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Simultaneous determination of aflatoxin B_1, fumonisin B_1 and deoxynivalenol in beer samples with a label-free monolithically integrated optoelectronic biosensor

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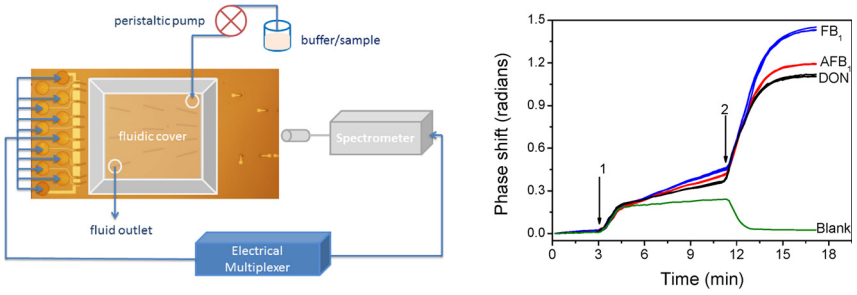
GRAPHICAL ABSTRACT



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ABSTRACT

A label-free optical biosensor for the fast simultaneous determination of three mycotoxins, aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1) and deoxynivalenol (DON), in beer samples is presented. The biosensor is based on an array of ten Mach-Zehnder interferometers (MZIs) monolithically integrated along with their respective broad-band silicon light sources onto a single chip. Multi-analyte determination is accomplished by functionalizing the sensing arms of individual MZIs with mycotoxin-protein conjugates. Assay is performed by pumping over the chip mixtures of calibrators or samples with a mixture of specific monoclonal antibodies, followed by reaction with a secondary anti-mouse IgG antibody. Reactions are monitored in real-time by continuously recording the MZI output spectra, which are then subjected to Discrete Fourier Transform to convert spectrum shifts to phase shifts. The detection limits achieved for AFB_1, FB_1 and DON were 0.8, 5.6 and 24 ng/ml, respectively, while the assay duration was 12 min. Recovery values ranging from 85 to 115% were determined in beer samples spiked with known concentrations of the three mycotoxins. In addition, beers of different types and origin were analysed with the biosensor developed and the results were compared with those provided by established laboratory methods, further supporting the accuracy of the proposed device.

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1. Introduction

Mycotoxins are low molecular weight compounds produced as secondary toxic metabolites by different fungi, belonging mainly to the *Aspergillus*, *Penicillium* and *Fusarium* species [1,2]. Over the years more than 400 mycotoxins have been identified, but only a few of them are found in hazardous quantities in food and animal feedstuff [3,4]. In this category belong amongst others aflatoxins, fumonisins and trichothecenes [1–4]. Aflatoxins are categorized as B or G, with B_1 to be considered as the most potent natural carcinogen [5]. Fumonisins constitute a group of structurally similar compounds, with fumonisin B_1 (FB_1) to account for about 80% of the fumonisins found in contaminated foods [6]. Fumonisins are less toxic than aflatoxins; nevertheless FB_1 has been classified as Group 2B carcinogen, i.e., suspected carcinogen to humans, by the International Agency for Research on Cancer. On the other hand, trichothecenes, and especially their main representative deoxynivalenol (DON), are not classified as carcinogenic to humans. Nonotheless, acute exposure to DON can cause gastroenteritis, vomiting, diarrhea while chronic exposure was shown to impact immunotoxic effects in animal models [7]. In addition, studies in animals have revealed possible synergistic effects when more than one mycotoxin occur in the same sample [8,9]. To protect humans and animals by the acute or chronic exposure to mycotoxins, the European Commission has set maximum allowable limits for the major mycotoxin contaminants in cereals and grains since 2006 [10].

The need to detect mycotoxins in foods and feedstuffs motivated the development of analytical methods aiming to their quantification in such samples. The first rather simple and low-cost method developed was thin-layer chromatography (TLC), suffering however from low sensitivity and difficulty for automation [11,12]. Thus, it was soon replaced by instrumental chromatographic methods, like HPLC [11–14]. The early HPLC methods took advantages of the intrinsic fluorescence properties of some mycotoxins such as aflatoxins [15]. Nowadays, however, HPLC coupled to mass spectrometry (LC–MS/MS) is the method employed by most laboratories worldwide for multiplexed determination of mycotoxins in food matrices [11,12]. The great advantage of the LC–MS/MS methods is their ability to detect a large number of substances in complex matrices with high sensitivity [16]. On the other hand, these methods can only be applied in a laboratory environment by highly skilled personnel, and the analysis cost is relatively high. Thus, immunochemical methods have emerged as alternatives for mycotoxin determination with high detection sensitivity at lower analysis cost [11–13]. In addition, immunochemical methods offer increased potential for on-site determinations through appropriate miniaturization of the required instrumentation and development of rapid assays [17]. Moreover, when a qualitative or semi-quantitative result is sufficient, lateral flow immunoassay strips could be applied. The main argument against the use of immunochemical versus the instrumental ones is the low level of multiplex capacity. To address this issue, multiplexed immunoassays based on color-encoded bead-based suspension arrays, like the xMAP technology, have been developed [18–20]. These assays are usually performed employing dedicated flow-cytometry systems [19,20], although compact, portable and lower cost planar array imaging detectors have been also implemented [20]. Another approach towards the development of small-size devices suitable for on-site application is the implementation of biosensors [21]. To this end, several immunosensors, based mainly on electrochemical or optical transduction principles, have been developed and applied for the detection of mycotoxins in various food matrices demonstrating excellent analytical performance [22–24]. Nevertheless, most of these sensors refer to single-analyte determination, whereas very few implement multiplexed analysis [22,25].

According to World Health Organization (WHO), beer is the most consumed alcoholic beverage worldwide [33]. Beers can be contaminated by mycotoxins due to the use of contaminated cereals for their production while some additional portion or other mycotoxins might arise during the beer brewing process [26–32] raising significant concern for the possible effects on human health [33,34]. The investigations performed so far for the determination of mycotoxin levels in bottled or draft beers rely on LC–MS/MS methods [27–31], enzyme immunoassays [32] and bead-based immunoassays [34], all of them performed in laboratory environment. More recently, there is an increasing trend for multiplexed label-free detection of mycotoxins with portable devices [35] for the rapid on-site testing of mycotoxins occurrence in different stages of beer brewing since it will be beneficial for both the beer manufacturers and the consumers.

To this end, in the present work, we developed an immunosensor based on arrays of monolithically integrated onto silicon chip Mach-Zehnder Interferometers (MZI) [36,37] for the fast and label-free simultaneous determination of aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1) and deoxynivalenol (DON) in beer samples. The sensor implemented has the advantage of high degree of integration since each chip accommodates ten MZIs along with their respective broad-band light sources in an area of only 35 mm^2, providing for the miniaturization of the measurement apparatus. In addition, due to the fact that the whole transmission spectrum is used for the mining of the analytical information, the detection sensitivity is considerably improved with respect to other interferometric sensors [36,37]. To take advantage of these unique sensor features for the simultaneous determination of AFB_1, FB_1, and DON in beer samples, all the parameters including concentration of immunoreagents, assay time, regeneration and reuse of chip, have been optimized. The ability of the developed immunosensor to simultaneously detect the three mycotoxins was evaluated through analysis of beers of different types and origin and the results obtained were compared with those received for the same samples by established mycotoxin analysis methods.

2. Materials and methods

2.1. Materials and instruments

Mouse monoclonal antibodies against AFB_1, FB_1 and DON as well as mycotoxin-protein conjugates (AFB_1-BSA, DON-OVA and FB_1-OVA), AFB_1 and FB_1 were obtained from Aokin AG (Berlin, Germany). DON, deoxynivalenol-3-β-D-glucopyranoside (D3G), 3-acetyl-DON (3ADON), 15-acetyl-DON (15ADON) and nivalenol (NIV) were purchased from Romer Labs (Tulln, Austria). Aflatoxin B_2, Fumonisin B_2, (3-aminopropyl)triethoxysilane (APTES) and highly pure methanol (CHROMASOLV® for HPLC, ≥99.9%) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Acros Organics (Geel, Belgium), goat anti-mouse IgG (affinity purified) and sodium dodecyl sulphate (SDS) were from Merck Millipore (Darmstadt, Germany), and absolute ethanol from Carlo Erba SpA (Milano, Italy). The water used throughout the study was doubly distilled.

Sensor chips were fabricated as it has been described in previous publications [36–39]. The measuring apparatus entailed a docking station that supported both the fluidic and the electrical connections. Aligned to the docking station was an optical fiber that provided for the collection of the transmission spectrum and its transfer to a miniaturized spectrometer (QE65000, Ocean Optics; Dunedin, FL) for the collection of the transmission spectra. The operation of the spectrometer was synchronized with a dedicated electrical multiplexer that turned on/off sequentially the 10 light sources of a single chip every 1 s. In this way, one spectrum from each one BB-MZI of the chip was recorded every 10 s by the PC which controlled the measuring apparatus through a dedicated Labview application.

2.2. Preparation of calibrators and samples

Stock solutions with a concentration of 1 mg/mL were prepared in an 80:20 methanol/water mixture for AFB_1 and DON and in 10:90 methanol/water mixture for FB_1. These solutions were stored in

aliquots at -20 °C in the dark. Calibrators were prepared in assay buffer containing 0.625% (v/v) absolute ethanol. All calibrators were kept aliquoted at 4 °C for up to 2 months. Beer samples were degassed in an ultrasonic bath for 10 min (Elmasonic S30 H; Elma Schmidbauer GmbH, Singen, Germany) and diluted 8 times with assay buffer, prior to the analysis.

2.3. Biochip preparation and assay performance

Chips were hydrophylized by a 30-s O_2 plasma treatment, and then immersed for 2 min in a 0.5% (v/v) APTES solution, followed by gentle washing with water and curing at 120 °C for 20 min. This protocol allowed for the creation of an APTES monolayer that provided for homogeneous protein immobilization through adsorption [40]. The APTES-modified chips were spotted with mycotoxin-protein conjugates (100 μg/ml in 0.05 M carbonate buffer, pH 9.2) using the BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories, Inc.). According to the spotting scheme, depicted in Fig. 1a, three out of ten BB-MZIs were spotted with each one of the three mycotoxin-protein conjugates and the one remaining MZI was spotted with an ovalbumin solution (100 μg/ml in 0.05 M carbonate buffer, pH 9.2) to serve as blank. Coverage of the depicted chip areas with the different solutions was accomplished by deposition of multiple overlapping spots (each one of ~400 microns in diameter). During spotting the chamber humidity was set at 75% and the temperature at 15 °C to avoid drying of the deposited solution. After spotting, the chips were incubated overnight at RT under controlled humidity conditions (75%), so as the spotted conjugates to be immobilized onto the surfaces through physical adsorption. Then, they were rinsed with washing buffer (0.01 M Tris-HCl, pH 8.25, containing 0.9% NaCl), blocked for 1 h (1% (w/v) BSA in 0.1 M $NaHCO_3$, pH 8.5), rinsed again with washing buffer and distilled water and dried under a N_2 stream. Dried biochips were used either immediately or kept at 4 °C in a desiccator until use. For the assay, the biochip was assembled with the fluidic module and was then placed on the docking station. A schematic presentation of the main part of the experimental set-up is depicted in Fig. 1b. At first, the calibrators or the diluted beer samples were mixed at equal volumes with a solution containing the three monoclonal antibodies (6 μg/mL of anti-AFB_1 Mab, 1.5 μg/mL of anti-FB_1 Mab and 3.0 μg/mL anti-DON Mab) in assay buffer (0.05 M Tris-HCl, pH 7.8, containing 9 g/L NaCl, 5 g/L BSA and 0.5 g/L NaN_3). The biochip was equilibrated with assay buffer, and then the mixtures of the calibrators or the samples with the antibodies were run over the chip for 8 min at a flow rate of 40 μL/min. Then, a 10 μg/ml solution of goat anti-mouse IgG in assay buffer was supplied for 4 min at the same flow rate. Regeneration of the chip was performed by passing a solution of 0.5% (w/v) SDS, pH 1.9, for 2 min followed by equilibration of the biochip with assay buffer. After the assay completion, the collected output spectra were subjected to Discrete Fourier Transform and the phase shift in TE polarization due to the reaction was calculated [37]. Thus, for the preparation of the calibration curves, the phase shift in radians corresponding to different calibrators (S_x) were expressed as percent ratios of the response obtained for the zero calibrator (S_0) and plotted versus each mycotoxin concentration in the calibrators in linear/log scale.

3. Results and discussion

3.1. Optimization of the on-chip assays

The assay format employed in the present study consisted of two steps: the primary immunoreaction of the immobilized mycotoxin-protein conjugates with the anti-mycotoxin specific Mabs and the secondary immunoreaction between the immunoadsorbed Mabs and the goat anti-mouse IgG antibody. The secondary immunoreaction was introduced as a signal enhancement step in order to expand the working range of the assays and to shorten the assay duration. Some of the assays conditions, like the composition of the different buffers employed, have been selected through experiments in microtiter plates taking into account the fact that the three assays will have to run simultaneously on chip. Nevertheless, most of the assay parameters, and especially the concentration of conjugates used for coating, the concentration of the antigen-specific antibodies and the duration of primary and secondary immunoreaction steps had to be optimized on-chip. In Fig. 2, the results from the on-chip optimization of the different assay parameters for DON are depicted. Regarding the concentration of the DON-OVA conjugate used for coating, maximum plateau values were received for concentration equal to or higher than 200 μg/mL (Fig. 2a), since however more than 90% of the maximum plateau value is achieved for DON-OVA concentration of 100 μg/mL, this concentration was finally selected. To select the anti-DON Mab concentration, the values provided for the zero calibrator and a calibrator containing 17.5 ng/mL of DON were determined for different anti-DON Mab concentrations. As shown in Fig. 2b, both signals increased as the anti-DON Mab concentration increased; however, the ratio of the signal corresponding to calibrator containing 17.5 ng/mL of DON to that of the zero calibrator, which is an indication of the assay sensitivity, deteriorated when the anti-DON Mab was used at concentrations higher than 1 μg/mL. Thus, in order to combine high zero calibrator signal and assay sensitivity, a 1 μg/mL anti-DON Mab concentration was selected. The duration of the two immunoassay steps was also determined with respect to maximum plateau signal values. The conditions selected were 8 min for the primary immunoreaction (Fig. 2c) and 4 min for the secondary (Fig. 2d) since more than 85% of the maximum plateau signal was obtained for this assay time. Similar experiments were carried out for the other two analytes and it was found that for all the assays, satisfactory zero calibrator signals were obtained for 8-min reaction with the anti-analyte specific MAbs and 4-min reaction with the secondary antibody.

Another parameter optimized was the composition of the regeneration solution. Five different solutions have been evaluated with regard to their ability to remove the bound antibody molecules after the completion of immunoreaction and render the biochip ready for a new assay cycle: a) a commercially available IgG elution buffer for immunoaffinity chromatography; b) a 0.1 M glycine-HCl buffer, pH 2.5; c)

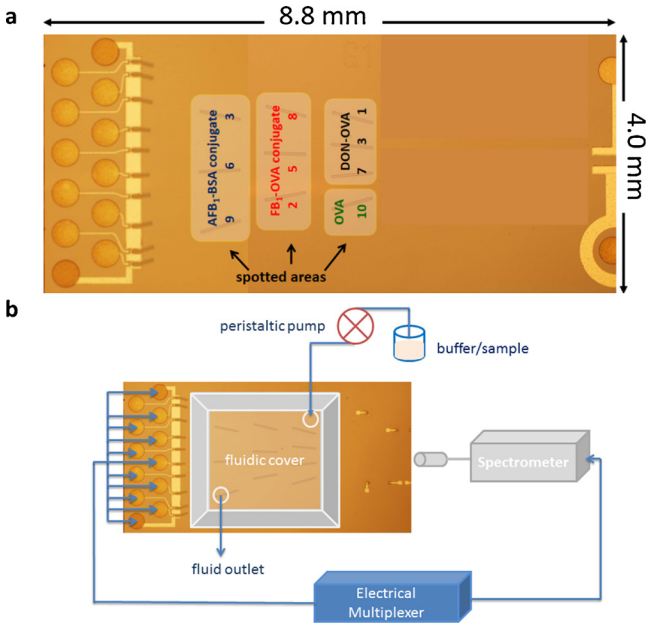

Fig. 1. (a) Photograph of the chip depicting the areas functionalized with the different mycotoxin-protein (sensors 1–9) conjugates and the non-reactive protein (sensor 10). (b) Schematic showing the basic parts of the instrumentation used for the measurements with the developed immunosensor.

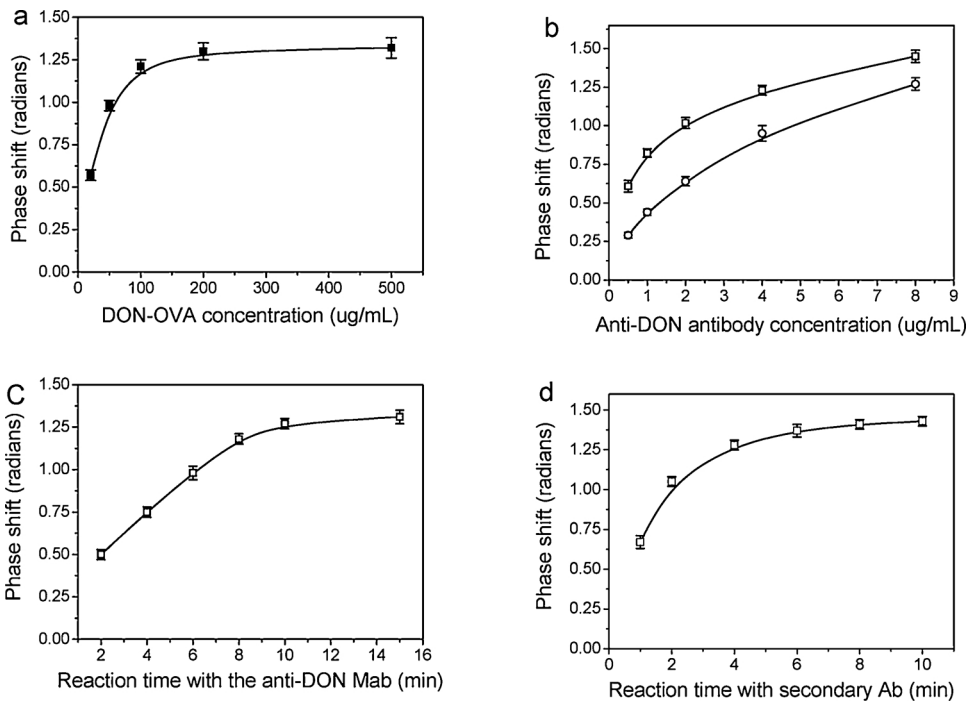

Fig. 2. (a) Effect of DON-OVA conjugate concentration of the zero calibrator signal. (b) Effect of anti-DON Mab concentration on signal obtained for the zero calibrator (□) and a calibrator containing 17.5 ng/mL DON (○) from BB-MZIs coated with 100 μg/mL of DON-OVA conjugate. (c) Effect of primary immunoreaction duration on the zero calibrator signal obtained from BB-MZIs coated with 100 μg/mL of DON-OVA conjugate and assayed with a 1 μg/mL anti-DON Mab solution followed by 4-min reaction with the secondary Ab. (d) Effect of secondary immunoreaction duration on the zero calibrator signal obtained from BB-MZIs coated with 100 μg/mL of DON-OVA conjugate and assayed for 8 min with 1 μg/mL anti-DON Mab solution. Each point is the mean of 3 chips (7 replicates each) ± SD.

a 50 mM NaOH solution; d) a 100 mM HCl solution, and e) a 0.5% (w/v) SDS solution adjusted to pH 1.9 with HCl. From preliminary experiments it was found that the maximum regeneration efficiency for all the above mentioned regeneration buffers was achieved when they were run over the chip for at least 2 min and thus, this time was adopted in all cases. At first, the amount of analyte-specific Mab remaining on the surface after regeneration was assessed through incubation with secondary antibody and expression of the signal obtained as percent ratio of the initial zero calibrator signal. As shown in Fig. 3a, for the AFB_1 and FB_1 assay the higher regeneration efficiencies were received using either 100 mM HCl or 0.5% (w/v) SDS, pH 1.9; for DON, 50 mM NaOH and 0.5% (w/v) SDS, pH 1.9, were the best performing regeneration solutions. In the frame of assays harmonization, 0.5% (w/v) SDS, pH 1.9, was selected as regeneration solution for further experimentation. The stability of the biochip towards regeneration was assessed through repetitive assay/regeneration cycles. As it can be concluded from the results presented in Fig. 3(b–d), the biochips were stable allowing for at least 20 assay/regeneration cycles without significant drop in the zero calibrator signal for all three assays. A small signal drop is observed for FB_1 and DON assays after 15 assay/

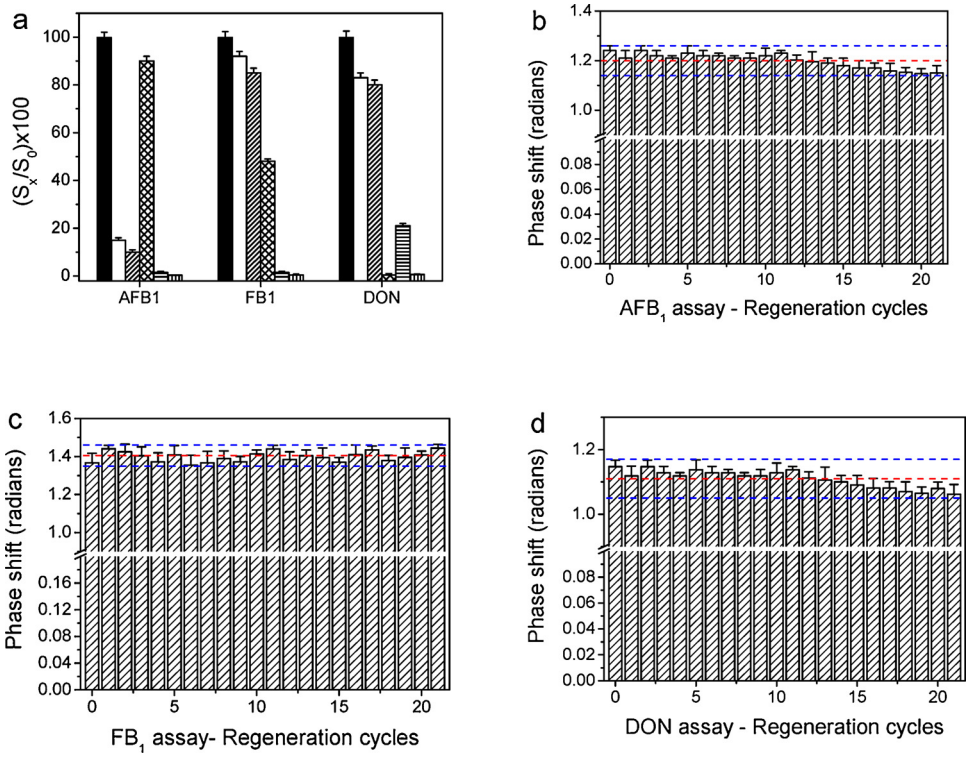

Fig. 3. (a) Percentage of signal obtained by running secondary antibody after regeneration with IgG elution buffer (white columns), 0.1 M glycine-HCl buffer, pH 2.5 (striped columns), 50 mM NaOH (cross-stitched columns), 100 mM HCl (horizontally striped columns), or 0.5% (w/v) SDS, pH 1.9 (vertically striped columns) with respect to the initial zero calibrator signal (black columns). (b–d) Zero calibrator values obtained after repetitive assay/regeneration cycles for AFB_1 (b), FB_1 (c) and DON (d). Each point is the mean of 3 chips (7 replicates each) ± SD. Solid red lines correspond to mean values of the 22 measurements; dashed blue values correspond to mean ± 2SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regeneration cycles which is however less than 10% of the zero calibrator signal obtained during the first run. These results further support the effectiveness of the selected regeneration solution and demonstrate the stability of immobilized onto biochips molecules.

3.2. Selection of matrix for preparation of calibrators

The effect of beer on the zero calibrator signal was determined for each one of the three mycotoxins using both undiluted beer and beer diluted 2, 4, 8 or 16 times with assay buffer. The beer used did not contain detectable amounts of the three mycotoxins as was verified through analysis by LC–MS/MS. The values obtained were compared to those provided by the zero calibrator in assay buffer and the results are presented in Fig. 4a–c for the AFB_1 (a), FB_1 (b), and DON (c) assays, respectively. As shown, the presence of beer affects differently each assay. In case of AFB_1, the zero calibrator signals for the undiluted as well as for the 2- and 4-times diluted beer samples were the same with those provided by the assay buffer, while further dilution resulted in about 10–15% drop of the signal compared to assay buffer. In FB_1 assay, undiluted beer provided significantly higher zero calibrator signals (almost 30%) as compared to assay buffer; a difference that was gradually reduced as the beer dilution increased leading to values equal to those obtained with the assay buffer for 8- or 16-times diluted beer. The opposite occurred in case of DON; the undiluted beer provided approximately 35% lower zero calibrator signal compared to assay buffer; however, this difference was gradually reduced by dilution levelling out for a dilution factor equal to or higher than 8. Although in all cases, an 8-times dilution seemed to minimize the effect of beer matrix, the zero calibrator values still differed from those of buffer. Thus, assay buffer containing 0.625% (v/v) ethanol was also tested, which corresponded to a mean ethanol content of a typical beer diluted 8-times with assay buffer. It was found that the addition of 0.625% (v/v) ethanol to the assay buffer led to zero calibrator values equal to those obtained for 8-times diluted beer. To further test the suitability of this solution for calibrators' preparation, the calibration curves obtained using solutions prepared in beer diluted 8 times with assay buffer, assay buffer containing 0.625% ethanol, or assay buffer without ethanol were compared. As shown in Fig. 4d, for AB_1, the calibration curves obtained with solutions prepared in 8-times diluted beer or assay buffer containing 0.625% (v/v) ethanol were identical. Similar results were obtained for the other two assays, leading to adoption of assay buffer containing 0.625% (v/v) ethanol as the matrix for calibrators' preparation. It should be noted that by using these calibrators different types of beers (lager, dark, ale, etc.) could be analysed without noticeable matrix effect.

3.3. Evaluation of assay specificities

The development of a chip for the simultaneous determination of the three mycotoxins necessitates that the response obtained from each sensor in the array is highly specific. Thus, the possible cross-reaction of the anti-mycotoxin-specific antibodies with the immobilized mycotoxin-protein conjugates was tested using chips modified as indicated in Fig. 1a by running over the chip separately each one of the monoclonal anti-mycotoxin antibodies followed by reaction with secondary antibody and regeneration. As shown in Fig. 5a, response distinguishable from that of the blank sensor (spotted with OVA) was obtained for each one of the three sensor groups only when the respective specific Mab was run over them. In addition, the responses obtained from a chip also modified with the three mycotoxin-protein conjugates upon running a mixture of the three anti-mycotoxin specific Mabs followed by secondary antibody (Fig. 5b) are identical to those provided from the sensors when probed only with the respective Mab, further confirming the specificity of the response. In Fig. 5b, the response of the blank sensor is also indicated. As shown, there is an increase in the signal upon introduction of the calibrator/Mabs solution mixture that is ascribed to the difference in the refractive index of the assay buffer, with which the sensor has been equilibrated, to that of the calibrator/Mabs

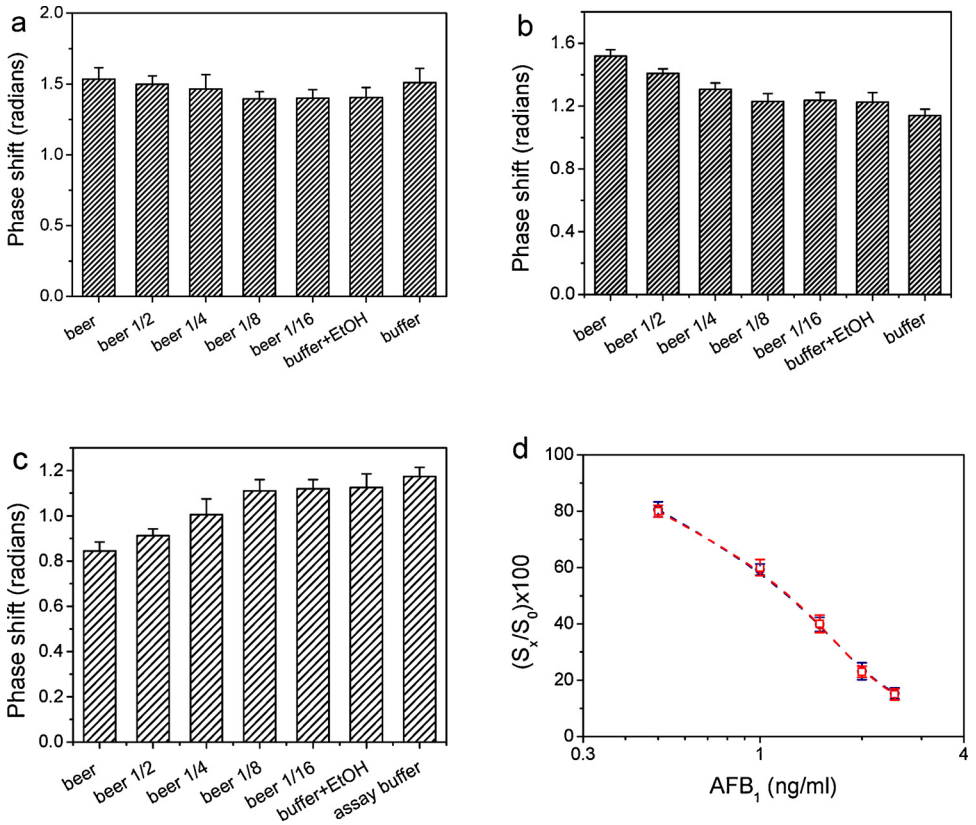

Fig. 4. Zero calibrator signals for AFB_1 (a), FB_1 (b), and DON assay (c) obtained with calibrators prepared in different matrices. (d) Typical AFB_1 calibration curves obtained with calibrators prepared in beer diluted 8 times with assay buffer (□) or assay buffer containing 0.625% ethanol (○). Each point is the mean of 3 chips (7 replicates each) ± SD.

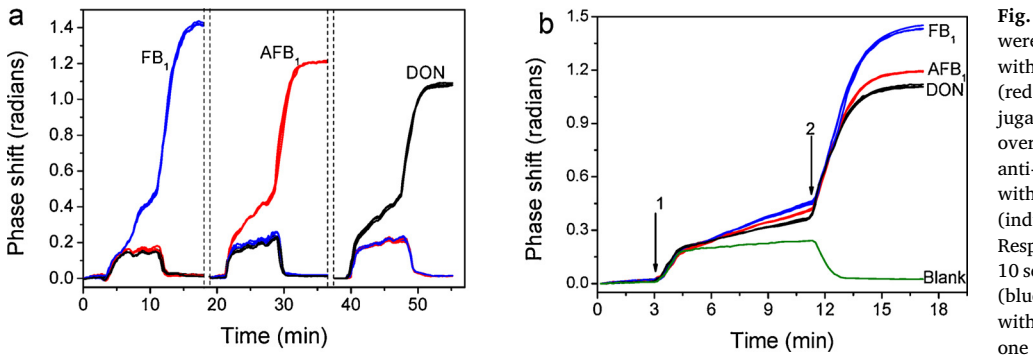

Fig. 5. (a) Responses obtained from a chip were 3 out of 10 sensors have been modified with FB_1-OVA (blue lines), 3 with AFB_1-BSA (red lines) and the rest 4 with DON-OVA conjugate (black lines) when running sequentially over the chip the anti-FB_1, the anti-AFB_1 and anti-DON Mab each one followed by reaction with secondary antibody and regeneration (indicated by the two dashed vertical lines). (b) Responses obtained from a chip where 3 out of 10 sensors have been modified with FB_1-OVA (blue lines), 3 with AFB_1-BSA (red lines), 3 with DON-OVA conjugate and the remaining one by OVA (green line) when running over the chip a mixture of anti-FB_1, anti-AFB_1, and anti-DON Mabs (arrow 1 to 2) followed by reaction with secondary antibody (arrow 2 to end of run). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

solution mixture. When, however, the secondary antibody solution which is also prepared in assay buffer is introduced the blank sensor response returns almost to its initial value demonstrating the low non-specific binding signal.

The specificity of Mabs implemented in the study towards analytes and/or metabolites of the three targeted analytes was also evaluated. For the AFB_1 assay cross-reactivity towards AFB_2 was checked, for FB_1 cross-reactivity towards FB_2, whereas for DON the compounds tested were D3G, 3ADON, 15ADON and NIV. In all cases, calibrators of each one of the potential cross-reactants with concentration ranging from 1000 to 0.1 ng/mL were prepared (by 10-fold serial dilution) in assay buffer, and run as single-analyte assays. Cross-reactivity (CR) was calculated by the following formula: %CR = (IC_{50} target analyte/IC_{50} cross-reactant)x100, where IC_{50} target analyte is the concentration of AFB_1, FB_1 or DON, respectively, corresponding to 50% inhibition and IC_{50} cross-reactant is the concentration of the tested compound which provided 50% inhibition of the respective zero calibrator signal. The percentage of cross-reactivity determined for AFB_2 to AFB_1 assay was approximately 1%, and thus no significant effect on the AFB_1 is expected taking into account that AFB_1 is the aflatoxin predominantly found in cereals. The Mab against FB_1 exhibited a cross-reactivity of 55% towards FB_2, which is desirable to a degree since the respective EU regulation refers to the sum of the two analytes [19]. Finally, the Mab against DON showed a high cross-reactivity with 3AcDON (~480%), less cross-reactivity with D3G (19.6%) and almost negligible cross-reactivity with 15AcDON and nivalenol (< 0.1%). The high cross-reactivity with 3AcDON is not expected to interfere greatly with the DON analysis since 3AcDON is generally encountered at much lower concentrations (20–100-times lower) than DON [34,41]. On the other hand, D3G could be found in amount of about 20% of the amount of DON in cereals, and in higher amounts in beer, and can therefore interfere to some extent with DON analysis [34].

3.4. Analytical characteristics of the assays

Characteristic real-time responses corresponding to calibrators containing different concentrations of the three analytes are provided in Fig. 6a–c for AFB_1 (a), FB_1 (b), and DON (c), respectively. The corresponding calibration curves are illustrated in Fig. 6d. The assays limit of detection (LOD) was calculated as the concentration corresponding to

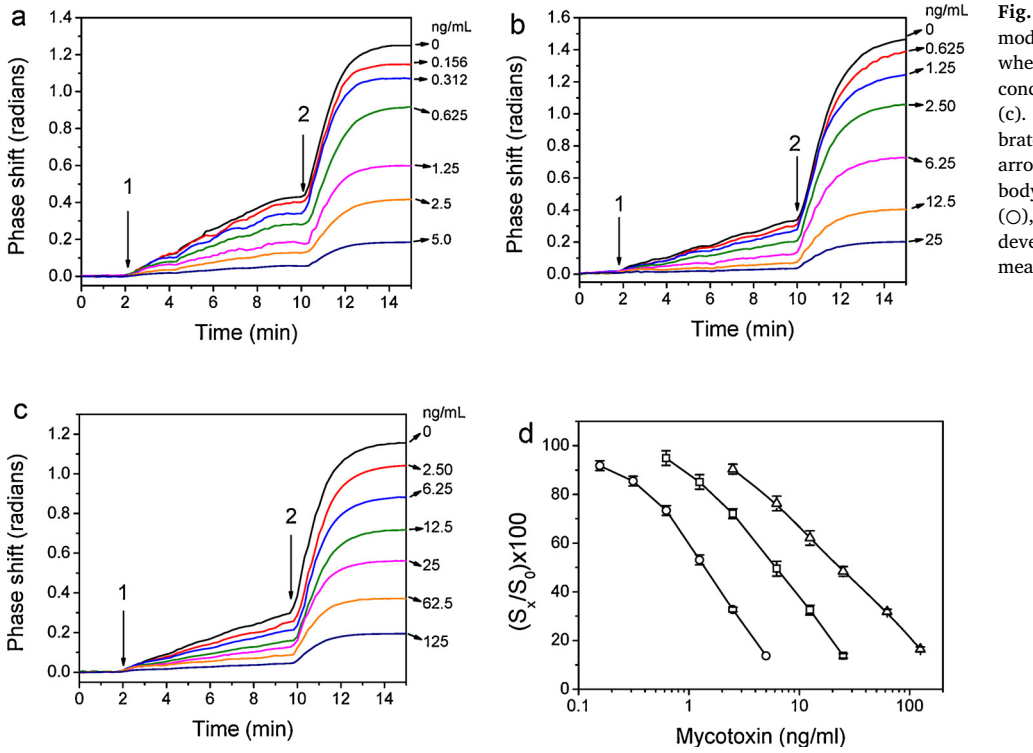

Fig. 6. (a–c) Responses obtained from chips modified with mycotoxin-protein conjugates when running calibrators containing different concentrations of AFB_1 (a), FB_1 (b), and DON (c). Arrow 1 indicates the time point of calibrator/antibody mixture introduction and arrow 2 the introduction of secondary antibody. (d) Typical calibration curves of AFB_1 (○), FB_1 (□), and DON (△) obtained with the developed immunosensor. Each point is the mean of 3 chips (3 replicates each) ± SD.

the mean value of 21 replicate determinations of the zero calibrator signal -3 standard deviations (SD) and were 0.1, 0.7 and 2.5 ng/mL, for AFB_1, FB_1, and DON, respectively, in assay buffer which corresponded to 0.8, 5.6 and 20 ng/mL, respectively, in undiluted beer taking into account the 8-times dilution. The limit of quantification (LOQ) was determined as the concentration corresponding to mean value of zero calibrator -6SDs (n = 21), and was 1.6, 11.2 and 40 ng/mL for AFB_1, FB_1, and DON, respectively, in beer, with linear working ranges up to 5.0, 25 and 125 ng/mL, respectively, in buffer or 40, 200, and 1000 ng/mL in undiluted beer.

The assay repeatability was determined by analyzing three control samples prepared in a lager beer and spiked with the three mycotoxins at different concentration levels that covered the whole range of the calibrators. The intra-assay coefficient of variation (CV) was determined by running 3 replicates of each control in the same day while the inter-assay CV was determined by duplicate measurements performed in 10 consecutive days and their values for the three controls were less than 12% and 16%, respectively. The assay accuracy was also evaluated through recovery experiments using beers that did not contain detectable amounts of the three mycotoxins as determined through analysis with LC–MS/MS [34]. For this purpose, six beers corresponding to different types, i.e., lager, pilsner, ale, dark, etc., were fortified with 3 different concentrations of each one of the three mycotoxins (see Table 1). All beer samples were analysed in triplicate both prior to and after the addition of mycotoxins and the recovery values were calculated by the following equation:

$$\% \text{ recovery} = [\text{amount determined/amount added}] \times 100.$$

As shown in Table 1, the recovery values determined for the three mycotoxins and the 6 different beer samples ranged between 85.0 and 115%, indicating the good accuracy of the developed immunosensor.

3.5. Analysis of beer samples

In total 29 beers, of different types and origin, have been analysed with the developed immunosensor. AFB_1 was not found in detectable concentrations in any of the samples analysed either with the immunosensor, the ELISA or the LC–MS/MS method. Detectable concentrations of FB_1 have been determined in 7 samples whereas most of them contained DON (Table S1). All samples were analysed by the respective ELISAs whereas selected samples have been also analysed by LC–MS/MS methods following a previously published protocol [34]. As shown in Table S1, the results obtained with the immunosensor are in general in good agreement with those obtained with the ELISA methods developed using the same immunoreagents. It should be noted, that the differences in the DON values obtained for some samples (like 12, 614, 991, 1026, U7, and U8) could be ascribed to the fact that these values are lower than the LOQ of the immunosensor, and therefore more prone to signals variation, whereas they are well above the LOQ of ELISA. For this reason we provided these values, mainly, for indicative purposes. A further source of discrepancy of the values determined for the same samples with the two methods could be the dilution of the samples prior to analysis which multiplies the deviation in the values calculated for the undiluted samples.

Comparison of the results obtained from samples analysed with the immunochemical (immunosensor and ELISA) and the LC–MS/MS method is provided in Table 2. In this case, discrepancies were also observed in the values determined for some samples. These discrepancies between the immunochemical and the LC–MS/MS methods can be ascribed at least for some cases to the cross-reactivity of the antibodies used towards metabolites of FB_1 and DON that can be present in the beer samples. This cross-reactivity could led to higher mycotoxin values when a sample is analysed with the immunochemical methods as compared to LC–MS/MS. Another possible source of discrepancies in the values determined by the two methods, especially for samples 941 and 11, for which higher values are determined by LC–MS/MS than with the immunochemical methods, could be matrix effect since these particular samples were specialty dark beers with high alcoholic content (~15%). Despite this fact, due to the simple procedure followed and the short analysis time we anticipate that the immunosensor developed could be employed as a screening tool for the on-site identification of suspicious samples decreasing thus considerably the work volume required for analysis by LC–MS/MS.

Although maximum allowable limits for mycotoxins in beer have not yet been officially established, the European Food Safety Authority (EFSA) has set a tolerable daily intake (TDI) of 2 μg/kg of body weight per day for the sum of FB_1, FB_2 and FB_3 [42] and 1 μg/kg of body weight per day for DON [43]. Thus, an individual that weights 70 kg should consume at least 2 litres of beer with a FB_1 concentration of

Table 1
Recovery values of AFB_1, FB_1 and DON determined in 6 different fortified beer samples.

Beer sample	Aflatoxin B_1			Fumonisin B_1			Deoxynivalenol		
	Amount added (ng/mL)	Amount determined (ng/mL)	% Recovery	Amount added (ng/mL)	Amount determined (ng/mL)	% Recovery	Amount added (ng/mL)	Amount determined (ng/mL)	% Recovery
Amstel lager	5.0	5.2	104	20	18.9	94.5	150	141	94.0
	7.5	8.2	109	50	52.5	105	300	278	92.7
	10.0	9.5	95.0	100	114	114	750	820	109
ZEOS pilsner	5.0	4.8	96.0	20	21.6	108	150	133	88.7
	7.5	8.3	111	50	46.4	89.8	300	345	**115**[a]
	10.0	10.9	109	100	96.0	96	750	710	94.7
Fix dark	5.0	5.6	112	20	22.3	112	150	167	111
	7.5	6.9	92.0	50	43.4	86.8	300	270	90.0
	10.0	9.3	93.0	100	113	113	750	790	105
Vergina pale ale	5.0	5.3	106	20	17.0	**85.0**[a]	150	162	108
	7.5	7.0	93.3	50	52.5	105	300	320	107
	10.0	10.6	106	100	109	109	750	730	97.3
Brink's dark	5.0	5.7	114	20	18.6	93.0	150	134	89.3
	7.5	8.3	111	50	54.5	109	300	260	86.7
	10.0	10.3	103	100	97.0	97.0	750	810	112
KEO lager	5.0	4.7	94.0	20	21.0	105	150	155	103
	7.5	7.2	96.0	50	51.5	103	300	290	96.7
	10.0	9.6	96.0	100	95.5	95.5	750	770	103

[a] Lowest and highest recovery values marked in bold.

Table 2
Concentrations of DON and FB_1 determined in beer samples by the developed immunosensor and comparison methods. Values in parentheses correspond to D3G concentration determined in the samples by the LC–MS/MS method.

Analyte	Sample no.	LC–MS/MS (ng/mL)	Immunosensor developed (ng/mL)	ELISA (ng/mL)
FB_1	48	20	20 ± 1.2	19 ± 0.9
	280	11	10 ± 0.8	11 ± 0.6
	398	28	25 ± 1.0	24 ± 0.8
	423	36	68 ± 3.4	70 ± 2.7
	488	51	54 ± 3.1	53 ± 1.7
	614	< LOD	20 ± 1.1	22 ± 0.9
	941	69	38 ± 2.3	38 ± 1.6
DON	11	104 (56)	64 ± 4.7	68 ± 2.7
	18	11 (21)	26 ± 2.3	25 ± 0.9
	124	41 (68)	92 ± 4.3	90 ± 3.5
	132	24 (36)	56 ± 2.2	55 ± 1.8
	138	< LOQ (42)	80 ± 4.7	82 ± 3.1

approximately 70 ng/mL (like sample 423) or 1 L of a beer with equal concentration of DON (e.g., samples 124 or 138) in order to exceed TDI. These concentrations are within the linear working range of the immunosensor developed which, to the best of our knowledge, is the only immunosensor reported in the literature for the simultaneous determination of the three targeted mycotoxins in beer. The most relevant literature reports include determination with GC– or LC–MS/MS [27–31], ELISA [32] or multiplex microsphere-based immunoassays [34] of one or two of the targeted mycotoxins, in some cases, in combination with other mycotoxins not included in the present study. In general, all these methods are more sensitive than the proposed immunosensor; nonetheless, they require multi-step sample pre-treatment that includes liquid extraction [27,28,30,32] or immunoaffinity preconcentration of the analytes prior to analysis [29,31], pH adjustment and/or centrifugation and much longer sample preparation and analysis time, with the exception of the multiplex microsphere-based immunoassay [34], than the sensor developed. Regarding determination of mycotoxins in beer samples with label-free optical sensors, an imaging surface plasmon resonance sensor (SPR) which detected DON and ochratoxin A has been recently reported [35]. The LOD in beer for DON was 17 ng/mL and the assay duration was 15 min which are comparable to that of the immunosensor developed. Thus, the proposed immunosensor could be a useful tool for the fast and cost-efficient screening of beer samples at the point-of-need (e.g., microbreweries, analysis of craft beers, etc.) with all the associated benefits for both beer producers and consumers. To this direction, steps towards further miniaturization of the set-up by employing micropumps and microvalves and a more elaborated fluidic network that will allow for automated delivery of the different reagents over the chip surface are currently under development.

4. Conclusions

The development of an immunosensor, based on monolithically integrated arrays of Broad-Band Mach-Zenhder interferometers, for the simultaneous label-free determination of mycotoxins AFB_1, FB_1 and DON in beer samples was demonstrated. The three analytes could be determined in less than 15 min with detection limits of 0.8, 5.6 and 24 ng/mL for AFB_1, FB_1, and DON, respectively, in undiluted beer. The assays were characterized by high repeatability and accuracy, while the values determined in beer samples were in good agreement with those provided for the same samples by already established laboratory methods, supporting further the accuracy of the measurements performed by the developed immunosensor. Moreover, the proposed immunosensor could be regenerated and re-used at least 20 times without marked loss of immobilized immunoreagents activity. These characteristics along with the small sensor size strengthen its potential for incorporation into a portable instrument for on-site determinations aiming to mycotoxin screening of multiple samples.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.07.080.

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