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Development and integration of an immunoaffinity monolithic disk for the on-line solid-phase extraction and HPLC determination with fluorescence detection of aflatoxin B1 in aqueous solutions

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Abstract

The development and characterization of an anti-aflatoxin B1 (anti-AFB1) immunoaffinity monolithic disk is reported. Polyclonal anti-AFB1 was covalently immobilized in batch on an epoxy-activated monolithic Convective Interaction Media (CIM) disk (12 mm × 3 mm i.d.) by a one-step reaction via epoxy groups of the polymer surface. 0.96 mg of antibody were immobilized and the binding capacity of the CIM disk was determined by frontal analysis. The CIM disk was coupled through a switching valve to a reversed-phase column, namely Chromolith Performance RP-18e. A fully automated HPLC method with fluorescence detection for the determination of aflatoxin B1 in aqueous solution was developed. The total analysis time with the integrated system is 46 min and the retention time of AFB1 is approximately 29 min. The binding capacity of the immunoaffinity disk was evaluated in terms of linearity, precision and accuracy of the extraction procedure. The immunoaffinity support was stable after repeated runs.
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Keywords: Affinity chromatography; Monolithic disks; Aflatoxin B1; Polyclonal antibody

1. Introduction

Mycotoxins are toxic metabolites produced by different genera of fungi. Such toxins are found world-wide in food and feed and exhibit a wide array of biological effects. They have been demonstrated to be genotoxic, carcinogenic, embryotoxic and teratogenic in humans and animals [1–3]. Aflatoxins (AF) are mycotoxins derived by *Aspergillus flavus* and *Aspergillus parasiticus* and are listed as Group I carcinogens by the International Agency for Research on Cancer (IARC), a body of the World Health Organization [3]. The main aflatoxins are the B1 (AFB1), B2, G1 and G2 together with their metabolites, among which the most important is the aflatoxin M1. AFB1 (Fig. 1) is the molecule with the highest toxic significance. It is commonly found in any foodstuff or animal feed which can support fungal growth during growth, harvest, or storage, although the main production has been reported in grains, nuts, copra and cottonseeds. The contamination of foods and animal feed with these mycotoxins is controlled worldwide by legal limits and, depending on the toxicity of these mycotoxins, in the countries of the EU equal limits are valid for aflatoxins: namely 2 μg/kg for AFB1 and 4 μg/kg for all AF in total [4].

The analysis of AF is a challenging task and a variety of well established methodologies already exist for analysing AF in many different food commodities [5].

Immunoassay methods such as ELISA are well suited for the rapid, routine diagnostic application of AF detection, although they show low reproducibility for concentrations very close to the EU legal limits. Although ELISA methods are generally good for rapid qualitative screenings, they fell short in providing a definitive confirmation of the toxin and an accurate quantitative determination [6]. Another semi-quantitative assay is flow-through immunoassay [7] while some quite promising results have been obtained with surface plasmon resonance technology [8].

Better suited for quantitative determination are the conventional analytical techniques such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) [9].

At present normal-phase HPLC is not often used anymore for aflatoxin determination and has been replaced by reversed-phase

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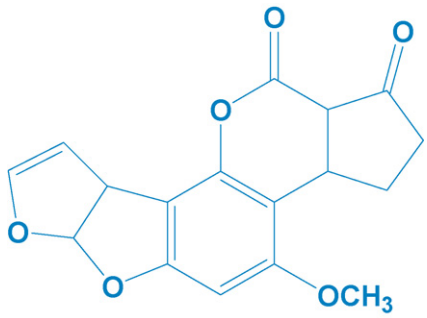

Fig. 1. Chemical structure of AFB1.

HPLC methods with fluorescence detection [10]. Many efforts have been made to enhance the natural fluorescence signal of AF in order to improve the limit of detection, including pre-column derivatisation with trifluoracetic acid or post-column derivatisation with active bromine or iodine derivatising agents [11,12]. Beta-cyclodextrins have been reported to increase the fluorescence intensity of unsaturated furan ring aflatoxins such as AFB1 when used as post-column derivatisation agents [13] or as additive to the eluent in HPLC [14].

Sample clean-up and enrichment are essential in the analysis of mycotoxins, particularly when chromatography is used for final determination. Immunoaffinity chromatography (IAC) is by far the method of choice [15,16]. The limitation of these columns is their lack of reusability as one sample preparation per column is recommended by the supplier. However, regeneration of an IAC for AF immunoextraction by washing with phosphate buffer saline (PBS) solution at pH 7.4 was first reported by Groopman et al. [17] and more recently, some authors suggested to regenerate immunoaffinity columns, by passing 5 ml, pH 7.4 through the column and keeping the column wet for at least 24 h at ca. 4 °C. Unfortunately, no results are reported about the binding capacity recovered after this treatment for repeated use of the column [18].

Hyphenated techniques involving immunoaffinity extraction and chromatographic separations are quite promising because of the theoretical potential for high sensitivity and selectivity [19]. The on-line coupling of immunoextraction with HPLC is of particular interest for automated sample processing and reduction of the turn-around time. Moreover, the high precision of HPLC pumps and injection systems allows the realization of automated immunoextraction systems with better precision than off-line methods. A liquid chromatographic column-switching system containing an anti-aflatoxin immunoaffinity pre-column was described for the determination of aflatoxin M1 in sample milk [20], while as far as AFB1 is concerned, only the automated AFB1 immunoextraction with disposable cartridges coupled on line with HPLC quantitation was reported [21].

The support material for antibody immobilization is one important item to consider. A rigid and high efficiency material should be employed to develop a high-performance immunoaffinity chromatography (HPIAC) method [22]. The recently developed macroporous polymeric disks based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (GMA-EDMA), commercially available under the name of Convective Interaction Media (CIM^{TM}) disks, have been used for immobilization of ligands in affinity chromatography [23–25]. The peculiar characteristic of these supports is that the mass transport is highly improved, since it occurs mainly by convection and not by total or partial diffusion as in conventional liquid chromatography. The improved mass transfer mechanism allows to consider only the biospecific pairing as a time limiting step [26,27].

On the basis of these preliminary remarks, the aim of this study was to set up a fully automated HPLC system in which an immunoaffinity disk containing anti-AFB1 antibodies is developed and coupled with a reversed-phase HPLC column. An epoxy-activated monolithic CIM disk was selected for the covalent immobilization of anti-AFB1 antibodies and the immunoaffinity-disk was coupled, through a switching valve, to a reversed-phase column. β-Cyclodextrin, added to the mobile phase, was used as fluorescence enhancer.

Issues considered in this study included the development and characterization of the immunoaffinity disk, the set-up of the elution conditions for the HPIAC and RPLC systems and the integration of the immunoextraction step to the analytical separation.

2. Materials and methods

2.1. Materials and chemicals

Epoxy-modified macroporous GMA-EDTA monolithic disks (CIM^{TM} Epoxy disk) used for antibody immobilization were from BIA Separation (Ljubliana, Slovenia). The disks had the following properties: diameter of 12 mm, thickness of 3 mm, volume of 0.34 cm^3, dry mass of 0.34 g, porosity of 70%, mean pore radius of 0.7 μm, specific surface of 10 m^2/g, and initial concentration of epoxy groups (3–5) × 10^{-3} mol/g. The macroporous supports were installed in a cartridge specially designed by the producer. Anti-AFB1 antibody obtained by rabbit fractionated antiserum, AFB1, β-cyclodextrin (purity min. 98%), glycine, sodium chloride, potassium chloride, potassium dihydrogenphosphate and disodium hydrogenphosphate were purchased from Sigma (St. Louis, MO, USA). Chromolith Performance (RP-18e, 100 mm × 4.6 mm i.d.) column was kindly provided by Dr. D. Lubda from Merck (Darmstadt, Germany). Water was deionized by passing through a Direct-Q system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and methanol were from Carlo Erba (Milan, Italy).

2.2. Solutions

Phosphate buffered saline (PBS) solution was prepared by dissolving 0.2 g potassium chloride, 0.2 g potassium dihydrogenphosphate, 1.16 g disodium hydrogenphosphate and 8.0 g sodium chloride in 1 l water.

AFB1 1 mg/ml stock standard solution was prepared by weighting 25 mg of AFB1 into 25 ml volumetric flask and bringing the flask to volume with acetonitrile. This stock standard solution was stored at −20 °C until use. Calibration standards

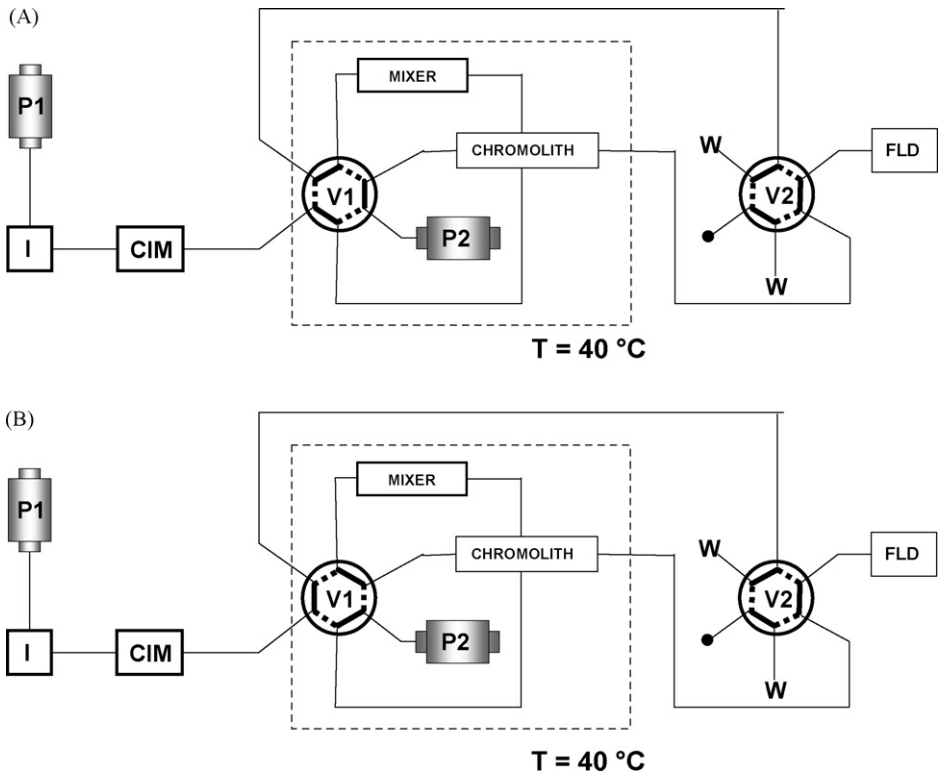

Fig. 2. Chromatographic system coupling the immunoaffinity-disk with the reversed-phase analytical column. (A) Valve 1 is in position A and (B) valve 1 is in position B.

were prepared daily from this stock solution by dilution with water.

2.3. Safety precautions

The solutions were prepared under a vented hood and all of the work areas and glassware which came in contact with the mycotoxin were cleaned with 5% sodium hypochloride solution. Waste solutions were disposed in bleach.

2.4. Chromatographic equipment

A schematic drawing of the column-switching system is given in Fig. 2. Chromatographic experiments were performed with two Agilent HP-1100 series (Palo Alto, CA, USA) modular systems, one for the immunoaffinity chromatography and one for the analytical separation. System 1 consisted of a quaternary gradient pump (P1), a vacuum degasser, a model 7725i Rheodyne manual injector (CA, USA) equipped with a 100 μl loop, and the CIM-disk. System 2 consisted of a quaternary gradient pump (P2), a vacuum degasser, a "mixer" realized with an empty stainless-steel LC column (250 mm × 4.6 mm i.d.) and a Chromolith Performance RP-18e column (100 mm × 4.6 mm i.d.) connected to a HP Model 1046 A fluorimetric detector (FLD). Both the mixer and analytical column were inserted in a thermostated column oven set at 40 ± 0.5 °C.

Systems 1 and 2 could be used independently, or the eluent from System 1 could be directed onto System 2 through a Rheodyne six-port switching valve model 2201 (V1) as shown in Fig. 2. A second valve was used for column-to-detector selection during method development (V2).

Automation of the hyphenated system was controlled by a Hewlett-Packard Vectra VE 6/350 personal computer equipped with a ChemStation software version A.06.03 operating in a Windows 2000 system environment.

2.5. Preparation and characterization of the immunoaffinity monolithic disk

Rabbit anti-AFB1 antibody was immobilized on a pre-activated CIM epoxy support. High reactivity of the epoxy groups of macroporous GMA-EDMA materials together with their porous channel-like morphology allows covalent binding in a single step under mild conditions. CIM-epoxy was washed in batch with 4 ml fractions of methanol, methanol:water (50:50), water and 50 mM phosphate buffer pH 8.0. Each washing step lasted 30 min. Sigma Rabbit anti-AFB1 fractionated antiserum (0.5 ml, concentration 6.8 mg/ml) was diluted to 2 ml with 50 mM phosphate buffer pH 8.0 in order to obtain a solution with a final concentration of about 1.7 mg/ml. The immobilization was carried out by a single-step reaction without any stirring (static conditions). The binding reaction was allowed to proceed at room temperature for 24 h. After the immobilization, the disk was washed for 1 h with 2 ml 50 mM phosphate buffer at pH 8.0 to remove the excess of unreacted antibody and the remaining epoxy groups were blocked with 4 ml ethanolamine 1 M overnight at ambient temperature. The disk was then washed with 4 ml of 50 mM phosphate buffer 1 M NaCl (pH 8.0) and with 4 ml of 50 mM phosphate buffer for 30 min. The disk was

Table 1
Time schedule of the integrated procedure

Pump 1[a]				Pump 2			Valve 1 position
Time (min)	Water	PBS[b]	Methanol	M2A[c]	M2B[d]	Flow-rate	
0.00–6.00	100.0	–	–	–	100.0	2.0	A
6.00–9.00	–	–	100.0	–	100.0	2.0	A
9.00–12.00	–	–	100.0	100.0	–	3.0	B
12.00–16.00	100.0	–	–	100.0	–	3.0	B
16.00–17.00	–	100.0	–	100.0	–	3.0	B
17.00–27.00	–	100.0	–	100.0	–	3.0	A
27.00–35.00	–	100.0	–	–	100.0	2.0	A
35.00–46.00	–	100.0	–	–	100.0	0.2	A

Solvent deliveries are expressed as percentage.
[a] The flow-rate of pump 1 was maintained at 0.8 ml/min during all the analytical procedure.
[b] Phosphate buffer saline, pH 7.4.
[c] Water:methanol (90:10), 6 mM beta-CD.
[d] Water:methanol (60:40), 6 mM beta-CD.

then inserted in its holder, connected to the HPLC system, and conditioned with a mobile phase consisting of PBS at a flow-rate of 0.8 ml/min. When not in use, the disk was stored at 4 °C in PBS containing 0.01% sodium azide. The amount of immobilized antibody was determined by the UV absorbance decrease of the antibody solution at 280 nm before and after the immobilization procedure. The amount of immobilized antibody was found to be 0.96 mg giving a reaction yield of 28.2%. A blank monolithic disk was also prepared following the same experimental procedure without antibody.

The binding activity of the immunoaffinity disk was evaluated by frontal analysis. In this procedure, AFB1 concentrations of 0.250–1 μg/ml were continuously applied to the disk at a flow-rate of 0.8 ml/min. After each set of experiments the retained AFB1 was eluted by applying methanol (5 ml) to the disk. The breakthrough times were determined from the resulting saturation curves after correcting for the void time of the system. Corrections for non-specific binding were made by performing similar experiments on the blank disk.

From the resulting breakthrough times, the apparent association equilibrium constants and binding capacities for AFB1 were determined as previously described for other affinity chromatographic systems [28–31]. The frontal analysis experiments allowed measuring the apparent moles of the solute required to saturate the antibody-disk at different analyte concentrations. When the results were plotted in terms of $1/m_{Lapp}$ versus 1/[analyte] a linear relationship characteristic of a solute with one class of binding sites was found. The binding capacity of the column, expressed by the total number of active sites was determined from the intercepts of this plot and was approximately 1.79 nmol. By using both the intercept and slope of this plot, it was possible to estimate the apparent association equilibrium constant for AFB1 on the immunoaffinity disk.

2.6. Chromatographic analysis

2.6.1. Immunoaffinity chromatography

The immunoaffinity chromatography was performed as a continuous step-wise adsorption–desorption process using the mobile phase delivered by pump 1 as reported in Table 1. After sample loading, the disk was washed with 4.8 ml of water (0–6.00 min) in order to remove salts. Quantitative elution of AFB1 was achieved with 4.8 ml pure methanol (6.00–12.00 min). Before starting the regeneration step, 3.2 ml of water (12.00–16.00 min) was passed through the disk. A typical elution profile of AFB1 from the immunoaffinity-disk occurs as shown in Fig. 3. The release of AFB1 occurred at 7.9 min. After AFB1 elution the antibody regeneration was accomplished by flushing the disk with 24 ml of PBS (16.00–46.00 min).

2.6.2. Analytical chromatography

The mobile phase delivered by pump 2 was prepared daily by dissolving the appropriate amount of β-CD in water and by adding methanol to give the desired final concentration.

Mobile phases were as follows: water:methanol 90:10 (v/v), 6 mM β-CD (M2A) and water:methanol 60:40 (v/v), 6 mM beta-CD (M2B). The elution of AFB1 starts at 27 min with mobile phase M2B delivered at 2.0 ml/min.

2.6.3. Integrated analytical procedure

The instrumental set-up is illustrated in Fig. 2 and the time schedule of the integrated procedure is reported in Table 1. The disk is preconditioned with 24 ml of PBS (16.00–46.00 min) and the sample is then injected. The disk is washed with 4.8 ml of water (0–6.00 min) to remove the salts before applying 4.8 ml methanol (6.00–12.00 min) as displacement solvent. The analyte elution is achieved in the first 2.4 ml methanol fraction (6.00–9.00 min) that is directed to the mixer, located after the CIM disk in this configuration (Fig. 2A). In the meantime, the analytical column is conditioned with M2B via pump 2.

Valve 1 changes position (from A to B) at 9.0 min. In this configuration (Fig. 2B) the mixer and the analytical column are in line and 54 ml of M2A is delivered by P2 (9.00–27.00 min). The analytical separation is finally obtained by changing valve 1 to position A and flushing the column with M2B. The total analysis time in this format is 46 min and the retention time of AFB1 is approximately 29 min.

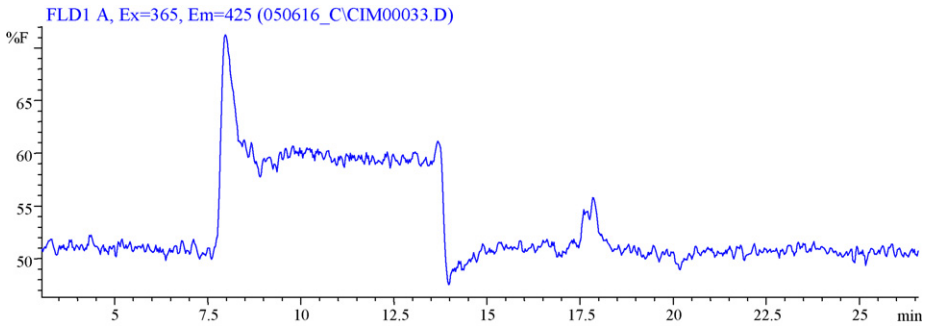

Fig. 3. Chromatogram showing a typical elution profile of AFB1 from the immunoaffinity-disk (200 ng/ml).

3. Results and discussion

3.1. Immunoaffinity chromatography

Polyclonal anti-AFB1 was successfully immobilized on epoxy-CIM. The amount of anti-AFB1 immobilized was found to be consistent with previously reported data regarding the immobilization of antibody on CIM monolithic disk [32]. The total number of binding site and the apparent association equilibrium constant for AFB1 on the immunoaffinity disk were 1.79 nmol and $0.68 \times 10^6\ M^{-1}$, respectively.

As a compromise between highest assay speed, better extraction efficiency and low backpressure (8–12 bar) a flow-rate of 0.8 ml/min was selected. The complete cycle of analysis, i.e. disk conditioning, injection, washing, elution/release takes 46 min. With the aim to reuse the anti-AFB1-disk a regeneration protocol was developed. The disk can be easily regenerated by flushing it with 24 ml of PBS for 30 min. In these conditions, immobilized anti-AFB1 trapped the target analyte with high efficiency and the repeatability of the entire experiment carried out on 1 μg/ml AFB1 standard solutions was good (concentration found ± S.D. = 1.00 ± 0.03 μg/ml, $n = 3$).

The time required for the regeneration of the immunoaffinity disk was reduced in comparison to the time suggested for the cartridges [18]. The different behavior can be ascribed to the different experimental protocol used. It can be hypothesized that the dynamic conditions, obtained in a flow-system can speed up the regeneration process. As suggested by the supplier of the disposable cartridges, AFB1 can be easily desorbed with a large volume of methanol, therefore the same organic solvent was selected for the on-line AFB1 release.

3.2. Immunoextraction and analytical separation on-line coupling: design and system optimization

One-way to destroy the antigen–antibody interaction is to submit the immunosorbent to a large volume of organic solvent. With some analytes, particularly low molecular mass solutes, it can sometimes be hard to find elution conditions that allow for the rapid dissociation of analyte from the immunocolumn. The result is a broad peak for the retained analyte that can be difficult to detect and/or that elutes over an extended period of time. One solution to this problem is to capture and concentrate the analyte during its elution from the immunoaffinity support by coupling a second on-line column, such as a reversed-phase column. This hyphenated approach can improve both sensitivity and selectivity with excellent potential for automation. Moreover, the relatively high precision of the LC pump and injection system also provides on-line immunoextraction with better precision than the off-line mode [33].

A significant part of this work has been dedicated to the study of the best conditions for coupling the immunoaffinity extraction to the analytical separation. The instrumental set-up developed in this study is shown in Fig. 2 and the time programme of the integrated method is given in Table 1. After preconditioning the disk with 24 ml of PBS, sample injection is started. The direct coupling of the CIM disk to the reverse-phase column was not possible because the methanol, required for the release of AFB1, would not allow the focusing of the analyte on the head of the analytical column. For this reason after CIM-disk a mixer, previously filled with water, was inserted. In the mixer, the mobile phase is blended in order to lower the eluotropic strength of the eluent yielding a final mobile phase composition close to the one flowing on the analytical column (M2B). The volume of the mixer was carefully selected in order to retain AFB1 during the transfer of the 2.4 ml methanol fraction containing AFB1. After that the position of valve 1 changes from A to B at 9.0 min. In this configuration (position B) the analytical column and the mixer are put in series and 54 ml of M2A are delivered by P2 in order to achieve the focussing of the analyte on the head of the column. This step was demonstrated to be crucial for the subsequent partition and chromatography of AFB1 onto the Chromolith column. The peculiar features of the Chromolith support allow to carry out this step at a high flow-rate (3 ml/min). The separation is finally obtained changing valve 1 to the A position and flushing the column with M2B between 27 and 35 min. The flow-rate is then lowered to 0.2 ml/min while the CIM-disk is reconditioning.

3.3. Selective and non-selective interactions

The solid support selected for the immobilization of an antibody is a key factor. A good sorbent should be mechanically stable, have a large pore size, and it should be hydrophilic enough in order to limit any non-specific interactions with the analyte and the sample matrix. Most commonly, hydrophobic forces are responsible of undesirable adsorption which results in co-

extraction of interfering hydrophobic molecules, decreasing the selectivity and limit of detection.

Nevertheless, it was clear from the preliminary studies that the blank disk was able to retain AFB1 in aqueous buffer. After the injection of AFB1 on the blank disk, AFB1 was not eluted even after large volume of aqueous buffer, indicating that a significant aspecific hydrophobic interaction between the analyte and the polymeric support takes place.

In order to demonstrate the presence of a specific binding activity on the immunosupport the retention mechanism of the analyte was studied, both on the immunoaffinity disk and on the blank disk, running the two CIM disks in parallel. The evaluation of the retention mechanism was performed by injecting AFB1 (1 μg/ml). The mobile phase used in this experiment are the one reported in Table 1, with the exception of the desorption conditions. Actually, different desorption conditions at increasing concentrations of methanol (from 50% to 100% methanol) were tested (from 6 to 12 min) and the areas of the AFB1 peaks obtained running the two disks in the same elution conditions were compared.

A methanol percentage of 100% was able to destroy both the selective and non-selective interactions and it was not possible to get an indication on the binding capacity of the immobilized antibody. The release of AFB1 from the blank CIM was quantitative in the range of methanol percentage used, unlike the immunoaffinity disk. In fact, by decreasing the methanol percentage, the amount of AFB1 realized from the immunoaffinity disk progressively decreases from 117% (5.5% R.S.D.) to 56.83% (6.48% R.S.D.) with 100% methanol and 50:50 water:methanol (v/v), respectively. The comparison of the two retention mechanisms obtained with the two columns demonstrated the occurrence of non-selective interactions between AFB1 and the polymeric sorbent matrix, thus evidencing that polymethacrylate monolithic support is indeed capable of hydrophobic/reversed-phase aspecific interaction with the analyte.

Table 2
Summary of calibration data for aflatoxin B1 extraction with the integrated system

Concentration range (μg/ml)	0.1, 0.25, 0.5, 1
Regression curve	$y = 7.4105x + 47.289$
Slope ± R.S.D. (%)	7.41 ± 10.27
N^a	3
Mean r^2	0.995
LOD (μg/ml)	0.05
LOQ (μg/ml)	0.1

[a] Number of experiments.

The AFB1 percentage eluted at 50% methanol was used as test to evaluate the disk performance during time. The AFB1 release decreased approximately to 32.93% after 100 immunoextractions.

3.4. System evaluation

The system was initially evaluated towards its ability to extract AFB1 in a linear fashion. Calibration curves were based on the analysis of aqueous standards at four concentration levels and three determinations at each level were performed. The linearity study was carried out in the range 100–1000 ng/ml and the linear calibration graphs were obtained by plotting the peak areas against the AFB1 concentration injected. The calibration curve for AFB1 was linear in the range studied with a correlation coefficient of 0.995 and had a limit of detection of 50 ng/ml (S/N = 3) and a limit of quantitation of 100 ng/ml. The calibration parameters are reported in Table 2. Example of chromatograms that were obtained with the integrated system at different AFB1 concentrations are reported in Fig. 4.

It should be mentioned that at present the limits of detection and quantitation of the method do not meet the requirements for AF determination in real samples but can be improved. In fact, the sensitiv-

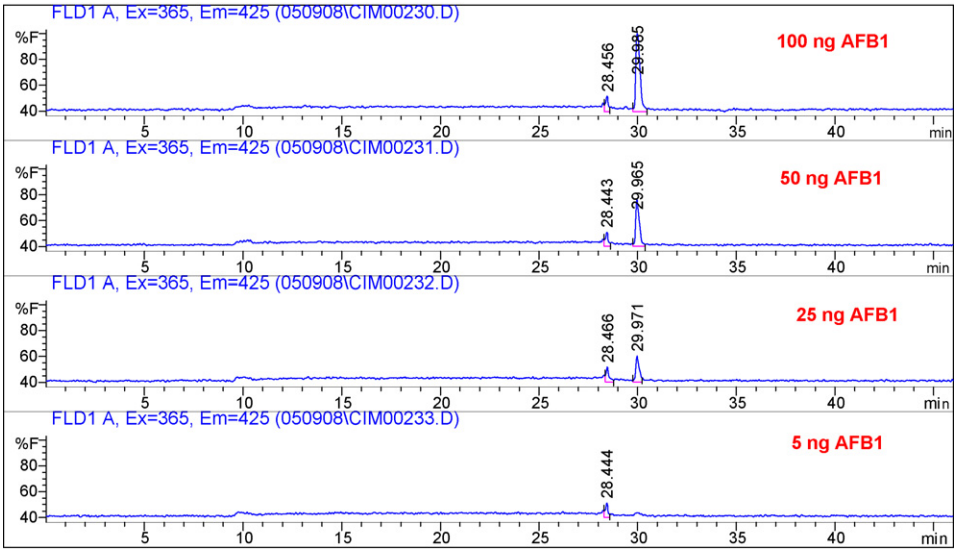

Fig. 4. Chromatograms showing typical elution profiles obtained with the integrated system. Injection volume 100 μl.

Table 3
Results of day-to-day repeatability

Concentration (μg/ml)	Area, mean value ± S.D. (μg/ml)	n[a]	R.S.D. (%)
0.1	106.57 ± 1.10	3	1.03
0.25	241.81 ± 26.98	3	11.16
0.5	430.61 ± 41.07	3	9.54
1	781.11 ± 62.83	3	8.04

[a] Number of experiments.

ity of the fluorimetric detector employed. Because fluorimetric detectors of more recent models than the one used in this work are known to have sensitivities one order of magnitude better, it will be possible to improve the sensitivity accordingly. Moreover the detectability of AFB1 could also be greatly improved by using other type of CDs as more efficient fluorescence enhancers such as succynil-β-cyclodextrin [14].

The within-day precision of the method was evaluated by making three injections of a 1 μg/ml solution and a precision of ±6.97% R.S.D. was obtained. The day-to-day precision was evaluated on three different days over a period of 3 months (Table 3).

The accuracy (recovery) (Table 4) of the HPIAC/RPLC method was evaluated by constructing two regression curves, one using the immunoaffinity-disk and the other using the blank-disk. The amount of AFB1 released from the blank CIM was assumed as 100%. Accordingly, the recovery of AFB1 for each point was calculated and expressed as percentage. The recovery ranged from 78.76% to 104.32%.

One item which should be considered with this type of systems is the capability of concentrating the analyte from large sample volumes in order to lower the detection limits. Although using larger sample volumes can increase the time required for sample injection, the corresponding increase in overall analysis time can be minimized by also increasing the flow-rate used for sample application. However, care must be exercised in doing this since the binding efficiency of the analyte may decrease when less time is allowed for the sample to interact with the immunoaffinity sorbent. Actually, the loading flow can affect recoveries of the analyte, in particular during the sample-loading step, high flow-rates may prevent analyte from binding to the immobilized antibody. The extent of this effect was examined by injecting fixed sample volumes (50 ng AFB1 in 10 ml) to the immunoaffinity-disk at different flow-rates (0.1, 0.5 and 1.0 ml/min). Recoveries of the analyte calculated by comparison with a 100 μl injection of the same amount of AFB1 are reported in Table 5. Taking into account the precision of the method, the recovery data indicate that sample binding efficiency remained relatively constant over the flow-rate range considered.

Table 4
Results of accuracy study

Concentration (μg/ml)	Blank-disk, area	Immunoaffinity-disk, area	Recovery (%)
0.1	102.45	80.69	78.76
0.25	227.48	194.99	85.72
0.5	401.74	419.09	104.32
1	781.79	770.01	98.49

Table 5
Effect of loading flow and volume on recovery

Loading flow effect[a], flow (ml/min)	Volume effect[b]		
	Recovery (%)	Volume (ml)	Recovery (%)
0.1	83.71	50	45.56
0.5	103.55	10	99.30
1.0	99.29	0.1[c]	100.00

[a] Volume loaded = 10 ml; AFB1 = 50 ng.
[b] Flow = 1 ml/min; AFB1 = 100 ng.
[c] Recovery from 0.1 ml sample volume assumed as 100%.

The maximum sample volume that can be loaded without loss in recovery is dependent on the affinity of the antibody. A fixed amount of AFB1 corresponding to 100 ng was injected in different sample volumes (0.1, 10 and 50 ml). Ten and 50 ml sample loading was obtained by using channel D of pump 1 with a constant flow-rate (1 ml/min). Accordingly, the time required for sample injection increased. The results reported in Table 5 indicate that the AFB1 extraction depends on the loading volume. The recovery obtained with 10 ml was quantitative and this result clearly indicate that the disk is able to concentrate the analyte a 100 times fold. Injecting a larger sample volume (50 ml), the recovery decreases to 45.56% indicating a decrease in extraction efficiency. The extraction efficiency was also evaluated for different AFB1 concentrations using a constant sample volume of 10 ml. The recovery ranged from 90.31% to 107.54% and a linear correlation was obtained ($y = 7.55x + 1.12$, $r^2 = 0.983$).

Over the course of this study, the column was used for more than 100 injections and desorption cycles. Only a gradual decrease in the binding was noted over this time. This slow loss of activity was not a problem since the immunoaffinity-disk originally contained a large excess of binding sites relative to the amount of injected AFB1.

4. Conclusions

Polyclonal anti-AFB1 was successfully immobilized on epoxy-CIM disk and the immunosupport was able to retain specifically AFB1. The immunoaffinity-disk was integrated in a chromatographic system. A column-switching configuration that requires two six-port valve and two HPLC pumping systems was employed for on-line immunoextraction and subsequent quantitation of AFB1.

The immunoaffinity-disk was characterized in terms of linearity, precision and accuracy, and the effects of sample volume and loading flow-rate were investigated. The immobilized antibody was stable after repeated runs.

The sensitivity of the method can be improved by optimizing different experimental parameters such as detector type, type of cyclodextrin added to the mobile phase and finally by increasing the sample volume loaded on the disk.

Future research will be performed to clarify to which extent the automatic system presented in this study can be used to process authentic samples contaminated by AFB1. In fact, it

was demonstrated that such monolithic support are capable of aspecific absorption and that the extraction of AFB1 is governed both by non-specific and specific interactions. This factor could represent a critical point in the analysis of real samples.

However, one of the advantages in using monolithic disks is the possibility to combine multiple disks in series in one housing allowing the combination of different antibodies within one chromatographic cartridge. This suggests to try the immobilization of other anti-AF antibodies and the complete development of the presented study will be to perform multi-residue analysis of several aflatoxins by putting in series disks onto which the specific antibodies for each aflatoxin have been immobilized. Due to the flexibility of the integrated chromatographic system, new methods can be developed and applied to analysis of aflatoxins in different matrixes.

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