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Rapid screening of aflatoxin B1 in beer by fluorescence polarization immunoassay

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ABSTRACT

This manuscript describes the development of a sensitive, fast and easily-performed fluorescence polarization immunoassay (FPIA) for the mycotoxin aflatoxin B1 (AFB1) in various beer samples, both lager and dark. The highest sensitivity was determined for six poly- and monoclonal antibodies selective towards aflatoxins. The sample pretreatment design was emphasized since beer samples are characterized by extremely diverse matrices. Herein, the choice of sorbent for effective removal of matrix interferences prior to analysis was crucial. The samples were diluted with a borate buffer solution containing 1% PEG 6000 and passed through the clean-up column packed with NH2-derivated silica. This sample pretreatment technique was perfectly suitable for the FPIA of lager beer samples, but for dark beer and ale it did not suffice. An artificial matrix was constructed to plot a calibration curve and quantify the results of the latter samples. The developed immunoassay was characterized by a limit of detection of 1 ng mL^{-1}. Apparent recovery values of 89–114% for lager and 80–125% for dark beer were established. The FPIA data for AFB1 was characterized by elevated linear regression coefficients, 0.9953 for spiked lager and 0.9895 for dark beer samples respectively.

1. Introduction

Alcoholic beverages play an important role in the social life of many contemporary societies. This is especially true for low alcohol content drinks such as beer. Depending on the region and culture, the consumption frequency and quantities of these beverages can be high. Besides the known adverse effects of alcohol, beer could also be the source of several toxins and pollutants transmitted from grains during the brewing process. The agricultural products mainly used for beer production, i.e. wheat [1,2], barley [3,4], and corn [5,6], could be contaminated by extremely toxic mycotoxins such as aflatoxins. The presence of mycotoxins in beverages, such as beer, is already described more than once [7–10].

Aflatoxins are secondary metabolites formed by certain *Aspergillus* spp., in particular *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus*, and belong to the most predominant mycotoxins. They can be considered as one of the most important chronic dietary risk factor [11–13]. Nowadays, 17 aflatoxins have been isolated, but only four of them (B1, B2, G1 and G2) are considered as significant food contaminants. Aflatoxins have been shown to be teratogenic, mutagenic, genotoxic and hepatocarcinogenic to humans and animals, depending on the duration and level of exposure [14–16]. Aflatoxins B1, B2, G1, and G2 have been classified as the *group I* human carcinogens [17]. AFB1 can survive the beer brewing process and is only partially removed [18,19]. In fact, considerable amounts are transferred into beer [20–22].

A great variety of chromatographic techniques for AFB1 detection were published. Since aflatoxins possess intrinsic fluorescence, they could be determined by HPLC with fluorescence detection [23–25]. The use of amperometric detection was also tested [26]. Although liquid chromatography coupled with (tandem) mass spectrometry (LC–MS/MS) is one of the most common technique for aflatoxin determination [27–29], the determination of aflatoxins in beer is not widely described. The LC–MS/MS techniques designed by Ventura et al. [30] and Al-Taher et al. [31] are a few examples. And despite a big diversity of the published immunochemical approaches for rapid screening of aflatoxin B1 [32–35], only a few examples devoted to the determination in beer samples [9,36].

Fluorescence polarization immunoassay (FPIA) is an extensively used immunochemical detection strategy for mycotoxins' determination [37,38]. This homogeneous technique perfectly meets the requirements of an easy-to-operate, reliable, fast and cost-effective analysis. FPIA is based on a difference in fluorescence

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polarization of labeled antigen and labeled analyte–antibody complex, a low and high molecular weight compound, respectively. The FPIA determination of AFB1 in different matrices was already more than once described [39,40], but no protocols devoted to the FPIA determination of this mycotoxin in beer are available.

One of the most valuable characteristics of FPIA from an operational perspective is the lack of need for a separation step to isolate bound from unbound compounds. However, this advantage generates the requirement of a purification procedure. This procedure should be rapid and easy, in order to retain the advantages of FPIA, and simultaneously it should effectively eliminate matrix components that can interfere in the analysis, to avoid a decrease in sensitivity.

The manuscript presents an optimized clean-up procedure with the followed highly sensitive FPIA for AFB1 detection in beer samples. To the best of our knowledge, there is no prior art available in the literature.

2. Material and methods

2.1. Reagents and materials

AFB1, aflatoxin M1 (AFM1), fluorescein isothiocyanate (FITC) isomer I, ethylenediamine dihydrochloride, N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), triethylamine (TEA), O-(carboxymethyl)hydroxylamine hemihydrochloride (CMO), sodium tetraborate, and sodium chloride were purchased from Sigma-Aldrich (Bornem, Belgium). Aminopropyl derived silica, Bondesil NH_2 (diameter 40 μm, pore size 90 Å), Bond Elut SAX-columns (500 mg), Bond Elut R Si columns (500 mg), tubes (Bond Elut reservoir, 1 mL) and polyethylene frits (1/4 diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium). Bakerbond™ SPE C18 columns were purchased from JT Baker (Deventer, Holland). Kieselgel 60 (0.063–0.200 mm) (SiO_2) was from Merck (Darmstadt, Germany).

The monoclonal anti-AFB1 antibody (MAb no. 1, 1.7 mg mL^{-1}) and anti-AFM1 antibody (MAb no. 2, 1.5 mg mL^{-1}) were kindly provided by Prof. Ch. Xu (School of Food science and Technology, Southern Yangtze University, WuXi, China). The rabbit antisera containing polyclonal antibody against AFB1 (PAb no. 3 and PAb no. 4) was lent by Prof. Duck-Hwa Chung (Gyeongsang National University, Jinju, South Korea). The specific anti-AFB1 antibody (MAb no. 5, 5.6 mg mL^{-1}) was catered by Prof. P.G. Sveshnikov (Russian research center for molecular diagnostics and therapy, Moscow, Russia). The rabbit antiserum containing polyclonal antibody against AFB1 (PAb no. 6) was granted by Yu (Chung Shan Medical University, Taichung, Taiwan). All other chemicals and solvents were of analytical grade. Ultrapure water was used throughout. Borate buffer (BB, 2.5 mM, pH~7.5, containing 1% sodium azide (w/v) as preservative) was used as a working buffer. Standard solutions of aflatoxins were prepared by diluting the reference stock solution (1 mg/mL in methanol) in the range of 0.001–100 ng mL^{-1} with BB.

All of the fluorescence polarization measurements for FPIA were performed on a TD × polarization fluorimeter (Abbott Lab., United States) in the PhotoCheck mode.

2.2. Synthesis of ethylenediamine fluoresceinthiocarbamyl (EDF)

EDF was synthesized using the modified technique described by Eremin et al. [41]. Ethylenediamine dihydrochloride (200 mg) was dissolved in the mixture of methanol (5 mL) and TEA (500 μL) and dropwise added to a FITC solution (117 mg) in methanol (10 mL) containing 100 μL of TEA. The solution was mixed for 1 h at RT, then the bright orange pellet was filtrated and dried.

2.3. Synthesis of AFB1–EDF

Because AFB1 does not contain active groups suitable for protein conjugates synthesis, a carboxymethyloxime derivative of the analyte (AFB1–CMO) was used. To synthesize the derivative, a modified technique, described by Thouvenot [42] was applied. AFB1 (10 mg) and O-(carboxymethyl)hydroxylamine hemihydrochloride (20 mg) were dissolved in 1 mL of pyridine and the reaction mixture was stirred for 24 h at RT. Then it was evaporated to dryness in a rotor evaporator at 50 °C and the residue was mixed with 5 mL of distilled water with NaOH to adjust the pH at 8, sonicated for 3 min to suspend the white residue, and the unreacted mycotoxin was extracted 5 times with 2 mL of chloroform each time (the chloroform fractions were removed). The hapten was precipitated in the aqueous phase by the addition of HCl (pH 3) and extracted four times with 10 mL of ethyl acetate. The extract was dried over anhydrous sodium sulfate, filtered and evaporated under vacuum at 50 °C.

The AFB1–EDF was synthesized according to the standard technique of N-hydroxysuccinimide/N, N'-dicyclohexylcarbodiimide activation of hapten's COOH-group. 10 μmol of AFB1 was dissolved in 500 μL of DMF containing 23 mg of NHS and 41 mg of DCC and was mixed. The molar ratio AFB1/NHS/DCC equal to 1/2/2 was used. The reaction mixture was stirred with an orbital shaker for 4 h at RT and was incubated overnight at 4 °C. The formed precipitate was removed by centrifugation (9167*g*, 10 min). Then 4.5 mg of EDF was added to the supernatants and the reaction mixtures were stirred for 2 h at RT in darkness, followed by an overnight incubation at 4 °C.

The synthesized tracer was separated and purified by the thin layer chromatography (TLC) on Silufol chromatographic plates (Czech Republic) with a silica gel layer thickness of 0.25 cm to remove impurities and starting reagents. As an eluent a mixture of methanol and chloroform in a volume ratio of 1/4 was used. The main yellow bands which luminescent in UV light (λ=365 nm) were collected from the chromatographic plate and extracted with methanol. The tracers were kept at 4 °C.

2.4. Fluorescence polarization immunoassay

The optimal concentrations of the immunoreagents were determined prior to optimization of the analysis procedure. The tracer working concentration was defined as the solution that possesses a total final fluorescence intensity ten times higher than the background signal [43]. The working concentrations of antibodies were identified from the graphical relation between their dilutions and the degree of fluorescence polarization (FP). Dilutions were performed with a borate buffer solution in the range 1/100–1/102, 400 (the final volume was 500 μL). Tracer was added to all the antibody dilutions in the optimal concentration (500 μL), and the FP was measured. The dilution curve was built in a semi logarithmic scale, and the optimal antisera dilutions corresponded to 70% of the tracer's binding response to the antibodies.

To construct an FPIA calibration curve, standard solutions of the target analyte were prepared in BB. 50 μL of each standard solution, 500 μL of a tracer working solution and 500 μL of an antibody solution were added, stirred, and the FP was measured and expressed in “milli-polarization” units (mP). Standard FPIA curves were plotted in a semi logarithmic scale with relative FP values (mP/mPmax) on the *y*-axis, and the logarithm of the analyte concentration in the *x*-axis. These curves showed a sigmoidal behavior. The limit of detection (LOD) was defined as the concentration that caused the analytical signal to decrease more than three times the signal-to-noise ratio (based on the results of 20 measurements). The IC_{50} value, the value of 50% binding inhibition, represents the sensitivity of the assay. The dynamic range

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