospect 33, 119071 Moscow, Russia. Tel: +7-495-954-31-42, Fax: +7-495-954-28-04. E-mail: dzantiev@inbi.ras.ru.

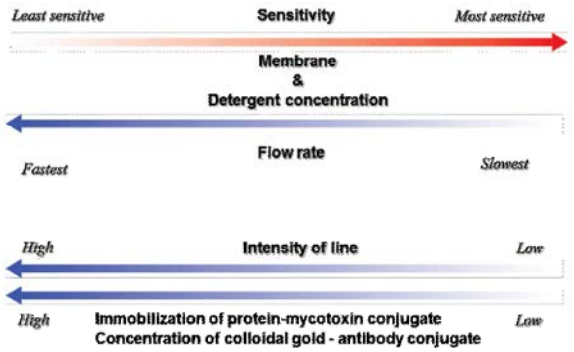


Fig. 1: Influence of various factors on the parameters of the immunochromatographic analysis.

Of note, the records of all potentially variable parameters cannot be reduced to a serial or parallel selection of optimal values for each individual factor. The requirements for immunoassays are to achieve a low detection limit, to have a short analysis time, with good reproducibility and intense staining for test and control zones of the strip; however, these requirements cannot be obtained when tests are performed under the same conditions. In this investigation we have described these difficulties briefly in a previous review [1]. For example, membranes with a low flow rate can be used to lower the detection limit. However, the analysis time might be lengthened to an hour or more. In contrast, immunochromatography can be performed in less than 5 minutes, but the intensity of staining in the test and analytical zones is often insufficient for the accurate interpretation of results.

Figure 1 based on previous data and analysis presented in [1] gives a systematic description of changes in factors that affect the analytical characteristics of the immunochromatographic test-system.

In the present article, the following parameters were studied to assess the impact of these factors on the performance of immunochromatographic tests: 1) composition and concentration of antibody conjugates with colloidal marker; 2) amount of reagent in the analytical zone; 3) choice of working membrane and membrane for the tested sample; and 4) composition of working buffer and concentration of its components.

An immune detection study was performed using mycotoxin [9] aflatoxin B1 (AFB1), a secondary metabolite of mould fungi of the genus *Aspergillus* [10], as an example. When ingested into animals or humans, it inhibits protein synthesis and induces immunosuppression, carcinogenic activity, genotoxicity and nephrotoxicity [11-16]. Therefore, the control of AFB1 is required for the characterization of agricultural products, food and animal feed [17-21].

MATERIALS AND METHODS

Materials and Reagents: Anti-AFB1 monoclonal antibodies were obtained from Puschino-Test Ltd (Puschino, Russia, www.test-p.ru). Their characterization in enzyme immunoassay systems was previously published [15]. The antibodies do not interact with mycotoxins from other chemical groups, namely ochratoxin A, zearalenone and T2 toxin (cross-reactivity in enzyme-linked immunosorbent assay [ELISA] =0.1%). Cross-reactivity with other aflatoxins were =10% for aflatoxin B2 and aflatoxin G2 and 75% for aflatoxin G1. Goat anti-mouse polyclonal antibodies were obtained from Arista Biologicals (Allentown, PA, USA, www.aristabiologicals.com).

Bovine serum albumin (BSA) was obtained from MP Biomedicals (Santa Ana, CA, USA, www.mpbio.com). AFB1, the aflatoxin conjugate AFB1-BSA, sodium azide, Triton X-100 and chloroauric acid were obtained from Sigma-Aldrich (St. Louis, Missouri, USA, www.sial.com).

All Other Reagents Were of Analytical Grade or Higher: Deionized water (18 MΩ/cm at 25°C, Simplicity Millipore, Billerica, MA, USA, www.millipore.com) was used for all the prepared solutions.

ELISA were performed with Costar 9018 (Corning, New York, NY, USA, www.corning.com) and Medpolymer (St. Petersburg, Russia, www.medp.spb.ru) microplates. When conducting ELISAs, absorbance of the reaction product was detected with a Zenyth 3100 microplate reader (Anthos Labtec Instruments, Salzburg, Austria, www.biochrom.co.uk).

The immunochromatographic test strips were fabricated from MDI Easypack membranes (Advanced Microdevices, Industrial Area, Ambala Cantt, India, www.mdimembrane.com) and Hi-Flow Plus membranes (Millipore, Billerica, MA, USA, www.millipore.com).

The application of reagents on the membranes was carried out IsoFlow dispenser Imagene Technology (Hanover, NH, USA, www.imagenetechnology.com) and Index Cutter-1 A-Point Technologies (Arista Biologicals, Allentown, PA, USA, www.aristabiologicals.com) was used for cutting.

Synthesis of Gold Nanoparticles: Colloidal gold [22, 23] was obtained according to the method of Frens [24]. A 1% aqueous $HAuCl_4$ solution (1 mL) was added to water (100 mL), the reaction mixture was heated to boiling and then a 1% aqueous sodium citrate solution (1.5 mL) was added with vigorous stirring. The solution was boiled for 15 min, cooled and stored at +4 °C. The preparation can be used for the immobilization of antibodies for at least one year.

Measurements of the Flocculation Curve: A modified method described in [25] was used. A series of dilute solutions of antibodies at concentrations from 300 to 0.5 μg/mL were prepared. Drops of these solutions (20μL) were injected into microplate wells containing 200 μL of colloidal gold (A_{520}=1). After incubation at room temperature for 10 min, a 10% NaCl solution (20 μL) was pipetted into each well. After 10 min, $À_{580}$ was measured and the plot of the absorbance versus the concentration of antibodies was constructed.

Immobilization of Antibodies on Colloidal Gold Particles: Colloidal gold was diluted to D_{580}=1. The pH was adjusted to between 8.5 and 9.0 with potassium carbonate, followed by the addition of anti-AFB1 antibodies (4.5-23.0 μg per mL of CG with D_{580}=1) diluted in 10-mM Tris buffer, pH 8.5. The resulting mixture was incubated for 15 min at room temperature, then 10% aqueous solution of BSA (V_{CG}:V_{BSA}=40:1) was added and the mixture was stirred vigorously for 10 min. The gold particles were pelleted by centrifugation at 15,000 ×*g* for 15 min at 4°C. The precipitate was collected and re-suspended in 10-mM Tris buffer, pH 8.5, containing 1% BSA and 1% sucrose (TBSU). The re-precipitation procedure was repeated twice. The pellet was re-suspended in TBSU and sodium azide was added to a final concentration of 0.05%. The obtained solution was stored at 4 °C [25, 26].

Preparation of Immunochromatographic Test-Strips: The AFB1-BSA conjugate was dissolved in PBS (50-mM potassium phosphate buffer, pH 7.4, supplemented with 0.1-M NaCl) to a concentration of 1 mg/mL and was applied to nitrocellulose membranes fixed on a plastic support, at a rate of 0.1 μl/mm, using an IsoFlow dispenser. Antibodies conjugated with gold nanoparticles were dissolved in TBSU and applied to the glass fibre conjugate pad (4.5 μL/mm).

The working membranes and three pads (for sample, for conjugate and absorbent pads) were assembled and cut into test-strips with a width of 3.5 mm by using an Index Cutter-1, placed in a sealed package containing silica gel, hermetically sealed under relative humidity of 30% and stored at room temperature, 20-22 °C.

Immunochromatographic Analysis of AFB1: Plant extracts were performed as previously described [27], with some modifications. Milled grains were mixed with an extraction solution (70% methanol, 30% water) at a ratio of 1:5 and incubated with gentle stirring at room temperature. After centrifugation, the supernatant was collected and stored at 4°C. The extracts were analysed by HPLC as previously described [28] and no AFB1 was detected.

For contamination, AFB1 in the range 0.1-20 ng/mL was added in 1 ml of extract (containing 70% methanol) and then mixed in a 1:1 ratio with PBS containing 0.1% of Triton X-100. Lower edges of the test strips were dipped into AFB1 dilutions in FBST or into samples of plant extracts. After 10 min of incubation and a 5-minute drying, the strips were scanned on a flatbed scanner Canon Lide 90 with a resolution of 600 dpi, without modes using contrast and colour correction.

Digital images of test strips were processed using Total Lab Quant (TotalLab, Keel House, UK, www.totallab.com). A rectangular area less than 90% of the width and length of the working membrane, was selected. This region was analysed using Total Lab for the presence and intensity of the coloured zone(s) and the level of background staining. Based on the obtained values of the integrated intensity of the staining, calibration curves were drawn and then processed.

Mathematical Processing of Assay Data: Dependences of the integrated coloration (y) on the concentration of an antigen in the sample (x) were estimated using the Origin 7.5 software package (Origin Lab, Northhampton, MA, USA, www.originlab.com) by employing the four-parameter sigmoid function (1).

$$y = \frac{A_1 - A_2}{1 + \left(\frac{x}{c}\right)^b} + A_2 \quad (1)$$

where A1 is the maximum value of the signal, A2 is the minimum value of the signal, c is the antigen concentration which inhibits binding of the antibodies to the immobilized antigen by 50% (IC_{50}) and b is the slope of the curve at IC_{50}.

This function was used to determine the antigen detection limit, which corresponds to 10% inhibition of binding - IC_{10} [29, 30].

RESULTS AND DISCUSSION

Choice of Antibodies: Ten antibodies against AFB1 were screened. Initially, ELISA was used to assess their reactivity, i.e. interactions with AFB1-BSA conjugate immobilized on the surface of the microtiter plate. The results of these experiments are shown in Figure 2. Based on the data obtained, four clones of the most high-affinity antibodies were selected. Achieving optical density by ELISA >1.0 at antibody concentrations less than 200 ng/mL (red lines in Figure 2) was a threshold criterion for the screening. To evaluate the interaction directly with AFB1, a competitive ELISA with immobilized BSA-conjugate of AFB1 and AFB1 in free solution was conducted (Figure 3). The minimum detection limit was reached for clone K1P.00. The antibody was characterized by ELISA with a detection limit for AFB1 equal to 0.05 ng/mL. The high affinity of the selected clone indicated it was the most suitable reagent to be used for development of the immunochromatographic system.

Comparison of Antibody-colloidal Gold Conjugates of Different Compositions for Immunochromatographic Analysis: As a marker, we used gold nanoparticles [31] with a mean diameter of 30 nm, synthesized by the Frens method [24]. Particles of this size provided a bright coloration and are stable both in solution and in the dry state on membranes and therefore are recommended as optimal markers for immunochromatography [24].

Typically for the synthesis of antibody-colloidal gold conjugates, the application of a flocculation curve is recommended [32-34]. This reflects the stabilization of the colloid by antibodies and the resulting stability of the preparation with respect to high salt concentrations. For these reagents, as follows from Figure 4 the aggregation of non-stabilized colloid was prevented using an antibody concentration equal to 4.5-5.0 mg/mL [25].

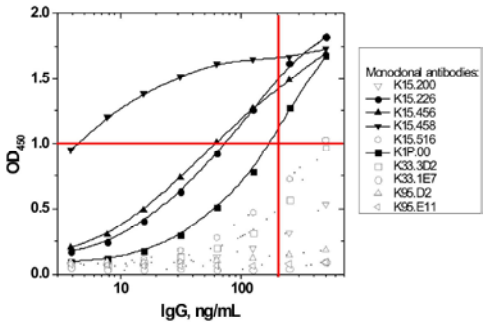


Fig. 2: Concentration dependent antibody binding to immobilized AFB1-BSA conjugate measured by ELISA (AFB1, aflatoxin B1; BSA, bovine serum albumin).

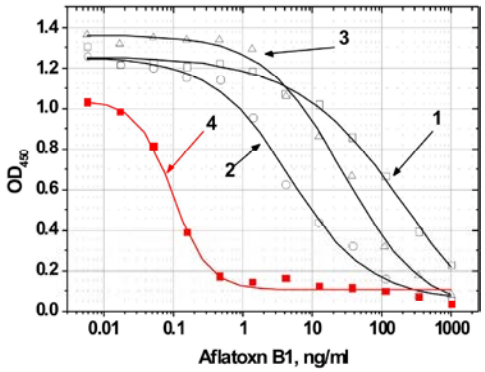


Fig .3: ELISA of AFB1 using various antibodies. Antibodies used were: 1 - K15.226; 2 - K15.456; 3 - K15.458; and 4 - K1P.00 (concentration of each antibody = 400 ng/mL).

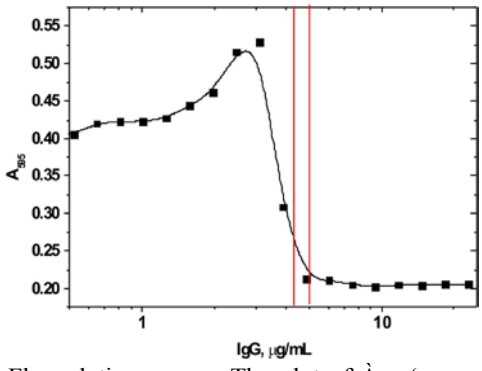


Fig. 4: Flocculation curve. The plot of $\grave{A}_{580}$ (measure of the coagulation of colloidal particles) versus the concentration of antibodies added to a colloidal gold preparation.

However, as noted previously [26, 35], this choice is not optimal for a competitive immunoassay. When high levels of antibodies are present in the conjugate, a greater

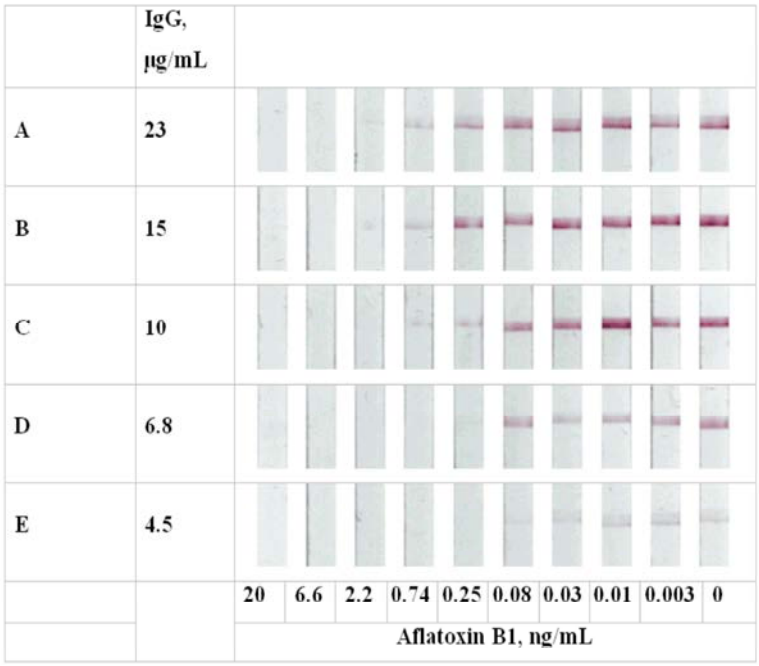
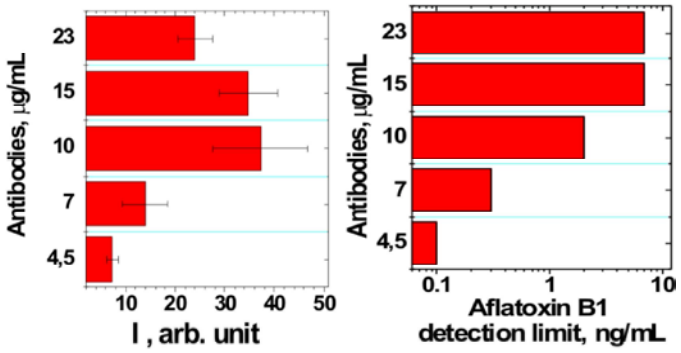


Fig. 5: Comparison of antibody-colloidal gold conjugates of different compositions used for immunochromatographic determination of AFB1. Lines A-E correspond to conjugates synthesized by the addition of 23, 15, 10, 6.8 and 4.5 μg of antibodies/mL of the colloidal suspension. AFB1, aflatoxin B1.



(a) (b)

Fig. 6: The intensity of coloration, I, in test zone (A) and visual detection limit (B) of immunochromatographic assays of AFB1 using antibody conjugates of different composition. The ordinate axis indicates the number of antibodies per mL of the colloidal gold suspension in the synthesis of the conjugates.

amount of antigen is required to induce inhibition of binding of the conjugate to the strip test zone. Therefore, we compared conjugates prepared by the addition of 4.5 to 23.0 mg of antibody/mL of the colloidal suspension during the synthesis.

The results of immunochromatographic testing is shown in Figure 5 and the values of test zone staining in the absence of antigen in the sample and the detection limits of AFB1 in Figures 5 and 6.

The limit of detection reached a maximum for conjugates with the highest content of antibodies (15 and 23 mg/mL colloidal gold suspension in the synthesis), whereas staining of the test zone intensity began to decrease. However, the minimum number of antibodies (4.5-mg/mL colloidal gold suspension in the synthesis) allowed the a decrease of the detection limit to 0.1 ng/mL, but due to a sharp decline in the intensity of staining, visual detection became non-reliable.

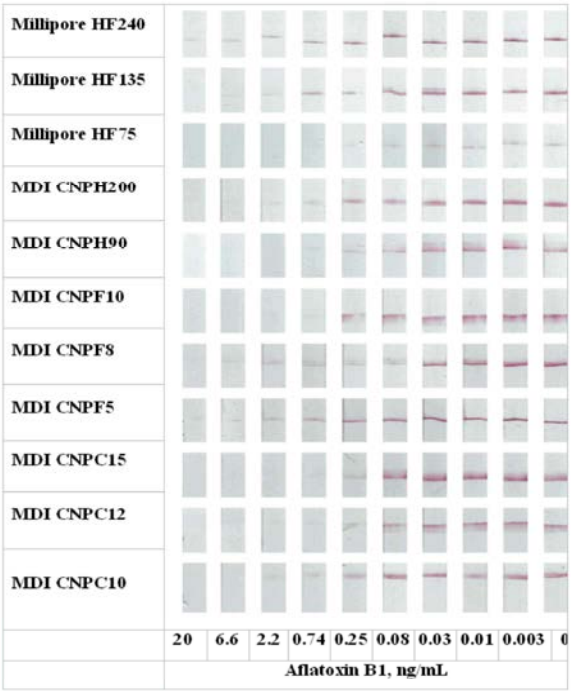
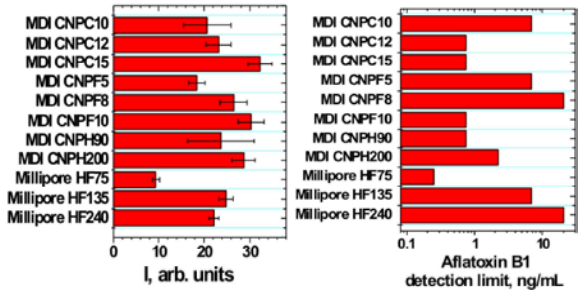


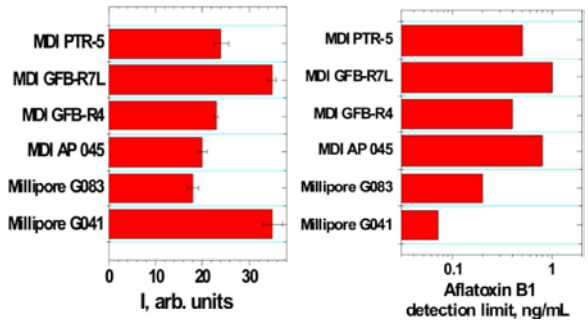
Fig. 7: The effect of different working membranes on the immunochromatographic determination of aflatoxin B1.



(a) (b)

Fig. 8: The intensity of coloration in test zone (A) and visual detection limit (B) of immunochromatographic assays of aflatoxin B1 using different working membranes

Considering these two factors, i.e. intensity of staining and detection limit, the optimal conjugate was prepared by immobilization of 7 mg of antibody/mL in the colloidal gold suspension. This protocol allows a shift in the detection limit to lower concentrations with small losses in colour intensity of the bands. Of note, the conjugate obtained in the immobilization of 4.5 mg of antibody (as it followed from the flocculation curve), was significantly inferior to this selection in its analytical characteristics.



(a) (b)

Fig. 9: The intensity of coloration in test zone (A) and visual detection limit (B) of immunochromatographic assays of aflatoxin B1 using different sample pads.

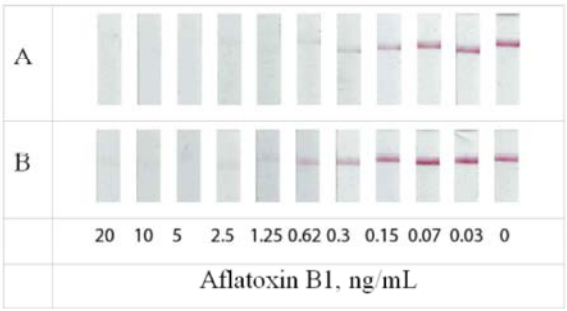


Fig. 10: The effect of 0% (A) and 35% (B) methanol on the immunochromatographic determination of aflatoxin B1.

An optimal membrane should provide a high intensity colour in the analytical zone and have a sufficiently low detection limit, low background signal and an even flow of liquid. Millipore membranes provided a more stable flow of fluid, but they were inferior to MDI membranes by either detection limit (HF240 and HF135), or by colour intensity of the test zone (HF75). MDI membranes have a small pore size and low sorption capacity (CNPF5 and CNPF8) and are unsuitable for analysis because of high background and low sensitivity in the case of visual detection. However, the CNPF10 membrane had a high staining intensity and fairly low detection limit. CNPH90 membranes with a stated high sorption capacity gave a low detection limit, but this was accompanied by low intensity of bands. CNPH200 membranes with a smaller pore size were characterized by very high intensity of test zone staining in the analytical zone and the detection limit was increased by more than an order compared with the HF75 membranes. Membranes with an average sorption capacity showed different

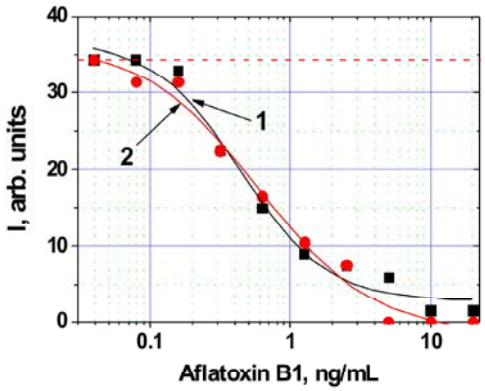


Fig. 11: Calibration curves of immunochromatographic determination of aflatoxin B1 in plant extracts. Plant extracts were 1 - barley and 2 - nuts.

results. For the CNPC12 membrane high background signal and low specific staining was noted. CNPC15 membranes were the best for this application, combining the best intensity of zone coloration, low detection limit and no background signal.

Selection of Sample Pad: The structure of the sample pad membrane has a significant effect on both the passage of the liquid front and on interactions during immunochromatography. We compared a number of membranes: Millipore G041 - glass membrane without any additives; Millipore Ñ083 - cellulose membrane, often used as an absorbent; MDI AP 045 - cellulose membrane with a high absorption capacity; MDI PTR-5 - polyester membrane for applying the conjugate; MDI GFB-R7L - fibreglass membrane with buffer and detergent applied by manufacturer; MDI GFB-R4 - glass fibre membrane, without pre-treatment.

As seen in Figure 9 the variation of membranes for the sample pad did not have a significant effect compared with the choice of working membrane. For further study, membrane G041 was selected, which gave the highest intensity and lowest detection limit for instrumental detection. In addition, as this membrane was manufactured without detergents or stabilizers, we added Tween-20 (final content 0.25%) into the solution used for sample dilution.

Validation of the Developed Test-System in Water-Organic Media: A mixture of water and methanol is commonly used for AFB1 extraction from solids, grain, flour and other items [36]. Therefore, the test system should operate effectively in water-organic media. We used a number of AFB1 dilutions in solutions with different methanol content, up to 70% (the initial concentration used in extraction). The optimal concentration was 35%. It did not require a significant dilution of the initial sample (and thus deterioration of analytical characteristics) and soothe methanol concentration was not too high to adversely affect the antigen-binding properties of antibodies. We observed similar binding of the marker in 0% and 35% of methanol, whereas higher methanol concentrations caused a decrease in the intensity of coloration (Figure 10).

Application of Lateral Flow Assay for Determining AFB1 in Plant Extracts: The developed system was tested for AFB1 detection in plant extracts using the "added-found" method; i.e. spiking unclarified, crude extracts of barley and nuts determined to be mycotoxin-free based on HPLC analysis, with various known concentrations of AFB1. When determining AFB1 in extracts of barley and nuts, the detection limit was 10 ng/mL by visual detection and 0.2 ng/mL by using the registration instrument (Figure 11). The analysis time was 10 min. The working range for AFB1 detection was 0.2-2.0 ng/mL (2-20 µg/kg of product). According to European Commission regulations (Commission Regulation [EU] No 165/2010), the maximum permissible level of AFB1 in different foodstuffs varies from 2 to 12 µg/kg. The proposed system therefore provides sufficient assay sensitivity for practical applications.

CONCLUSIONS

In summary, the immunochromatographic test-system was developed for AFB1 detection. It requires simple sample preparation and is suitable for the assay of crude plant extracts with a detection limit of 0.2 ng/mL for instrumental measurements. The duration of the assay is 10 min. The universal nature of the proposed approach allows its use in the development of other systems for immunochromatographic assay.

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