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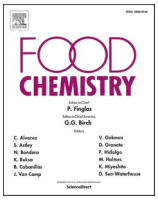

Single-step noncompetitive immunocomplex immunoassay for rapid aflatoxin detection

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ABSTRACT

Owing to the high carcinogenicity of aflatoxins, these toxic secondary metabolites pose a severe risk to human and animal health and can have major economic implications. Herein, we report the development of a noncompetitive immunoassay for aflatoxins based on a monoclonal capture antibody and a unique anti-immunocomplex (anti-IC) antibody fragment (scFv) isolated from a synthetic antibody repertoire. The anti-IC scFv recognizes the immunocomplex and enables the development of noncompetitive sandwich-type assays despite the small size of the analyte. The single-step assay developed in this work, with a detection limit of 70 pg mL^{-1}, could detect aflatoxins within 15 min. The assay was applied to the analysis of spiked food samples, and the results showed that the method could provide a rapid and simple tool for aflatoxin detection. Moreover, the work demonstrates the potential of anti-IC antibodies and non-competitive immunoassays for the analysis of small molecule contaminants.

1. Introduction

Mycotoxins are among the most notorious contaminants in agricultural products due to their wide spread and high toxicity. These toxic compounds are produced as secondary metabolites by various fungi that are ubiquitous pathogens in many plants and crops. Mycotoxin contamination in food or feedstuffs poses a serious threat for human and animal health but also contributes to massive economic consequences (Bennett & Klich, 2003; Bräse, Encinas, Keck, & Nising, 2009). Among mycotoxins, aflatoxins are of the greatest concern for human and animal health. In fact, aflatoxins have been evaluated to be among the most potent known mutagenic and carcinogenic substances (IARC, 1993; WHO, 2018). Aflatoxin producing *Aspergillus* species, such as *A. flavus* and *A. parasiticus*, commonly infect food crops, and aflatoxins can be found in cereals, legumes, and nuts, but also in a variety of other agricultural crops, such as coffee and spices. Several national and international authorities, including the European Commission, have established strict regulatory limits for controlling aflatoxins as well as other major mycotoxins in order to restrict the intake of these toxins (European Commission, 2006, 2010).

Sensitive methods for mycotoxin monitoring ensure efficient and reliable analysis, and there is a growing trend towards fast analytical tools (Krska, Welzig, Berthiller, Molinelli, & Mizaikoff, 2005; Renaud, Miller, & Sumarah, 2019; Shephard, 2016). While chromatographic techniques, such as high-performance liquid chromatography (HPLC) combined with fluorescence detection or mass spectrometry, provide accurate identification of mycotoxins, these approaches inevitably require long analysis times, expensive instrumentation, specially trained personnel, and often laborious sample preparation. On the other hand, fast screening methods, most notably enzyme-linked immunoassay (ELISA) and lateral flow tests, offer fast and low-cost alternatives for on-site mycotoxin detection (Köppen et al., 2010; Zhou, Xu, Kuang, Xiao, & Xu, 2020). These simple and affordable methods find wide application for field use and low-resource settings in developing countries where the true health burden of mycotoxin exposure occurs (Shephard, 2008).

Immunoassays and other immunological techniques are highly suited for fast yet sensitive analysis of mycotoxins and other small molecule contaminants. Plethora of methods based on competitive immunoassays have been developed for aflatoxins to meet a range of analytical requirements (Huang et al., 2021; Jia et al., 2021; Nolan, Auer, Spehar, Elliott, & Campbell, 2019; Wang, Niessner, Tang, & Knopp, 2016; Zhou et al., 2020). However, these approaches are almost exclusively based on the competitive immunoassay format despite some fundamental issues with this assay format. In fact, noncompetitive

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assays are known to be superior to the competitive ones according to both theoretical consideration and experimental design (Jackson & Ekins, 1986; Li et al., 2018). A major factor hampering the development of noncompetitive assays for mycotoxins is the small size of the analyte that impedes the simultaneous binding of two antibodies. Nevertheless, noncompetitive immunoassays would be ideal to detect also trace amounts of small molecules due to the increased sensitivity, wider dynamic range, higher precision, and shorter incubation times in comparison with the competitive assay (Kobayashi & Goto, 2001; Kobayashi & Oyama, 2011).

The vast progress in the field of antibody engineering have brought forth the discovery of unconventional antibody binders that enable the development of noncompetitive assays also for small molecules (Li et al., 2018). For example, so-called anti-immunocomplex (anti-IC) antibodies bind specifically to the antibody − antigen complex, and they can be used for the development of two-site noncompetitive sandwich-type immunoassays. Though the discovery of such binders is problematic by traditional immunization-based techniques, phage display methodology provides a more controlled strategy for generating antibody binders beyond the polyclonal and monoclonal ones. Owing to the remarkable characteristics of anti-IC antibodies, also referred to as anti-metatype antibodies (Voss & Mummert, 1997), they have been applied to the development of sensitive immunoassays for several small molecules in various assay formats, for example, morphine (Pulli, Höyhtyä, Söderlund, & Takkinen, 2005), microcystins (Akter et al., 2016), nodularin (Akter, Vehniäinen, Kankaanpää, & Lamminmäki, 2017), HT-2 toxin (Arola, Tullila, Nathanail, & Nevanen, 2017; Arola et al., 2016), and estradiol (Leivo, Kivimäki, Juntunen, Pettersson, & Lamminmäki, 2019).

In this work, we report the development of a rapid noncompetitive immunoassay for aflatoxins. The assay is based on the combination of an aflatoxin-specific monoclonal capture antibody and a recombinant anti-IC antibody fragment selected from a synthetic antibody repertoire. This straightforward assay includes only a one single incubation step where all the three antibody reagents (biotinylated monoclonal antibody, anti-IC binder, and europium-labelled secondary antibody) are added at the same time. With a total assay time of 15 min the developed method is highly suitable for rapid testing or high throughput screening from various food products. Moreover, the work demonstrates the potential of anti-IC binders for the development of noncompetitive immunoassays for the analysis of small molecules.

2. Materials and methods

2.1. Materials

The mouse monoclonal anti-AFB antibody was purchased from HyTest Ltd. (Turku, Finland) and biotinylated with biotin isothiocyanate as previously described (Akter et al., 2016). Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and M_1 (AFM_1) together with deoxynivalenol (DON) and ochratoxin A (OTA) were from Fermentek (Jerusalem, Israel), and aflatoxin G_2 (AFG_2) was from Sigma-Aldrich (Saint Louis, MI, USA). Helper phage VCS M13 and the *Escherichia coli* XL1-Blue cells were from Agilent Technologies (Santa Clara, CA, USA). Anti-alkaline phosphatase (anti-AP) polyclonal antibody was purchased from LifeSpan Biosciences, Inc (Seattle, WA, USA), and anti-phage antibody was from Sigma-Aldrich. These secondary antibodies were labelled with a europium chelate as previously described (Akter et al., 2016). Dynabeads M−280 Streptavidin (SA-beads) and Dynabeads Antibody Coupling Kit together with the HisPur Ni-NTA spin columns were from Thermo Fisher Scientific (Waltham, MA, USA).

Superb broth (SB, 2% yeast extract, 3% peptone, 1% MOPS, pH 7) was used as the culture medium, and Luria broth (0.5% yeast extract, 1% peptone, 1% NaCl) supplemented with 1.5% agar, 0.5% glucose, and 100 μg mL^{-1} ampicillin (LA–Amp) or 10 μg mL^{-1} tetracycline and 25 μg mL^{-1} chloramphenicol (LA−Tet/Cam) was used for preparing agar plates.

Tris-buffered saline (TBS) contained 50 mM Tris (pH 7.5) and 150 mM NaCl, whereas TBT-0.05 consisted of TBS supplemented with 0.05% (*v/v*) Tween-20. For Tris saline azide (TSA) buffer, 0.02% (*w/v*) NaN_3 was added to TBS. The AP substrate *p*-nitrophenyl phosphate (pNPP) was from Sigma-Aldrich, and for the assay, it was dissolved in pNPP buffer (500 mM Tris, pH 9.0, 200 mM NaCl, 10 mM $MgCl_2$) to 1 mg mL^{-1}. Red assay buffer, washing buffer, and europium fluorescence intensifier (EFI) solution together with streptavidin coated microtiter plates were from Kaivogen Oy (Turku, Finland). Multilabel counter Victor 1420 for the fluorescence measurement was from Wallac/PerkinElmer Life Sciences, (Waltham, MA, USA), and Hidex Sense microplate reader was from Hidex (Turku, Finland).

2.2. Selection of anti-immunocomplex antibodies by phage display

Anti-immunocomplex (anti-IC) binders were isolated from a synthetic monovalent scFvP library (Brockmann et al., 2011; Huovinen et al., 2013) using the anti-AFB antibody and AFB_1 immobilized on magnetic beads as the target in consecutive selection rounds. Firstly, the phage library (4×10^{12} cfu diluted in 1 mL of TBT-0.05) was incubated with SA-beads (50 μg) for 1 h at room temperature to remove unwanted binders against streptavidin. Thereafter, the beads were separated with a magnet, and the library suspension was further pre-selected with the biotinylated anti-AFB (500 ng) coupled to the SA-beads (50 μg) during 1 h. After these subtractive steps, the pre-selected library suspension was mixed with unspecific native mouse IgG (100 μg) and free biotin (10 nmol). After 30 min incubation with the blockers, the suspension was mixed with the IC-saturated beads. IC-saturated beads were prepared in advance by incubating first the biotinylated anti-AFB (10 μg) with the SA-beads (500 μg diluted in 250 μL of TBT-0.05) for 30 min in rotation at room temperature. The antibody-coated beads were then blocked with free biotin (50 nmol) and washed three times with TBT-0.05. Then, the antibody-coated beads (in 250 μL of TBT-0.05) were incubated with AFB_1 (100 × molar excess) overnight in rotation at + 4 °C.

The pre-selected library was incubated with the anti-IC beads in the presence of the excess of AFB_1 for 4 h at + 4 °C. Then, after three washes with cold TBT-0.05 and one wash with TBS, the bound phages from the beads were eluted with trypsin (12 μg in 200 μL of TBS) during 30 min at room temperature. The reaction was stopped by adding soybean trypsin inhibitor (10 μg in 10 μL of TBS). The beads were then collected with a magnet, and the supernatant containing the eluted phages was used to infect the XL1-Blue *E. coli* cells grown to exponential phase (optical density at the wavelength of 600 nm, OD_{600} ~0.5) during 30 min at + 37 °C. The cells were plated on LA−Tet/Cam and grown overnight at + 30 °C. The cells were collected the next morning, and the plasmid DNA pool was extracted with a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For phage production, the collected cells were used to inoculate 20 mL of SB supplemented with 1% glucose, 10 μg mL^{-1} tetracycline, and 25 μg mL^{-1} chloramphenicol. The cells were grown at + 37 °C, 300 rpm until the exponential growth phase (OD_{600} = 0.3–0.6). The cells were then infected with VCS-M13 helper phage (~2.5×10^9 tfu) for 30 min at + 37 °C without shaking. The cells were collected by centrifugation (3200 *g*, 10 min, +4 °C) and resuspended in 20 mL of fresh SB medium containing tetracycline and chloramphenicol. After 1 h of growth at + 30 °C and 300 rpm, 100 μM IPTG and 50 μg mL^{-1} kanamycin were added to the cultures, and the phage production was continued overnight (+26 °C, 300 rpm). The bacteria were harvested by centrifugation (12000 *g*, 10 min, +4 °C), the phages in the supernatant were precipitated twice by adding 1/5 vol of PEG/NaCl (20% PEG, 2.5 M NaCl). Finally, the phages were dissolved in 500 μL of TSA buffer supplemented with 1% BSA, and the amplified phage stock was used for the second selection round. To avoid binders against streptavidin or biotin, in the second round anti-AFB was directly immobilized on magnetic beads according to the Dynabeads Antibody Coupling Kit instructions, but

apart from that, the selection process (with 6×10^{10} cfu phages from the 1st round and 50 μg IC-saturated beads) was completed as in the first round.

2.3. Phage-based immunoassay

After two rounds, the success of the selections was evaluated in a phage-based ELISA using the immobilized anti-AFB antibody and the polyclonal phage pools from each selection round together with Eu-labelled anti-phage secondary antibody. First, the biotinylated anti-AFB antibody (25 ng/well) was added to prewashed streptavidin-coated wells in the assay buffer and incubated for 30 min. For the control wells, no antibody or anti-AFM antibody (BioTez, Berlin, Germany) as a control was added. The wells were then washed three times with the washing buffer, and the amplified phages (2×10^{10} cfu mL^{-1} in assay buffer) were added to the wells without or with AFB_1 (100 ng mL^{-1}). All incubation steps were done in the total volume of 200 μL with slow shaking at room temperature. Finally, the plate was washed three times, and 200 μL of EFI solution was added to each well. After 5 min incubation, the time-resolved fluorescence signals were measured with a Victor 1420–fluorometer (340 nm excitation, 616 nm emission, 400 μs delay, and 400 μs measurement time).

2.4. Screening of anti-IC binders

For screening the positive scFv clones, the DNA pool from the second panning round was used to subclone the genes encoding for the antibody fragments in vector pLK06H (Huovinen et al., 2013) to produce the scFv as a fusion with bacterial alkaline phosphatase (scFv-AP). After transformation to *E. coli* XL1-Blue cells, single colonies were manually picked from LA−Amp plates to inoculate a small-scale culture in 160 μL of SB (supplemented with100 μg mL^{-1} ampicillin, 10 μg mL^{-1} tetracycline, 0.05% glucose) on 96-well microtiter plates. Protein expression was induced with 200 μM IPTG, and the culture was continued overnight at +26 °C, 900 rpm. The next morning the cells were lysed with lysozyme (200 μg per well) and freeze−thawing. The cell debris was collected by centrifugation (3200 *g*, 30 min, +4 °C), and the supernatant was used for the screening immunoassay.

For the immunoassay, biotinylated anti-AFB antibody (20 ng/well in assay buffer) was added to prewashed streptavidin-coated wells and incubated for 30 min. For the streptavidin-background wells, only buffer was added. The wells were then washed three times with the washing buffer, and 20 μL of the scFv-AP in the culture supernatant was added to each well without or with AFB_1 (50 ng mL^{-1} in assay buffer). After 1.5 h incubation, the plate was washed three times and pNPP was added. The incubation was continued for 1 h, and finally, absorbance at 405 nm was measured with Hidex Sense microplate reader. All incubation steps were done in the total volume of 100 μL with slow shaking at room temperature.

Later on, selected antibodies were produced in a larger scale and purified by metal affinity chromatography according to the manufacturer's instructions.

2.5. Noncompetitive single-step immunoassay

The reagent mixture (50 ng biotinylated anti-AFB, 1 μg scFv-AP, and 25 ng of Eu-labelled anti-AP per well in the assay) was mixed with the sample or toxin standards (final concentration per well between 0 and 300 ng mL^{-1}) and added to prewashed streptavidin wells in triplicates. The total volume in each well was 200 μL, and the dilutions were made in the assay buffer. After 10 min incubation at room temperature with slow shaking, the wells were washed four times with the washing buffer. Then, EFI solution was added (200 μL/well), and after 5 min incubation, the time-resolved fluorescence of the Eu-chelate label was measured as before.

2.6. Sample preparation and analysis

Rice flour, maize flour and hazelnut were purchased from a local supermarket. HPLC analysis confirmed that the samples did not contain detectable levels of AFB_1. For the sample analysis, 1 g of the sample was weighed and extracted using 5 mL of 70% methanol (*v/v* in water) for 20 min with rotation. Then, the mixture was centrifuged at 3200 *g* for 10 min, and the supernatant was used for sample analysis. Spiked samples were prepared by adding a known amount of AFB_1 to the sample (in triplicate) which was then allowed to equilibrate for 3 days at room temperature protected from light. The spiked samples were subjected to methanol extraction as described and analyzed with the single-step immunoassay after 10-fold dilution or with HPLC.

For the HPLC, the sample extracts were first filtered and cleaned using a commercial immunoaffinity column (Vicam, Milford, MA, USA) according to the manufacturer's protocol. Briefly, 10 mL of the sample (previously diluted 1:1 with distilled water) was passed through the column at a rate of 1–2 drops per second using a syringe attached to the aflatest column. The column was washed with 10 mL of water with the same flow rate. Subsequently, aflatoxins were eluted with 1 mL of methanol, and the eluate was evaporated to dryness on a hotplate at +60 °C. The AFB_1 stock solution in methanol (500 μL) was dried and treated in the same way as the derivatization procedure used for samples.

Before injection into the HPLC, the samples were derivatized by adding 200 μL of hexane and 50 μL of trifluoroacetic acid (TFA) and stirring with vortex for 30 s. The vial was let to stand 5 min before adding 900 μL of water:acetonitrile (9:1). The samples were vortexed for 30 s, and the aqueous layer was filtered through a syringe filter. HPLC was performed as previously reported (Abbas, Hussien, & Yli-Mattila, 2020) with minor modifications. Derivatized samples and standards (20 μL) were injected into the HPLC system (LiChroCART, Agilent Technologies, Waldbronn, Germany), equipped with a UV and fluorescence (excitation 360 nm, emission 440 nm) detectors. The column was C18 reversed phase (LiChrospher 100, 125 × 4 mm, 5 μm). The mobile phase consisted of water:methanol:acetonitrile (60:30:15, *v/v/v*). The total run time was 30 min with a flow rate of 500 μL min^{-1}. The AFB_1 concentrations in the sample were calculated based on the area of the standard peak.

2.7. Immunoassay data analysis

The measured signals were analyzed with Origin 2016 software (OriginLab Corp., Northampton, MA, USA). The half maximal effective concentration (EC_{50}) was calculated using the four parametric logistic fit. The detection limit (LOD) was calculated from the standard curve based on the average background signal + 3 × standard deviation. The apparent recovery from each spiked sample was calculated as the ratio between the observed toxin concentration and the spiked concentration.

3. Results and discussion

3.1. Selection of anti-immunocomplex binders

The synthetic antibody library was used to select scFv antibody fragments recognizing the immunocomplex (IC) consisting of the monoclonal anti-AFB capture antibody and AFB_1. For the first selection round, biotinylated anti-AFB was immobilized on the surface of streptavidin-coated magnetic beads and saturated with AFB_1. To avoid enrichment of background binders to streptavidin or biotin, in the second round, anti-AFB was directly coupled to epoxy-coated magnetic beads which were similarly saturated with AFB_1. Moreover, preselection against mere anti-AFB and addition of unspecific soluble mouse IgG were used to block the enrichment of binders recognizing the anti-AFB in the absence of AFB_1. The enrichment of binders towards the IC was confirmed by the phage-based immunoassay (**Fig. S1A**). No

significant background binding was seen to the streptavidin surface or the anti-AFM antibody that was used as a control. Somewhat high background signal, however, was observed to the anti-AFB antibody showing that the phage pool probably consisted of a significant amount of binders against the antibody alone. This also demonstrates the difficulty in completely blocking the enrichment of such unwanted binders even with the aforementioned preventive measures. Yet, in the second round the signals with the IC were significantly higher than with the antibody alone, and thus, the antibody gene from the pool from the second round was subcloned into a bacterial expression vector to screen for individual binders.

A total of 320 individual scFv binders fused with bacterial alkaline phosphatase (scFv-AP) were screened for their binding toward the IC consisting of the anti-AFB and AFB_1. Among the tested binders, 17% of them were identified as antibody binders as they showed binding to the immobilized anti-AFB but not the IC. Such binders might be anti-idiotypic or bind to the constant regions of the capture antibody. Only 18 binders were specific for the IC with low background binding to the anti-AFB antibody alone (**Fig. S1B**). Those binders that provided a signal-to-background ratio (in presence vs. absence of AFB_1) higher than two were sequenced and further tested for their binding capacity. From these, 12 unique DNA sequences were identified with clone 4H2 exhibiting the highest signal-to-background ratio of 14.2 in the assay (**Fig. S1C**).

The most promising binders (namely, 2A1, 2D5, 4G1, 2E4, 4G10, and 4H2) were produced in a larger scale and purified by affinity chromatography using the His-tag included in the scFv-AP fusion. Fig. 1 shows the response curves of these scFv-APs in the noncompetitive immunoassay. EC_{50}-values between 4.1 ng mL^{-1} (clone 4G10) and 7.8 ng mL^{-1} (clone 2A1) were observed, and all tested binders were concluded to function as anti-IC antibodies for AFB_1. The performance of the identified anti-IC binders was further tested with different aflatoxins to study their specificity. All binders had significant cross-reactivity with aflatoxins B_2 and G_1 (Fig. S2). Clone 4H2 was observed to be the best candidate for detecting total aflatoxin levels.

3.2. Noncompetitive single-step immunoassay

The anti-IC scFv clone 4H2 was used to establish a single-step noncompetitive immunoassay for aflatoxin detection. The assay was based on a biotinylated monoclonal capture antibody (anti-AFB) which was immobilized on a streptavidin-coated microtiter plate. In the same single step, the immunocomplex formed of the anti-AFB and the target analyte AFB_1 was recognized by the anti-IC scFv-AP binder which was further recognized by the Eu-labelled anti-AP antibody (Fig. 2**A**). After incubating the assay reagents together, the wells were washed, and the time-resolved fluorescence signals were measured after adding the europium fluorescence intensifier (EFI) solution. The amount of both immunocomplex participating antibodies in the assay was optimized (**Fig. S3**), and finally 50 ng of biotinylated anti-AFB antibody and 1 μg of scFv-AP were used in the subsequent experiments. Although higher antibody concentrations increased the absolute signals, the assay sensitivity in terms of the EC_{50} value was not further improved with more antibody. In fact, lower antibody concentrations could provide better detection limits as the background signals as well as the standard deviation in the absence of AFB_1 were lower (**Fig. S3B**).

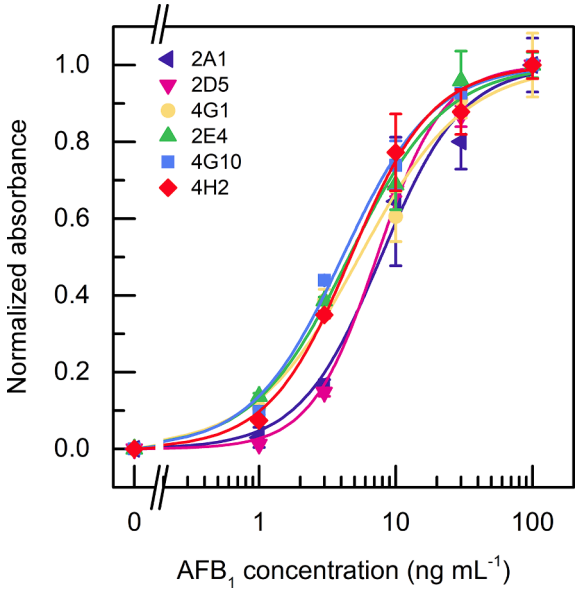

Fig. 1. Comparison of the most promising scFv anti-IC binders (namely, 2A1, 2D5, 4G1, 2E4, 4G10, and 4H2) in the noncompetitive immunoassay for AFB_1. The measured absorbance (normalized to the maximum and minimum values) is depicted as the average of three replicates ± standard deviation.

Moreover, the effect of the incubation time on the assay performance was tested. With increasing incubation times, the absolute fluorescence signals increased to some extent. However, longer incubation times did not provide significant benefits in the assay sensitivity, and similar EC_{50} values were obtained with different incubation times (**Fig. S4**). Similarly, the assay performance of the single-step assay was comparable to a two-step assay where the anti-AFB was added in the first step, and after a washing step, the scFv-AP, sample (AFB_1), and the secondary antibody were added (**Fig. S4A**). Closer comparison of short incubation times (10 and 20 min) showed that while the absolute signals were higher for 20-min incubation, in fact, 10-min incubation could provide a lower detection limit since also the background signals and the variation were lower in that case (**Fig. S4B**). For the optimized assay with 10-min incubation, the EC_{50} value was determined to be 3.5 ng mL^{-1} based on a four-parametric logistic fit, and the dynamic range (from EC_{10} to EC_{90}) was between 0.78 and 15.5 ng mL^{-1}, but with longer incubation times the range could be extended further. The sensitivity of the assay in terms of the LOD with 10 min incubation was 70 pg mL^{-1} (0.22 nM) calculated from blank signal + 3 × the standard deviation of the blank (Fig. 2**B**). Considering using a 10-fold dilution factor for the assay when analyzing sample extracts according to the experimental section, this LOD corresponds to 3.5 μg kg^{-1} aflatoxin in a food sample. Current European Commission Regulations (European Commission, 2010) vary from 4 to 10 μg kg^{-1} for the sum of aflatoxins depending on the foodstuffs in question. Moreover, the sensitivity of the novel immunoassay is comparable to previously reported immunoassays for aflatoxins. While some methods in the literature, for example based on photoelectrochemical detection (Pei et al., 2021), have reported significantly lower detection limits, those often depend on tedious assay protocols, long incubation times, or signal amplification steps, whereas our method benefits from the simple and rapid assay protocol. Comparison of the analytical performance of the single-step noncompetitive with recent examples from the literature is presented in **Table S1**.

3.3. Cross-reactivity

AFB_1 is generally the most predominant aflatoxin among the major aflatoxins (WHO, 2018). However, aflatoxins B_1, B_2, G_1 and G_2 can occur simultaneously in agricultural products, and thus, it can be beneficial if the method is capable of detecting also these aflatoxins. The specificity of the noncompetitive immunoassay was evaluated by determining the cross-reactivity with related aflatoxins. Cross-reactivities with aflatoxin variants ranged from 3% (AFM_1) to 89% (AFG_1). AFM_1 is the hydroxylated aflatoxin metabolite that can be found in milk of mammals fed with aflatoxin contaminated feed, yet, it is not found in grains or nuts. AFB_1 and AFG_1 can be considered to be equally potent regarding carcinogenicity. AFG_2 and AFB_2, on the other hand, are less toxic than AFB_1, and they occur less frequently in agricultural product and are generally not found in the absence of AFB_1 (European Commission,

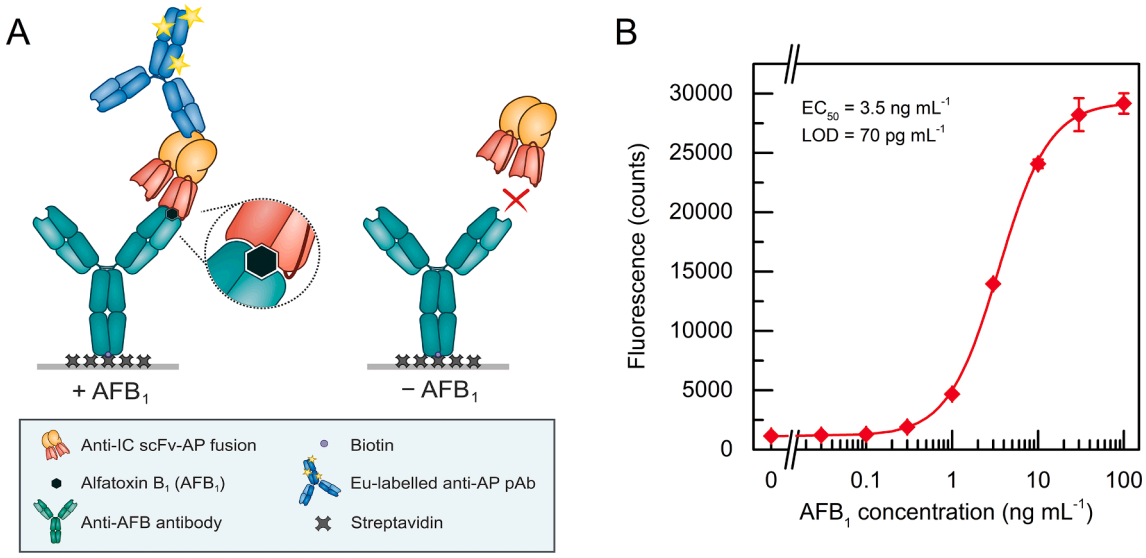

Fig. 2. Single-step noncompetitive immunoassay for AFB_1 detection. (A) Scheme of the assay based on the anti-immunocomplex antibody fragment in fusion with alkaline phosphatase (anti-IC scFv-AP) that recognizes the AFB_1 bound to the monoclonal anti-AFB antibody. The detection is based on the time-resolved fluorescence of the Eu-chelate labelled anti-alkaline phosphatase polyclonal antibody (anti-AP pAb). (B) Standard curve of the single-step assay with clone 4H2. The measured time-resolved fluorescence signals are depicted as the average of three replicates ± standard deviation. The EC_{50} value was determined by the four-parametric logistic fit and the limit of detection (LOD) was calculated as the signal of blank + 3 × standard deviation of the blank.

1996; European Food Safety Authorit, 2013; WHO, 2018; Wogan, 1975). Furthermore, other mycotoxins tested, ochratoxin A (OTA) and deoxynivalenol (DON), did not show any significant cross-reactivity with the tested concentrations (Fig. 3; **Table S2**) showing that the assay is highly specific for aflatoxins.

3.4. Matrix effect and sample analysis

The applicability of the noncompetitive anti-IC immunoassay to the real sample matrix was demonstrated with spiked food samples. Aflatoxins and many other mycotoxins are usually extracted from food and feed samples using high concentrations of organic solvents, such as methanol or acetonitrile. Yet, high concentrations of solvents can interfere significantly with the antibody binding and the immunoassay performance. To assess the influence of methanol in the single-step noncompetitive assay, a series of AFB_1 dilutions were prepared in 7%, 14%, and 27% methanol, and the response was compared to the one in assay in buffer (Fig. 4**A**). With 7% methanol, corresponding to 10-fold dilution of the sample extract in 70% methanol, the performance was comparable to the one of buffer, but higher concentrations significantly decreased the fluorescent response.

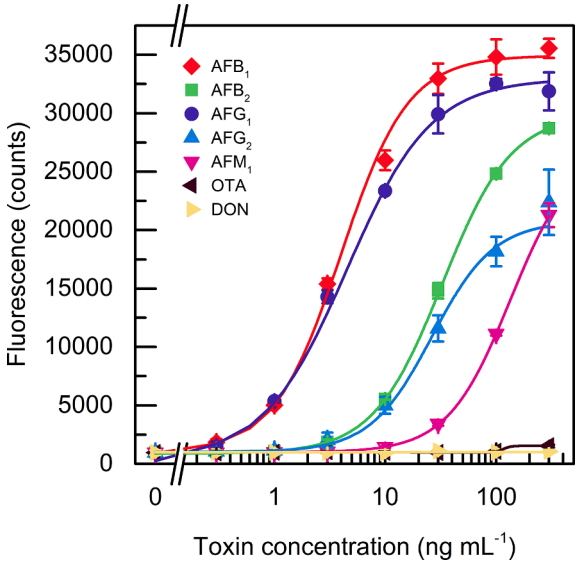

Fig. 3. Cross-reactivity of the single-step assay with different aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1) and other mycotoxins, ochratoxin A (OTA) and deoxynivalenol (DON). The measured time-resolved fluorescence signals are depicted as the average of three replicates ± standard deviation.

A problem associated with many analytical methods for mycotoxins is the potential matrix effect due to co-extractives that might interfere with the assay performance. To assess the potential matrix interference, the analysis was repeated with various food samples. Maize, rice, and hazelnut samples were purchased from a local supermarket, and they were confirmed not to contain AFB_1 at detectable levels by HPLC analysis. After sample extraction in 70% methanol, the assay was carried out using 10-fold diluted sample extracts (Fig. 4**B**). In comparison with the assay in buffer, the extracts seemed to lower the assay response. Most significantly, the maize extract resulted in higher background signals, necessitating a matrix-based standard curve. To avoid the matrix effects or the dilution step necessary for the analysis, one should include other cleanup steps to the protocol. In this work, with the aim of developing a simple and straightforward method, we decided to stick with the simple methanol extraction and dilution procedure since it could provide sufficient performance. Nevertheless, further optimization of the sample extraction protocols, perhaps individually for each sample type, in future might improve the detection limits in real samples.

Finally, the performance of the method was evaluated by determining the AFB_1 concentration in spiked maize, rice, and hazelnut samples. The recoveries ranged from 83% to 129% with relative standard deviations (RSD) of 1–4% (Table 1) demonstrating the applicability of the noncompetitive immunoassay for sample analysis. Confirmatory analysis of the same spiked samples was performed with the HPLC.

4. Conclusions

Analysis of small molecule contaminants remains a challenging yet important task for food and environmental safety. Aflatoxins are common contaminants in many agricultural products, and there is a need to develop rapid, low-cost, and accurate detection methods for these toxins

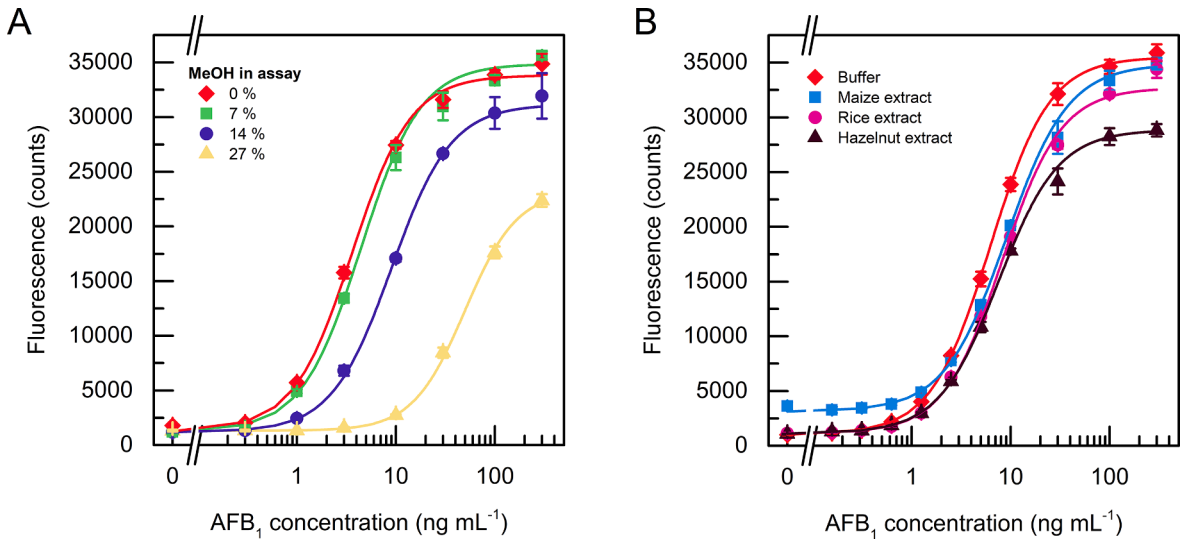

Fig. 4. The effect of methanol and sample matrix on the assay performance. (A) The assay was repeated with different percentages of methanol (7–27% in the final assay volume, corresponding to diluting the sample extract in 70% methanol between 10- and 2.5-fold, respectively). Assay in assay buffer (0% methanol) was used as a reference. (B) The assay was performed in the presence of maize, rice, or hazelnut sample extracts (10-fold dilution in the assay). The measured time-resolved fluorescence signals are depicted as the average of three replicates ± standard deviation.

Table 1
Analysis of spiked samples with the single-step immunoassay and HPLC.

Sample	Spiked AFB_1 (μg kg^{-1})	Immunoassay		HPLC	
		Detected AFB_1 ± SEM (μg kg^{-1})	Recovery	Detected AFB_1 ± SEM (μg kg^{-1})	Recovery
Maize flour	20	25.7 ± 0.7	129%	21 ± 7	106%
	30	30.9 ± 0.7	103%	31 ± 3	105%
	50	47 ± 1	94%	54 ± 17	107%
	100	112 ± 9	112%	113 ± 9	113%
Rice flour	20	19.4 ± 0.4	97%	16 ± 4	82%
	30	28 ± 1	94%	30 ± 13	101%
	50	51 ± 2	101%	67 ± 31	135%
	100	99 ± 1	99%	92 ± 14	92%
Hazelnut	20	16.6 ± 0.1	83%	28 ± 9	140%
	30	28.5 ± 0.5	95%	32 ± 2	106%
	50	45 ± 1	90%	52 ± 5	103%
	100	91 ± 4	91%	91 ± 6	91%

SEM, standard error of the mean ($n = 3$).

in the field and low-resource settings. In this work, we used phage display technology to select an anti-immunocomplex antibody for aflatoxins from a synthetic antibody library. The study clearly demonstrates the usefulness of phage display antibody library techniques in the development of binders against difficult targets, such as immunocomplexes. Based on the anti-IC antibody and the monoclonal capture antibody, a single-step noncompetitive immunoassay was developed using a europium-labelled secondary antibody. The immunoassay, with a total analysis time of 15 min, exhibited good analytical performance and was used to analyze spiked maize, rice and nut samples with promising results. Based on the cross-reactivity profiling, the assay, developed with AFB_1, also shows potential for the detection of the equally toxic AFG_1, while the less toxic variants AFB_2 and AFG_2 as well as the milk-borne AFM_1 are recognized much less efficiently. Furthermore, no significant cross-reactivity was observed with other mycotoxins often present in the same samples. This simple and fast method provides a practical tool for aflatoxin screening, and altogether, demonstrates the potential of single-step noncompetitive immunoassays for the analysis of small molecule contaminants. Future work aims to optimize further the sample extraction protocols to establish routine procedures for the robust analysis of different sample matrices.

CRediT authorship contribution statement

Riikka Peltomaa: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. **Asmaa Abbas:** Formal analysis, Investigation, Writing – review & editing. **Tapani Yli-Mattila:** Writing – review & editing, Resources, Supervision. **Urpo Lamminmäki:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133287.

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