Indirect determination of aflatoxin B1 in beer by a multicommitted optical sensor

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**Abstract:** This manuscript reports the determination of Aflatoxin B1 (AFB1), mycotoxin which is considered one of the most carcinogenic substances known. A multicommitted flow injection-solid phase spectroscopy (FI-SPS) system combined with photochemically-induced fluorescence (PIF) is developed, for the first time, for its determination with quantitative purposes. A strongly fluorescent degradation product is obtained on-line by irradiation with ultraviolet light. The determination is carried out by measuring the fluorescence intensity of the photoproduct at 353/424 (λex/λem), once retained on C18 silica gel filling the flow-cell. A linear dynamic range of 0.09-12 µg L⁻¹, detection limit as sensitive as 29 ng L⁻¹ and a relative standard deviation (RSD) of 1.4% were obtained. The method proposed has been satisfactorily applied to the determination of AFB1 in different types of beer (normal and non-alcoholic). Hydrophobic compounds are eliminated from beer samples and AFB1 is extracted with acetonitrile by solid-phase extraction on C18 sorbent. Recoveries of the target compound from spiked beers are between 94-106%. The results obtained in the analysis of real samples are in good agreement with those provided by a reference chromatographic method.
Indirect determination of aflatoxin B₁ in beer by a multi-commuted optical sensor

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Abstract
This paper reports the determination of aflatoxin B$_1$ (AFB$_1$), which is considered one of the most carcinogenic substances known. A multi-commuted flow injection-solid phase spectroscopy (FI-SPS) system combined with photochemically-induced fluorescence (PIF) was developed, for the first time, for its quantitative determination. A strongly fluorescent degradation product was obtained on-line by irradiation with ultraviolet light. The determination was carried out by measuring the fluorescence intensity of the photo-product at 353/424 (λ$_{ex}$/λ$_{em}$), once retained on C$_{18}$ silica gel filling the flow-cell. A linear dynamic range of 0.09-12 µg L$^{-1}$, detection limit as sensitive as 29 ng L$^{-1}$ and a relative standard deviation (RSD) of 1.4% were obtained. The method proposed was satisfactorily applied to the determination of AFB$_1$ in different types of beer (normal and non-alcoholic). Hydrophobic compounds were eliminated from beer samples and AFB$_1$ was extracted with acetonitrile by solid-phase extraction on C$_{18}$ sorbent. Recoveries of the target compound from spiked beers were between 94-106%. The results obtained in the analysis of real samples are in good agreement with those provided by a reference chromatographic method.

Keywords: Aflatoxin B$_1$, sensor, multi-commuted, photochemical induced fluorescence, beer
Introduction

Aflatoxins are the main toxic secondary metabolites of the genus *Aspergillus flavus* and *Aspergillus parasiticus* (Asis et al. 2002; Ali et al. 2005). Under favourable conditions of temperature and humidity, these fungi grow in foods and produce aflatoxins. Mutagenic and carcinogenic activity, teratogenic properties and hepatotoxic action are the most dangerous damage caused by these compounds to human health (Krska et al. 2008). The occurrence of aflatoxins in food can be caused by both direct contamination via grain and grain products or “carry over” of these compounds and their metabolites into animal tissues, milk and meat after intake of contaminated foodstuffs. The most toxic aflatoxin is aflatoxin B$_1$ (AFB$_1$), classified as Group I human carcinogen by the International Agency for Research on Cancer (IARC) (Wang et al. 1998; IARC 2003), resulting in median lethal dose (LD$_{50}$) values ranging from 0.3 to 9.0 mg kg$^{-1}$ body weight. It is regulated by legislation in foods (EC 2006) for direct human consumption at 2 ng g$^{-1}$.

The analytical determination of AFB$_1$ is complicated by two main factors: (1) the complexity of the sample matrix in which it normally appears (corn, peanuts, cottonseed, nuts, almond, figs, fruits and spices) (Gourama and Bulleman 1995; Miller 2008) (AFB$_1$ can colonize and contaminate grain before harvest or during the storage); and (2) the low levels present in these samples. Analytical methodologies must be designed to address these requirements. Firstly, in order to avoid matrix interferences, a typical analysis of AFB$_1$ involves a liquid–liquid extraction (using solvents such as methanol, acetonitrile, and/or their combinations), followed by a clean-up step, e.g., using multifunctional, Florisil or immunoaffinity columns (Nawaz et al. 1995; Giray et al. 2007; Fu et al. 2008; O’Riordan and Wilkinson 2008). Secondly, with respect to its detection, a reliable and sensitive method must be selected for the screening and determination of this compound. Different methods have been established (Turner 2009), including capillary electrophoresis (CE) (Peña et al. 2002), thin-layer chromatography (TLC) (Stroka et al. 2000; Papp et al. 2002; Braicu et al. 2008), high-performance liquid chromatography (HPLC) (Abdulkadar et al. 2000; Gilbert and Vargas 2003; Brera et al. 2007), and enzyme-linked immunosorbent assay (ELISA) (Garden and Strachan 2001; Lee et al. 2004). For positive confirmation, LC-MS methods have also been reported (Sforza et al. 2005; Cavaliere et al. 2007). Although sensitive and accurate, most of the chromatographic methods require expensive
equipment and derivatisation after extraction in order to remove interfering substances. Also, commercially available ELISA methods require enzymatic reactions, washing and separation of bound and free label, while CE has remained a research topic rather than finding application in routine analysis (Shepard 2009).

The intrinsic fluorescence of AFB$_1$, which is due to the presence of a chain of conjugated bonds and heteroatoms in its molecule, could be used for its determination. Nevertheless, the use of spectrofluorimetric analysis is difficult due to the complexity of the matrix, which shows a great variety of natural fluorescent compounds whose spectra often overlap the analyte signal. This situation demands tedious separation steps to enable the AFB$_1$ determination. Different approaches can be used in order to both avoid these inconveniences and increase the sensitivity of the spectrofluorimetric methods; for example: chemical modification of the molecule with chlorine or bromine (Mably et al. 2005), the use of β-cyclodextrin as fluorescence enhancer (Hashemi et al. 2008) or organised media based on surfactants (Shtykov 2002; Goryacheva et al. 2008), and the possibility of working with photochemically induced fluorescence (PIF).

The current work focuses on the development of a novel method for the detection of AFB$_1$ in beer. Its incidence in this product has been already described (Scott and Lawrence 1997; Mably et al. 2005; Zollner and Mayer 2006; Romero González et al. 2009; Pietri et al. 2010). This presence is due to its transfer from contaminated grain (barley and maize) during the brewing process. The use of adjuncts during the processing, normally maize grit, explains this contamination; moreover, AFB$_1$ can be also found in malted barley (Pietri et al. 2010). Sensors may be a good choice for the analysis of AFB$_1$ due to their fast, simple, and low-cost detection capabilities. Nevertheless, to date only electrochemical sensors (Ammida et al. 2004; Owino et al. 2008; Tan et al. 2009) can be found in literature for the determination of this aflatoxin in alcoholic beverages. No optical sensors have been developed for this purpose.

In this paper we evaluate the potential of combining PIF with multi-commutation to determine AFB$_1$ in the complex matrix of beer. The method is based on the on-line generation of a fluorescent photoprodut from AFB$_1$ by UV-irradiation and its monitoring when adsorbed onto C$_{18}$ silica gel. The measurement of the photoproduct retained on this solid support, packed in the flow-cell placed in the detection area, was adopted for improving both detection limit and selectivity. The enhancement in selectivity makes it possible to analyse complex samples, such as beer, whose analysis would not be possible in homogeneous solution due to the high number of interfering
species. This methodology, called flow injection-solid phase spectroscopy (FI-SPS) or flow-through optosensor, combines advantages of FI with the analyte pre-concentration on a small amount of a solid support (López Flores et al. 2005). The use of multi-commutation, as alternative to conventional FIA, is introduced due to its favourable intrinsic advantages such as low-cost equipment, high sample throughput, and simplicity, as long as automation is complete (Reis et al. 1994; Catalá-Icardo et al. 2002). To the authors’ knowledge, this is the first PIF determination of AFB$_1$ in beer to be reported. This is also the first application of multi-commuted-flow methodology to the determination of this aflatoxin. Spiked as well as real samples of beer were used to validate the results.

Materials and methods

Apparatus and Instrumentation

A Varian Cary-Eclipse Luminescence spectrometer (Varian Inc., Mulgrave, Australia) was used for recording spectra and making fluorescence measurements. It was controlled by a microprocessor fitted with a Cary-Eclipse (Varian) software package for data collection and treatment. The following instrumental parameters were used: excitation and emission slit widths were set at 10 and 20 nm, respectively and photomultiplier voltage was 630 V. The excitation and emission wavelengths established were 353/424 nm for the fluorescent photoproduct.

The multi-commuted flow system is shown in Fig 1. It was built with a four channel Gilson Minipuls-3 (Villiers Le Bell, France) peristaltic pump fitted with a rate selector and pump tubing type Solvflex (Elkay Products, Shrewsbury, MA, USA), three 161T031 NResearch three-way solenoid valves (Neptune Research, MA, USA) and an electronic interface based on ULN 2803 integrate circuits. The valves were operated at an electric potential of 12 V and a direct current of 100 mA. PTFE tubing (0.8 mm i.d.) and methacrylate connections were also used. The software for controlling the system was developed in Visual Basic 6.0 by our research group. A 176.752-QS Hellma flow cell (Müllheim, Baden, Germany) (inner volume, 25 µL; light path length, 1.5 mm) filled with C$_{18}$ silica gel was used in the detection area. The solid support was loaded as methanol slurry just up to a height which enabled the light beam to pass completely through the solid phase and the outlet was locked with glass wool, to avoid the beads movement and allow the continuous flow.
For the photochemical on-line AFB\textsubscript{1} conversion, a home-made photoreactor was prepared by loosely coiling 400 cm of PTFE tubing around a low-pressure mercury lamp (15 W, 254 nm). The UV lamp was wrapped in aluminium paper and it was introduced into an aluminium box for maximum light reflection and heat dissipation. The photoreactor was placed just between V\textsubscript{3} and the detection area (see Figure 1). All the experiments were carried out at room temperature.

Other apparatus consisted of a vacuum system 12-port Visiprep SPE Vacuum Manifold (Supelco, Bellefonte, PA), a Crison Model 2002 pH-meter with a glass/saturated calomel combination electrode (Crison, Barcelona, Spain) and a Selecta Ultrason ultrasonic bath (Barcelona, Spain).

**Figure 1**

**Reagents and solutions**

AFB\textsubscript{1} from *Aspergillus flavus* was purchased from Sigma-Aldrich, St. Louis, MO, USA. The stock standard solution (50 mg L\textsuperscript{-1}) was prepared by dissolving 5 mg of the analyte in a 50\% (v/v) methanol:water solution. It was kept away from light with aluminium foil and stored at 4°C in a refrigerator, remaining stable for at least four weeks. Working standard solutions were prepared daily by taking an aliquot of the stock solution and diluting with ultrapure water obtained from a Milli-Q system (Millipore, Bedford, MA). All reagents were analytical reagent grade. The carrier solution (25\% (v/v) methanol:water solution) was prepared by dissolving the required volume of methanol in water. C\textsubscript{18} bonded phase silica gel beads (55-105 \textmu m average particle size) (Waters, Milford, MA, USA) was used as active solid support in the detection area. Cation and anion exchangers (Sephadex SP C-25, Sephadex CM C-25, Sephadex QAE A-25 and Sephadex DEAE A-25, all of them having 40-120 \textmu m average particle size) (Sigma, Alcobendas, Madrid, Spain) were also tested for the retention of AFB\textsubscript{1} photoproduct.

Methanol, acetonitrile, toluene and cyclohexane were obtained from Panreac (Barcelonla, Spain). Octadecyl (C\textsubscript{18}) Bakerbond SPE cartridges of 6 mL with 500 mg of packing material (J.T. Baker, Phillipsburg, NJ, USA) and 0.20 \textmu m nylon filters (Millipore Corporation, Bedford, MA) were also used for solid phase extraction (SPE) procedure.

**Sample preparation**
Seventeen different Spanish beers were purchased from local markets. The sample was kept away from the daylight, as far as possible, with the aid of aluminium foil, along all the process.

Pre-cleaning of the sample. A previous hydrophobic compounds removal, with toluene and cyclohexane, was carried out to improve the subsequent extraction. Initially, beers were previously degassed by sonication and then aliquots of 1 mL were spiked with known amounts of AFB$_1$. Next, they were treated under gentle stirring, in consecutive 3 min steps, first with 1 mL of toluene (twice) and then with 1 mL of cyclohexane (twice). At the end of each step, the mixture was centrifuged for 5 min at 3500 rpm, and the supernatant was discarded. Finally, cleaned beer was dried under a gentle stream of nitrogen to get rid of residual solvent.

Extraction of AFB$_1$. A C$_{18}$ cartridge was conditioned by passing consecutively 10 mL of acetonitrile and 10 mL of water. Subsequently, cleaned beer was loaded onto the column at a flow-rate of 2-3 mL min$^{-1}$ under vacuum. Then, the cartridge was washed with 10 mL of water and vacuum dried for 1 min. Finally, AFB$_1$ retained in the cartridge was eluted with 2×1 mL of acetonitrile and the eluate was concentrated to dryness under a gentle stream of nitrogen. The residue was re-dissolved to a final volume of 10 mL in a 5% (v/v) methanol:water solution and the pH value was adjusted at 5.5 by adding an HNO$_3$ or NaOH solution.

Procedure

The schematic valve system diagram is shown in Fig. 1. All experiments were carried out in triplicate, and the results are expressed as peak height mean values. In the initial status, the UV lamp and the peristaltic pump were switched on to condition the flow system (constant flow rate 2 mL min$^{-1}$) until a stable baseline was recorded. Being all valves switched off, the carrier solution (25% (v/v) methanol:water solution) flowed through the system, while the sample solution was recycled to its vessel. Then, in order to insert the sample in the flow system, valves V$_1$ and V$_2$ were switched on for 75 s and V$_3$ for 15 s. In this way the sample solution circulated through the system, whereas the carrier solution was recycled to its recipient. For the first 15 s of this step, the sample plug was directed towards the waste through V$_3$, so cleaning the tubing between V$_1$ and V$_3$ with the new sample solution. Over the next 60 s, as V$_3$ was deactivated, the sample plug was pumped towards the photo-reactor. After this, all valves were switched off.
again for 35 s, the time required for the sample solution to stay into the photo-reactor. At this point, valve V\textsubscript{1} was switched on for 120 s, in order to recycle the carrier solution to its recipient and stop the flow, so allowing the photo-degradation process. Finally when valve V\textsubscript{1} was switched off and the photo-degradation product arrived to the flow-cell the analytical signal was measured at the corresponding wavelengths, 353/424 nm/nm. The lamp was always switched on during a whole experimental session in order to obtain the best precision.

Results and Discussion

Optimisation of AFB\textsubscript{1} extraction

The fluorescence photo-degradation product generated from AFB\textsubscript{1} is selectively adsorbed on the non-ionic exchanger C\textsubscript{18} silica gel in the working conditions. Nevertheless, owing to the fact that some mycotoxins found in cereals such as ochratoxin A, aflatoxins, fumonisins, and trichothecenes (deoxynivalenol, T-2, and HT-2 toxins) can survive the brewing process, they can also be detected in beer (Romero González et al. 2009). A previous clean-up step was necessary in order to ensure the elimination of others species, including these common mycotoxins present in beers, which could be retained on the solid-phase in the detection area and compete with the photo-degradation product for the active sites.

One of the most often used possibility as clean-up step and direct extraction of liquid samples, in mycotoxins analysis, is SPE. With the purpose of establishing a reliable SPE method for the extraction of AFB\textsubscript{1} from several types of beer, some considerations were taken into account according to the literature (Kralj Cigic and Prosen 2009). First, C\textsubscript{18} cartridges were selected for the extraction procedure due to the slightly better affinity for aflatoxins, compared with others mycotoxins. Second, to elute the target compound, acetonitrile was used because it has demonstrated a higher selectivity for aflatoxins (B\textsubscript{1}, B\textsubscript{2}, G\textsubscript{1}, and G\textsubscript{2}) and HT-2 and T-2 toxins, whilst fumonisins (B\textsubscript{1} and B\textsubscript{2}) were not extracted when this solvent was applied. In addition these two latter mycotoxins were eliminated in the washing step carried out, before the elution, due to their high solubility in water. The washing and elution volumes were optimised taking into account both the elimination of the most polar interfering substances without eluting AFB\textsubscript{1} and the most complete extraction of this latter. The volumes selected were 10 mL of water and 2 mL of acetonitrile, respectively.
Due to a large decrease in the fluorescence of the AFB$_1$ and its photo-product when they were diluted in acetonitrile, it was necessary to evaporate the extract and solubilise it in methanol. The percentage of methanol necessary for the solubilisation of the final residue, once the target compound extracted of the C$_{18}$ cartridge and evaporated, was tested in the range 0-30% (v/v) methanol:water solution. For dilutions with methanol in a percentage larger than 10% the fluorescence signal decreased. Therefore, the minimum amount of methanol capable of dissolving AFB$_1$ which was 5% (v/v) methanol:water solution was selected as optimum.

A pre-cleaning step, prior to the SPE procedure, was also necessary in order to remove hard and soft resins (Molina García et al. 2011). It was fulfilled in the way described in section 2.3. If it is not carried out AFB$_1$ is poorly recovered, probably due to the competition between the target compound and those high molecular weight substances by the active sites of the C$_{18}$ cartridge.

*Preliminary test of sorption*

The native fluorescence of AFB$_1$ can be enhanced by photochemical derivatisation procedures. This technique allows a significant increase in the fluorescence signal of AFB$_1$ due to the reaction with hydroxyl radical produced from water by ultraviolet radiation, which leads to new structures with stable and higher fluorescence (Joshua 1993). This is an interesting alternative to its chemical derivatisation in order to simplify the system, reduce the consumption of reagents and enhance selectivity. With the purpose of improving, even more, both sensitivity and selectivity of the determination of AFB$_1$ after its photo-chemical conversion with UV irradiation, several sorption tests of both the original compound and the fluorescent photo-product on different solid supports were carried out. The retention assays were performed at different pH values and with anion-exchangers (Sephadex QAE A-25, and Sephadex DEAE-25), cation-exchangers (Sephadex SP C-25 and Chelex 100) and non-ionic supports (C$_{18}$ silica gel). In all the cases a higher signal was obtained when a solid support was used, being practically insignificant in the case of cationic exchangers. However, the others (anionic and non-ionic exchangers) provided a significant retention. Anion-exchangers allowed a very strong retention of the AFB$_1$ photoproduct, but its complete elution was very difficult in a reasonable time. Finally, C$_{18}$ silica gel was selected since it provided both a strong retention and a quick elution of the AFB$_1$ photo-product.
Fluorescence spectra of AFB$_1$ and its photo-degradation product, recorded in homogeneous solution, showed maximum excitation/emission wavelengths at 359/446 nm and 359/435 nm, respectively (Fig. 2). This is in agreement with the theoretical blue fluorescence under UV light (430-530 nm for emission) belonging to AFB$_1$ and AFB$_2$, contrary to AFG$_1$ and AFG$_2$ which have green fluorescence (530-590 nm). The spectra of the AFB$_1$ photo-degradation product retained on C$_{18}$ resin showed maxima excitation/emission wavelengths at 353/424 nm (Fig. 2). As a result of the pre-concentration process of the AFB$_1$ photo-degradation product on the active sensing area, a 20-fold increase in the PIF signal was obtained when SPS methodology was used.

**Figure 2**

AFB$_2$ aflatoxin (AFB$_2$) is an important interfering species in the fluorimetric determination of AFB$_1$ due to the substantial overlapping of their spectra (Mohammad et al. 2007). The tolerated AFB$_2$/AFB$_1$ (w/w) ratio in homogeneous solution is about 0.1, which makes impossible to determine AFB$_1$ in presence of AFB$_2$ without a previous separation of this latter. As we could check, in the established working conditions AFB$_2$ did not photo-degrade under UV irradiation, which agrees with the results previously found by other authors in aflatoxin photo-degradation tests (Joshua 1993). On the other hand, although AFB$_2$ itself was retained on C$_{18}$ silica gel solid support it did not interfere in the AFB$_1$ determination at the concentration levels usually found in beer. This fact can be attributed to the increase of the AFB$_1$ fluorescence signal provided by both its photo-degradation and the sorption of the photoproduct generated on a solid support and to the improvement in selectivity originated by the measurement of the analytical signal in solid phase.

Taking into account the possible retention of other organic compounds of the matrix on C$_{18}$ silica gel, the emission and excitation slits and filters were established providing the best sensitivity and selectivity, respectively. The selected excitation and emission slits were 10 and 20 nm, respectively. Filter ranges were set at 250-395 nm for excitation and 420-1100 nm for emission. These values also supplied the best ratio between analytical signal and background noise. With the same criterion, the voltage of the photomultiplier tube was set at 630 V.

**Chemical variables**
Photo-chemical conversion of AFB$_1$, as well as the fluorescence properties of the obtained photo-product and its sorption on the solid support is governed by the pH value. Therefore, this is one of the most important variables to be taken into account. The influence of this variable was studied by inserting into the system different analyte solutions adjusted, using HNO$_3$ or NaOH solutions, to pH values ranging from 1 to 11.

The maximum fluorescence signal was obtained when pH 5.5 was used (Fig. 3). Higher or lower pH values caused a decrease in the intensity of fluorescence. Therefore, a pH value of 5.5 was selected as optimum for the next experiments. Several buffer solutions in a pH ranging 5.0-6.0 and concentration levels between 0.01-0.1 mol L$^{-1}$ were tested (citric acid/sodium hydroxide; sodium acetate/acetic acid; tartaric acid/sodium hydroxide). It was observed the same variation in all cases, fluorescence intensity decreased by half when comparing to the absence of buffer solution. This performance may be due to the interference of buffer ions in the photochemical conversion of AFB$_1$, so sample solution without buffering was finally chosen.

**Figure 3**

The nature of the carrier solution will affect mainly to the sorption of the photo-degradation product on the solid support. Taking into account the non-polar nature of the solid support used in this case, aqueous solutions with different methanol percentage, up to 45% (v/v), were tested in order to obtain the maximum retention of the AFB$_1$ photoproduc. A solution of 20% (v/v) methanol provided the highest analytical signal and a decrease in this latter was observed for higher and lower methanol percentages. On the other hand, an incomplete elution of the photo-product was achieved for methanol percentages lower than 25%. Therefore, as a compromise between sensitivity and complete regeneration of the solid support, a solution of 25% (v/v) methanol:water was selected as carrier solution.

**Flow system variables**

**Irradiation time.** The irradiation time is the residence time of the sample inside the photo-reactor under UV radiation. It is another critical variable in PIF methodology. In order to optimize this variable, 2 mL of 8 µg L$^{-1}$ AFB$_1$ solution were inserted in the system (2 mL min$^{-1}$), the flow was stopped just when the whole plug of sample was within the photo-reactor (400 cm) and this was irradiated for increasing periods of time. After turning off the lamp and re-establishing the flow, the fluorescence signal was
recorded. The results showed that the kinetic of photo-degradation of AFB₁ in the working conditions is slow (Figure 4). With an irradiation time of 120 s the maximum fluorescence signal was obtained. Therefore, this value was selected as optimum one.

**Figure 4**

**Flow rate and photo-reactor length.** In general, the residence time of the sample in the photochemical reactor and, consequently, the irradiation time can be controlled by the flow-rate and/or the length of the tubing around the lamp. As the time required for AFB₁ degradation is high, instead of using a long photo-reactor tubing or stopping the peristaltic pump, the carrier solution was recycled for the required time (120 s) once the sample plug was placed inside the photo-reactor, so stopping the circulation of the sample solution through the system. Consequently, the length of the photo-reactor was selected in order to be just the minimum one required to accommodate the whole sample plug (2 mL), namely 400 cm.

2 mL min⁻¹ was the flow-rate established. This was the maximum flow-rate allowed by the flow-system without overpressure problems and it also allowed the best throughput of the method.

**Sampling time.** In flow-through sensors is possible to improve sensitivity by only increasing the sample volume inserted in the system. This effect is a consequence of the sorption on a constant amount of solid support of increasing amounts of analyte in the detection area. This feature allows both the enhancement of sensitivity, by increasing the sample volume, and the reduction of strong matrix effects by means of a previous dilution of the sample before its insertion in the system.

In a multi-commuted system the sample volume can be controlled by varying the sampling time. With 8 µg L⁻¹ solution of AFB₁ and sampling times ranging from 10 s to 80 s, the fluorescence signal increased linearly until a value of 60 s. In addition, the throughput of the system was also affected by this variable. Figure 5 shows the study of the influence of sampling time. Sampling times lower than 60 s provided a quicker elution of the photoproduct from the solid support but they also originated a significant decrease in the analytical signal. Therefore, a 60 s sampling time was selected as a compromise between fluorescence intensity and peak time. Higher sampling times produced higher analytical signals than that obtained with 60 s. However, they also originated wider flow peaks, so decreasing the sampling frequency.

**Figure 5**
Figures of merit

In the above established optimum working conditions, the calibration curve was established for the photoprodut of AFB\textsubscript{1} after injecting in triplicate, sample solutions containing increasing concentrations of the analyte. Analytical figures of merit are given in Table I. Quantification was carried out by using peak height as analytical signal. Data were fitted by standard least-squares treatment. The proposed methodology responds linearly in the AFB\textsubscript{1} concentration range 0.09-12 $\mu$g L\textsuperscript{-1}. The standard deviations of intercept and slope were also calculated (average of three determinations). Detection limit (LOD) and quantification limit (LOQ) were estimated as the concentration that produced a fluorescence signal equal to three and ten times the standard deviation of background fluorescence, respectively (MacDougall 1980). The LOD obtained with the proposed method allows the determination of this aflatoxin in beer at trace levels. This value, 0.029 $\mu$g L\textsuperscript{-1}, is similar or lower, in most of the cases, than others previously reported (Table II). On the other hand, LOQ attained by the method is much lower than the maximum level fixed by European Commission regulation for all cereals and products derived from cereals (2 $\mu$g kg\textsuperscript{-1}), so making the method suitable for routine analysis.

Tables I, II

Intra-day repeatability was established by comparing the response of 10 independent determinations of solutions containing 5 $\mu$g L\textsuperscript{-1} of AFB\textsubscript{1}. Inter-day repeatability was also performed for 10 consecutive days. RSDs obtained, in both cases, were low, and even though the measurements are made in solid phase.

Analytical applications

The proposed flow-through optosensor was successfully applied to the determination of AFB\textsubscript{1} in beer. The pre-treatment and procedure described in Experimental were used in each instance. The slope of the calibration curve obtained by spiking the final beer extracts with AFB\textsubscript{1} was different to that obtained by spiking the original samples, and both of them were different than that obtained by external calibration. This demonstrated the existence of a light negative matrix effect. This latter is due to the presence in the final extracts of interfering species and the incomplete recovery of AFB\textsubscript{1} after the pre-treatment. Consequently, the calibration curves were constructed with matrix-matched standards, that is, the analysis was carried out by spiking different
aliquots of a beer sample with increasing amounts of the analyte. The matrix effect was
evaluated by comparing the slopes of aqueous standards and standard addition
calibration curves for different beer samples ($m_{\text{standard}}/m_{\text{standard-addition}} \approx 0.9$). Of the
seventeen beers analysed, including normal ($n=12$) and non-alcoholic ($n=5$) beers, only
three samples contained traces of the target compound (0.86, 0.28 and 0.17 µg L$^{-1}$).
AFB$_1$ was not detected in the rest of beer samples investigated, including non-alcoholic
beers.

A recovery study, at three concentration levels, was also performed on both the
three samples containing AFB$_1$ and two of the samples no-containing this latter. The
results obtained summarized in Table III. In all cases good recoveries, ranging from 94
to 106% were achieved. The applicability of the proposed method to the analysis of
AFB$_1$ in beer was demonstrated by comparison with a reference method (Romero
González et al. 2009), based on ultrahigh-performance liquid chromatography-tandem
mass spectrometry. The statistical study of precision and accuracy of both the proposed
and the reference method was performed from $F$ criterion and the $t$ test, respectively
(Saunders and Fleming 1971). The results obtained in every case (Table III) show that
there is no significant statistical difference between the results obtained by both
methods, indicating the utility of the proposed method for routine analytical control.

**Table III**

**Conclusions**

An automatic spectroscopic method for the analysis of AFB$_1$ in beer has been
developed, for the first time. A photochemical induced fluorescence based flow-through
sensor, making use of the multi-commuted assembly, has been used by this effect. The
fluorescent photo-product generated on-line was selectively retained on an active solid
support, originating a transitory fluorescent signal. This fact added to the clean-up step
and SPE extraction carried out on the beer samples, makes the presented method to have
characteristics such as high sensitivity and selectivity, low reagent consumption, easy to
handle, rapidity and simplicity and low cost. Although the determination of AFB$_1$ in
complex matrices by the proposed method needs a previous pre-treatment, the time
consumed is compensated with the rapidity in the measurement of the analytical signal.

On the other hand, the detection limits of the proposed method are similar or even
quite lower than those obtained using reported chromatographic methods for AFB$_1$
determination in beer and other matrices. Therefore, it can be considered as an interesting alternative to the chromatographic determination of this aflatoxin. In addition, the results obtained in the analysis of several types of beer compare well against those supplied by a reference liquid-chromatography method. All this shows the feasibility of the proposed optosensor as screening method for determination of AFB₁ in non-alcoholic and normal beer.

Acknowledgements

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References


Asis R, Romina D, Paola DI, Mario AJ. 2002. Determination of aflatoxin B₁ in highly contaminated peanut samples using HPLC and ELISA. Food and Agricultural Immunology 14:201-208.


**Figure captions**

Figure 1. Multi-commuted flow-injection system. Carrier solution (25% (v/v) methanol:water solution); $V_1$, $V_2$ and $V_3$ three-way solenoid valves; PH photo-reactor; Flow cell filled with C$_{18}$ silica gel. For each solenoid valve, the solid and dotted lines refer to “Off” and “On” positions, respectively. Flow rate, 2 mL min$^{-1}$. The scheme at the lower part shows the valve time program (sampling time, 60 s).

Figure 2. Excitation and emission fluorescence spectra of: (A) AFB$_1$ in homogeneous solution (0.2 mg L$^{-1}$); and (B) photoproduct of AFB$_1$ in homogeneous solution (continuous line) and retained on C$_{18}$ silica gel (dashed line). 0.1 mg L$^{-1}$ of AFB$_1$ (in solution); 12 µg L$^{-1}$ of AFB$_1$ (in solid phase); pH, 5.5; irradiation time, 120 s.

Figure 3. Influence of pH of the sample solution. 8 µg L$^{-1}$ AFB$_1$; carrier solution, 25 % MeOH (v/v); sampling time, 60 s; irradiation time, 120 s.

Figure 4. Influence of irradiation time in fluorescence signal of AFB$_1$. 8 µg L$^{-1}$ of AFB$_1$; sampling time, 60 s; flow-rate, 2 mL min$^{-1}$; photo-reactor length, 400 cm.

Figure 5. Influence of the sampling time in the fluorescence intensity (continuous line) and peak time (dashed lines). 8 µg L$^{-1}$ AFB$_1$; pH, 5.5; irradiation time, 120 s; flow-rate, 2 mL min$^{-1}$.
209x297mm (400 x 400 DPI)
Table I. Analytical parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear dynamic range / µg L⁻¹</td>
<td>0.09 – 12</td>
</tr>
<tr>
<td>Calibration graph</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>6.68 ± 0.04</td>
</tr>
<tr>
<td>Slope / L µg⁻¹</td>
<td>34.8 ± 0.07</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9994</td>
</tr>
<tr>
<td>Detection limit / µg L⁻¹</td>
<td>0.029</td>
</tr>
<tr>
<td>Quantification limit / µg L⁻¹</td>
<td>0.09</td>
</tr>
<tr>
<td>Intraday RSD (%)⁻¹</td>
<td>1.4ᵇ</td>
</tr>
<tr>
<td>Interday RSD (%)⁻¹</td>
<td>2.9ᵇ</td>
</tr>
<tr>
<td>Sampling frequency / h⁻¹</td>
<td>10</td>
</tr>
</tbody>
</table>

ᵃn=10, ᵇ[AFB₁]= 5 µg L⁻¹
Table II. Comparison of the proposed method with others reported in literature.

<table>
<thead>
<tr>
<th>Method</th>
<th>Linear range</th>
<th>LOD</th>
<th>Sample</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid Chromatography (UHPLC) (mass spectrometry)</td>
<td>0.5-100 (µg L⁻¹)</td>
<td>0.04 (µg L⁻¹)</td>
<td>Beers</td>
<td>Romero González et al. 2009</td>
</tr>
<tr>
<td>Liquid Chromatography (UPLC) (mass spectrometry)</td>
<td>1-15 (µg L⁻¹)</td>
<td>0.1 (µg L⁻¹)</td>
<td>Beers</td>
<td>Ventura et al. 2006</td>
</tr>
<tr>
<td>Liquid Chromatography (fluorescent detection)</td>
<td>-</td>
<td>0.019 (µg L⁻¹)</td>
<td>Beers</td>
<td>Scott and Lawrence 1997</td>
</tr>
<tr>
<td>Liquid Chromatography (HPLC) (fluorescence detection)</td>
<td>5-35 (µg Kg⁻¹)</td>
<td>0.06 (µg Kg⁻¹)</td>
<td>Animal feeds</td>
<td>Wejdan et al. 2010</td>
</tr>
<tr>
<td>Liquid Chromatography (HPLC) (fluorescence detection)</td>
<td>0.05-24 (µg L⁻¹)</td>
<td>0.015 (µg L⁻¹)</td>
<td>Food samples</td>
<td>Herzallah Saqer 2009</td>
</tr>
<tr>
<td>Liquid Chromatography (HPLC) (fluorescence detection)</td>
<td>0.5-4 (µg Kg⁻¹)</td>
<td>0.02 (µg Kg⁻¹)</td>
<td>Breakfast Cereals</td>
<td>Polixeni and Panagiota 2009</td>
</tr>
<tr>
<td>Liquid Chromatography (UV detection)</td>
<td>20-200 (µg L⁻¹)</td>
<td>0.32 (µg Kg⁻¹)</td>
<td>Corn and Peanuts</td>
<td>Fu et al. 2008</td>
</tr>
<tr>
<td>Liquid Chromatography (HPLC-PD-FD)</td>
<td>0.1-20 (µg Kg⁻¹)</td>
<td>0.035 (µg Kg⁻¹)</td>
<td>Cereal flours</td>
<td>Quinto et al. 2009</td>
</tr>
<tr>
<td>Liquid Chromatography (HPLC-PCD-FD)</td>
<td>-</td>
<td>0.1 (µg Kg⁻¹)</td>
<td>Rice</td>
<td>Mansooreh 2009</td>
</tr>
<tr>
<td>Spectrofluorimetry</td>
<td>0-18 (µg Kg⁻¹)</td>
<td>0.9 (µg Kg⁻¹)</td>
<td>Wheat</td>
<td>Hashemi et al. 2008</td>
</tr>
<tr>
<td>Spectrofluorimetry</td>
<td>0.1-15 (µg L⁻¹)</td>
<td>-</td>
<td>Pistachio</td>
<td>Mohammad et al. 2007</td>
</tr>
<tr>
<td>Spectrofluorimetry (one-and-two-photon-induced fluorescence)</td>
<td>-</td>
<td>46 (µg L⁻¹)</td>
<td>Wines and beers</td>
<td>Mably et al. 2005</td>
</tr>
<tr>
<td>Electrochemical immunosensor</td>
<td>0.1-10 (µg L⁻¹)</td>
<td>0.06 (µg L⁻¹)</td>
<td>Rice</td>
<td>Tan et al. 2009</td>
</tr>
<tr>
<td>Voltammetry</td>
<td>0.4-40 (µg L⁻¹)</td>
<td>0.15 (µg L⁻¹)</td>
<td>Groundnut</td>
<td>Hajian and Ensafi 2009</td>
</tr>
</tbody>
</table>
Table III. Determination of AFB\textsubscript{1} in beers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Proposed method</th>
<th>Reference method</th>
<th>$t_{\text{calc}}$</th>
<th>$F_{\text{calc}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added (µg L\textsuperscript{-1})</td>
<td>Found ± σ\textsuperscript{a} (µg L\textsuperscript{-1})</td>
<td>Found ± σ\textsuperscript{a} (µg L\textsuperscript{-1})</td>
<td></td>
</tr>
<tr>
<td>Beer 1 (Normal)</td>
<td>-</td>
<td>0.86 ± 0.05</td>
<td>0.90 ± 0.02</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.90 ± 0.09</td>
<td>1.95 ± 0.05</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.83 ± 0.03</td>
<td>2.86 ± 0.10</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.84 ± 0.04</td>
<td>4.96 ± 0.04</td>
<td>2.12</td>
</tr>
<tr>
<td>Beer 2 (Normal)</td>
<td>-</td>
<td>0.28 ± 0.03</td>
<td>0.29 ± 0.08</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.33 ± 0.05</td>
<td>2.25 ± 0.06</td>
<td>1.77</td>
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<tr>
<td></td>
<td>4</td>
<td>4.24 ± 0.09</td>
<td>4.30 ± 0.02</td>
<td>1.22</td>
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<tr>
<td></td>
<td>6</td>
<td>6.30 ± 0.06</td>
<td>6.36 ± 0.08</td>
<td>1.04</td>
</tr>
<tr>
<td>Beer 3 (Normal)</td>
<td>-</td>
<td>0.17 ± 0.07</td>
<td>0.15 ± 0.06</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.22 ± 0.05</td>
<td>6.19 ± 0.02</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.28 ± 0.08</td>
<td>8.17 ± 0.09</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.95 ± 0.02</td>
<td>10.13 ± 0.03</td>
<td>2.16</td>
</tr>
<tr>
<td>Beer 4 (Normal)</td>
<td>-</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>-</td>
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<tr>
<td></td>
<td>1</td>
<td>0.94 ± 0.07</td>
<td>1.06 ± 0.04</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.08 ± 0.04</td>
<td>2.97 ± 0.01</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.11 ± 0.08</td>
<td>5.20 ± 0.06</td>
<td>1.56</td>
</tr>
<tr>
<td>Beer 5 (Non-alcoholic)</td>
<td>-</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6.84 ± 0.05</td>
<td>6.95 ± 0.03</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>9.19 ± 0.02</td>
<td>8.88 ± 0.01</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>11.74 ± 0.08</td>
<td>11.46 ± 0.08</td>
<td>2.25</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average of three replicates
\textsuperscript{b}Theoretical value 2.772 (p=0.05)
\textsuperscript{c}Theoretical value 39.00 (p=0.05)