Microchim Acta 153, 101–108 (2006)
DOI 10.1007/s00604-005-0456-y

Microchimica Acta
Printed in Austria

Original Paper

Development and Application of a Two-Phase Clean-Up System in Food Samples Prior to Fluorescence Analysis of Aflatoxins

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Received February 3, 2005; accepted October 17, 2005; published online December 21, 2005

Abstract. A new method based on the use of a two-phase separation system is proposed for the clean-up of aflatoxins after their extraction from food samples. After extraction from the sample with an appropriate solvent, aflatoxins contained in organic solvent diffuse to the aqueous phase. At that stage their relative concentration is measured fluorimetrically at 350 nm (λ_{exc}). Different organic solvents were tested, and the composition of the aqueous phase was varied. The data obtained showed that the best efficiencies were observed with toluene and PBS-65% methanol. The method was simpler and shorter, and capable of monitoring aflatoxins in samples with a LOD equal to 0.3 $\mu g\,kg^{-1}$ (AFB_1). However, the proposed method shows limited selectivity and could thus serve as a screening method for total aflatoxins or be combined with chromatographic techniques for individual aflatoxin determination.

Key words: Aflatoxin; two-phase system; clean-up; food.

Aflatoxins are a group of mycotoxins produced by the food spoilage fungi *Aspergillus flavus* and *Asp. parasiticus* [1]. Within this group, about 18 compounds have been identified, but only aflatoxin B_1, B_2, G_1, G_2 and M_1 are routinely monitored. They exhibit acute and chronic toxicity including mutagenic, carcinogenic and teratogenic effects in a wide range of organisms [2]. The order of aflatoxin toxicity ($AFB_1 > AFG_1 > AFB_2 > AFG_2$) seems to be due to the structure of the toxin, especially the terminal furan moiety [3–5]. These aflatoxins are present in several commodities such as peanuts, cereals and cottonseeds, whereas aflatoxin M_1, which is just as toxic as aflatoxin B_1 [6], is most often analyzed in milk and dairy products.

Aflatoxin B_1 has been listed as a carcinogen of group I by the International Agency for Research on Cancer [7], and aflatoxin M_1 has been classified a class 2B human carcinogen [8]. The European commission has sets current maximum levels for total AFB_1, AFB_2, AFG_1 and AFG_2 (4 $\mu g\,kg^{-1}$) and AFB_1 alone (2 $\mu g\,kg^{-1}$) in ground nuts, nuts, dried fruits and cereals ready for retail sale, and for AFM_1 (0.05 $\mu g\,kg^{-1}$). For foods that are to be processed, the legal limits are 8 $\mu g\,kg^{-1}$ and 15 $\mu g\,kg^{-1}$ for aflatoxin B_1 and total aflatoxins, respectively [9].

In the USA, the maximum permitted levels are about five times greater than those established for Europe.

Because of their occurrence in a wide range of foods and their harmful effects on humans and animals, the

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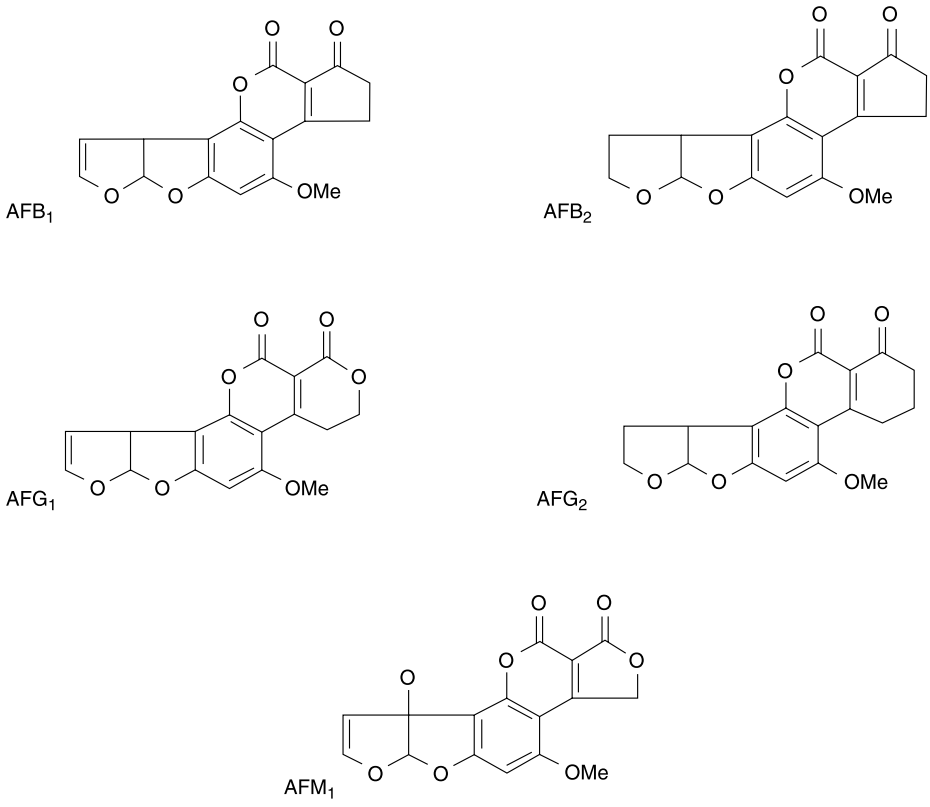

Fig. 1. Chemical structures of aflatoxins AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1

systematic monitoring of aflatoxins presents a real challenge in terms of both detection methods and adequate sample preparation. For this reason, several methods and techniques for aflatoxin determination have been developed during the last few years [10–15]. Most of these analytical methods are based on detection of either the native or the enhanced fluorescence of aflatoxins.

Chromatographic analyses, such as TLC (thin layer chromatography) [16–18] and HPLC (high performance liquid chromatography) [19–21], are the methods currently most widely used for aflatoxin detection. These techniques are usually preceded by complex operations that include sample preparation, extraction, purification, and concentration of the extract before the final determination of the analyte. In other cases, the clean-up step requires immunoaffinty columns [22–24], SPE cartridges (solid phase extraction) [25, 26] or multifunctional columns [27, 28] prior to chromatographic determination.

Immunoassay techniques have also been developed to monitor aflatoxins in several food samples [29–33]. These techniques, however, require specific antibodies and many steps (coating, incubation, washing, etc.) which increase the assay time. Consequently there is a need for the definition of techniques that are based on simple, available, and reliable analytical approaches in order to provide screening methods for the monitoring of food contamination by aflatoxins. These techniques must also take into consideration the cost and the time of analysis.

In this paper, we propose the introduction of a clean-up procedure which makes use of a two-phase system using organic and aqueous solvents. The purpose is to efficiently extract aflatoxins while excluding interferents so that the final sample is suitable for direct determination of total aflatoxins by the fluorescence method without the need of a derivatisation step [34–37].

Using this clean-up procedure, the screening of aflatoxins in food samples would be carried out in three steps:

Firstly, the toxins are extracted from real samples with an appropriate organic solvent. In the second step the extract is evaporated, and then redissolved in the highly hydrophobic solvent toluene. Finally this aflatoxin extract is shaken with a modified aqueous phase (PBS/methanol) to give two immiscible phases. These

series of operations are devised so that the aflatoxin is selectively transferred to the ''aqueous phase'' while many interfering substances can remain in the two organic solvents used in the several extraction steps. Finally, the aflatoxins recovered in the aqueous phase are determined by fluorescence emission using the appropriate excitation wavelength.

We have optimised the clean-up procedure through a systematic and iterative study of the different parameters that affect the partitioning. These include organic solvent, aqueous phase composition, the ratio of organic phase/aqueous phase, and the time of contact.

This paper reports the data from the first survey of the AFM_1 and AFB_1 content determined in spiked foods that have been subjected to the proposed two-phase clean-up system.

Experimental

Chemical and Reagents

Acetonitrile and acetone were supplied by Merck. Toluene, methanol, chloroform, and methylene chloride were from Sigma-Aldrich.

Water produced in a Milli-Q system (Millipore, Bedford, UK) was used for preparing all solutions. NaH_2PO_4 and Na_2HPO_4 were supplied by Carlo Erba, NaCl and KCl were obtained from Sigma-Aldrich.

The aflatoxins B_1, B_2, G_1 and G_2 were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Aflatoxin M_1 was obtained from Alexis Biochemicals (Carlsbad, CA, USA – www.alexis-corp.com). Stock standard solutions of aflatoxin B_1 ($1\,mg\,mL^{-1}$), aflatoxin B_2 ($0.25\,mg\,mL^{-1}$), aflatoxin G_1 and G_2 ($0.5\,mg\,mL^{-1}$) and aflatoxin M_1 ($62.5\,ng\,mL^{-1}$) were prepared in methanol and kept in safe conditions at $-20\,^{\circ}C$.

Instrumentation

The fluorimeter used was a computer-controlled Fluoro Max-2 (Jobin-Won) device. Measurement of the aflatoxins was performed in the PBS-65% methanol solution that resulted from the clean-up procedure using an excitation wavelength of 350 nm.

The shaker used for the extraction and the clean-up experiments was GLAS-COL (Terre Haule, USA).

Food Sample Preparation and Extraction of Aflatoxins

Milk and barley samples were collected from local markets; these products have shown to be free of aflatoxin according to the official method reported by the AOAC [38].

Barley was first ground in a household blender at high speed for 5 min. It was then artificially contaminated with defined volumes of AFB_1 solution and left to equilibrate 15 min under stirring. The final aflatoxin concentrations in spiked barley were equal to 0.3, 0.15 and $0.05\,nmoles\,g^{-1}$.

The extraction methods were a modification of those described by AOAC methods [29]. For this step, 15 mL of methanol was added to 5 g of sample. The mixture was blended for 10 min, and then the methanol was removed from the sample using a Pasteur pipette. The same procedure was repeated for the same sample with another aliquot of methanol. The total methanol volume (25 mL) was filtered and then evaporated at $50\,^{\circ}C$ under a gentle N_2 steam for approximatively 30 min.

Extraction was also carried out following the same procedure using chloroform. The dried extract was dissolved in 5 mL of toluene before the clean-up step.

For milk, the extraction of aflatoxin M_1 from spiked samples was similar to that of barley. 5 mL of sample containing aflatoxin M_1 was extracted with chloroform, and the extract was then evaporated at $50\,^{\circ}C$ under N_2 and then dissolved in 5 mL of toluene. The final aflatoxin concentrations in spiked milk were equal to 50, 25 and $5\,nmoles\,L^{-1}$.

Clean-Up Procedure

The clean-up step involves a two-phase system in which the ''aqueous'' phase is in fact a mixture of PBS and miscible hydro-organic solvent while the organic phase is a solvent that is immiscible with the aqueous phase. Equal volumes (5 mL each) or varying volume ratios (2:1, 3:1) of the organic and the aqueous phases are put in a shaker and left to be stirred for certain lengths of time (10, 30 and 120 min). During this time the aflatoxins partitioned out of the organic solvent and were recovered in the aqueous phase that was separated from the organic solvent using a separatory funnel. Finally, the aflatoxin composition was determined directly from the fluorescence spectra.

Different organic solvents have been tested in this scheme: toluene, benzene, chloroform and methylene chloride. The solvents used to modify the PBS aqueous phase were methanol, acetone, and acetonitrile. Previously, these same solvents had been shown to give an effective extraction of aflatoxin from different sources, and on this basis they were selected to facilitate the selective solubilisation of aflatoxin in the aqueous phase in the present investigation. AOAC official methods also propose methanol and acetonitrile in various percentages for the preparation of aflatoxin standard solutions [38].

For the optimisation studies reported here, the efficiency of the clean-up step has been calculated as follows:

$$\tau_{\text{eff.}}\ (\%) = I_{F1}/I_{F2} \times 100$$

where I_{F1} is the fluorescence intensity of the aflatoxins contained in the aqueous phase recovered from the clean-up step and I_{F2} is the fluorescence intensity of an aqueous phase containing the same amount of aflatoxin added directly.

Results and Discussion

Effect of the Aqueous Phase Composition on the Efficiency of the Clean-Up

The first experiments for the optimisation of the clean-up step were performed using toluene as the organic phase. Toluene was chosen on the basis of its strongly hydrophobic nature. Different ''aqueous'' mixtures were prepared using equal volumes of PBS with organic solvents (methanol, acetone, acetonitrile) that were miscible with water and that had been demonstrated to solubilise aflatoxins or elute them from an SPE column. In these experiments 5 mL of toluene

spiked with a known quantity of aflatoxin was mixed with an equal volume of the prepared aqueous phase and agitated. In these preliminary experiments the agitation was allowed to proceed for 2 h to ensure maximum transfer of aflatoxin into the aqueous phase.

The clean-up step was investigated using different organic solvents to modify the phosphate buffer. These first trials included acetone, acetonitrile and methanol. Clearly the best extraction efficiency of aflatoxin from the organic phase was achieved with methanol, which gives an extraction efficiency equal to 40% compared to acetone and acetonitrile, which gives efficiencies equal to 7 and 1%, respectively.

Selection of the Organic Phase

In a second phase of the investigation, the effect of the organic solvent used to solubilise the aflatoxin-containing residue was evaluated. Since aflatoxin is known to be soluble in organic solvents, there was a large number of candidate solvents of which those immiscible with the aqueous phase were studied. Lin and his co-workers reported the use of these different solvents or their mixture for investigations of aflatoxin analysis [39]. The aqueous phase employed in these experiments was a 50/50 mixture of methanol and PBS.

The extraction efficiencies were found to be equal to 40% for toluene, 15% for benzene, and 3% for both chloroform and methylene chloride. Among the organic solvents examined, toluene was selected for the following experiments since it gave the highest efficiency.

Effect of the Methanol Proportion in the Aqueous Phase

Once toluene had been established as the most suitable organic solvent for the clean-up procedure, we went back to investigate the effect of varying the proportion (0 to 80%) of methanol used in preparing the aqueous phase. The efficiency of extraction increased with increasing proportions of methanol in the aqueous phase. However, for levels over 65% of methanol, there was a visible increase in the miscibility of the toluene with the aqueous phase. For this reason, a methanol proportion of 65% which gave an efficiency of 60% was selected to ensure maximum immiscibility of the two phases; this value was used in subsequent experiments.

Effect of the Organic Solvent

With the newly optimised aqueous phase (PBS-65% methanol), the same four organic solvents were re-investigated. Efficiencies equal to 60, 30, 5, and 3% were obtained with toluene, benzene, chloroform, and methylene chloride, respectively. Consequently, toluene remained the best choice for enhancing the amount of aflatoxin that could be recovered in the aqueous phase.

Effect of the Extraction Time

To further improve the clean-up procedure, a crucial parameter to study was the contact time needed for the passage of aflatoxin in the aqueous phase. During 10 min extraction time, the efficiency of the clean-up approaches 52%. Over 30 min, the extraction efficiency remains the same and it does not exceed 60% even after 120 min.

Since the efficiency of extraction increased with time up to a plateau at thirty minutes, this relatively limited contact time was utilised for further investigations.

The data obtained using the proposed clean-up system showed a relative standard deviation (RSD) of 5.5% obtained from three replicate measurements.

Effect of the Ratio of Aqueous Phase/Organic Phase

A final variable that was examined was the ratio of the aqueous phase to the organic solvent. This parameter was varied from 1:1 to 3:1.

While the maximum efficiency obtained with equal volumes was around 60%, it was shown that this value could be increased by increasing the volume of the aqueous phase relative to that of the organic one with a value of 86% reached when the ratio was 2:1. In fact, total recovery of aflatoxin from the organic phase was achieved with a 3:1 ratio of PBS-65% methanol to toluene.

Extraction Efficiency of the Different Aflatoxins Under the Optimised Conditions

The clean-up procedure was then applied separately to different congeners of aflatoxin to evaluate the efficiency of the proposed method for the different forms of aflatoxin; those tested were aflatoxins M_1, B_1, B_2, G_1 and G_2.

The results obtained (data not shown) demonstrate that under the optimised conditions the proposed method allows essentially 100% extraction of each form.

Fluorescence Detection of Total Aflatoxin Content

The fluorescence spectra of the aflatoxin congeners are reported in Fig. 2. An aflatoxin concentration of 75 nM was used in each case with the excitation wavelength, $\lambda_{exc} = 350$ nm.

It can be seen that the different congeners of aflatoxin have different emission intensities, with aflatoxins B_2 and G_2 showing the highest intensity, followed by aflatoxins G_1 and B_1, and aflatoxin M_1 having the lowest emission. There was also a slight difference in the peak values of the fluorescence

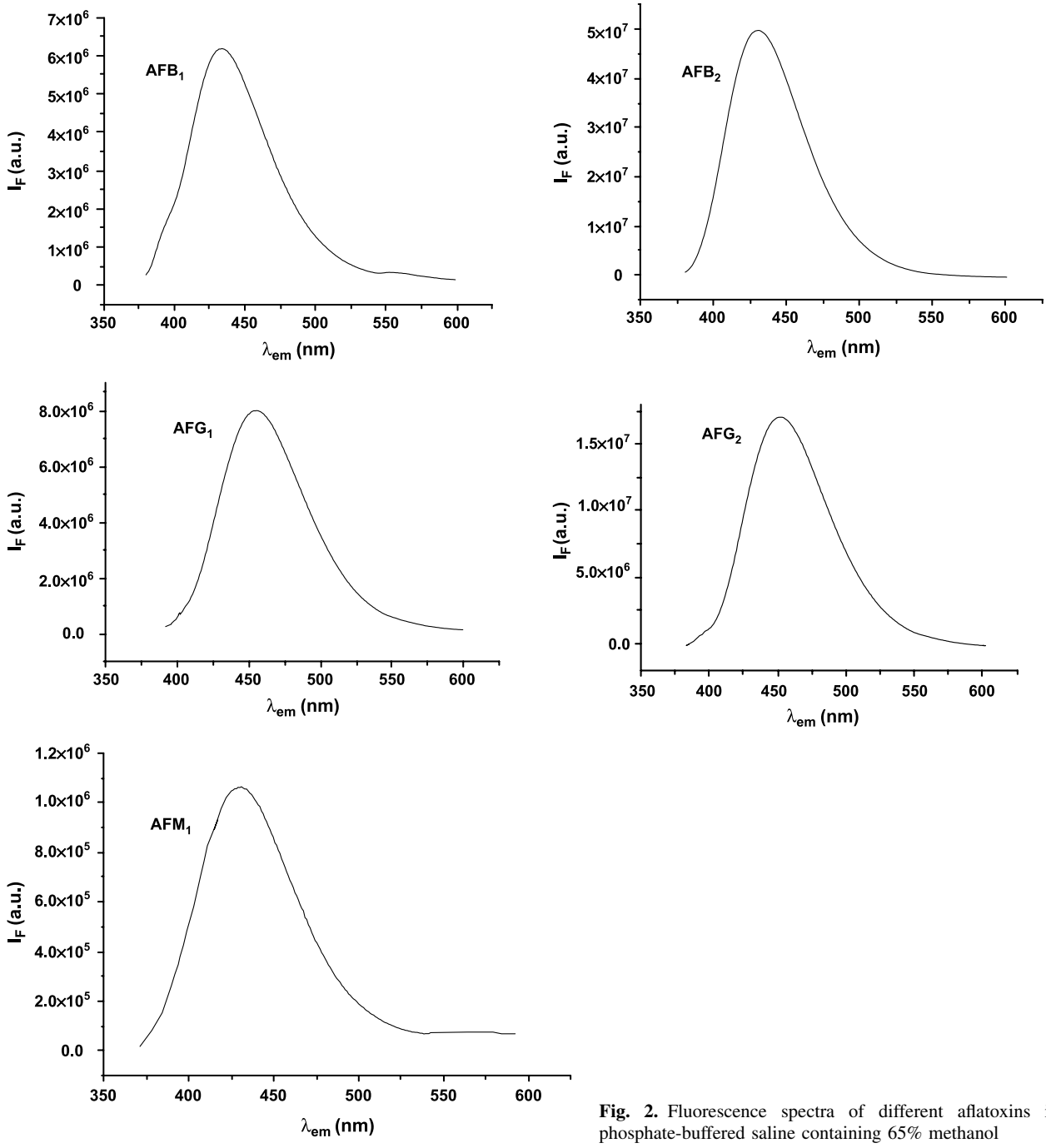

Fig. 2. Fluorescence spectra of different aflatoxins in phosphate-buffered saline containing 65% methanol

Table 1. Maximum fluorescence emission and intensities of 75 nmol L^{-1} aflatoxins B_1, B_2, G_1 and G_2 at $\lambda_{exc} = 350$ nm

Aflatoxin	Maximum λ_{em} (nm)	I_F ($\times 10^6$) (a.u.)
AFB_1	434	6.1
AFB_2	430	5
AFG_1	455	8
AFG_2	452	16.1
AFM_1	431	1.1

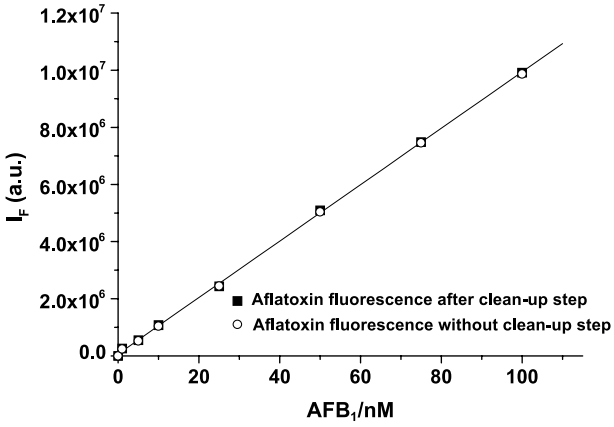

Fig. 3. Calibration curve of aflatoxin B_1 $\lambda_{exc} = 350$ nm/clean-up system: PBS-65% methanol/toluene (3:1), 30 min

emission; the B and M-aflatoxins showed maximum emission around $\lambda_{em} = 430$ nm while for the G-aflatoxins the value of λ_{em} was around 450 nm. The data for the various aflatoxin forms have been listed in Table 1.

It was also shown (Fig. 3) that the fluorescence of the aflatoxin B_1 in PBS-65% methanol is not affected by the toluene used in the clean-up system step since the intensity was practically unchanged.

The proposed method is thus able to detect concentrations of aflatoxin B_1 as low as 1 nM with a linear range of aflatoxin B_1 fluorescence, obtained under optimised conditions, from 1 to 100 nM. This corresponds to 0.3 μg kg^{-1} to 31.4 μg kg^{-1} for the samples prepared as described in the experimental section. The equation of the calibration curve is: $y = 68.64\,10^3 + 98.73\,10^3 x$, where y is the fluorescence intensity (I_F) and x is the AFB_1 concentration (nM), with a regression coefficient of 0.999.

Data Relative to the Fluorescence of the Total of Aflatoxins Excited at Various Wavelengths

Five millilitres of toluene was spiked with a mixture of all the aflatoxins, i.e. B_1, B_2, G_1, G_2 and M_1 (15 nM each) and shaken for 30 min together with the aqueous phase. After this extraction, the aqueous phase containing the aflatoxins was subjected to fluorescence measurement using different excitation wavelengths (320, 340, 350 and 360 nm). The fluorescence intensities obtained are shown in Table 2.

Table 2. Fluorescence intensity of the sum of aflatoxins in PBS-65% methanol at different wave lengths

Wavelength (nm)	I_F ($\times 10^6$) (a.u.)
320	71.7
340	60.2
350	48.2
360	28.3

The present investigations were carried out in order to explore the possibility of separating the fluorescence spectra of the different aflatoxins (B_1, B_2, G_1, G_2) based on the excitation wavelength.

The emission of the total aflatoxins increases when increasing the excitation wavelength without, however, revealing any differentiation between each aflatoxin.

Spiked Sample Experiments

The clean-up procedure was then applied to the analysis of some food samples. Different foods were spiked with aflatoxin B_1 (or M_1 for the milk sample) and left to equilibrate. The original extraction of aflatoxin from milk and barley, i.e. the organic solvent used, was carried out on the basis of the previous studies reported in the literature. Both chloroform and methanol were employed for the initial extraction.

Table 3. Recovery of aflatoxin B_1 extraction from spiked barley after the clean-up assay (n = 3)

Aflatoxin concentration (nmoles g^{-1})	$\tau_{eff.}$ (%)	
	Chloroform	Methanol
0.3	40 ± 7	86 ± 4
0.15	46 ± 5	104 ± 4
0.05	42 ± 4	95 ± 6

Table 4. Recovery of aflatoxin M_1 extraction from spiked milk (n = 3)

Aflatoxin concentration (nmoles L^{-1})	$\tau_{eff.}$ (%)
50	100 ± 5
25	95 ± 5
5	98 ± 4

Table 5. Figures of merit of comparable methods for direct fluorimetric determination of aflatoxins

Method	Analytical range, LOD	Comments	Reference
Two-phase clean-up system	0.3–31.4 $\mu g\,kg^{-1}$ LOD = 0.3 $\mu g\,kg^{-1}$ (AFB_1)	Rapid and simple technique for screening of total aflatoxins without a clean-up column. Recoveries over 86% and reaching 100% in certain cases. Time of analysis is about 30 minutes.	this article
Immunoaffinity column clean-up	0.05–0.5 $\mu g\,kg^{-1}$ LOD = 0.05 $\mu g\,kg^{-1}$ (AFM1)	Fast and simple method for determination of AFM1 in milk. Recoveries around 97%. Antibody affinity columns are very expensive. Time of analysis is about 8 minutes.	[40]
Automated immunoaffinity column clean-up	0.1–50 $\mu g\,kg^{-1}$ LOD = 0.1 $\mu g\,L^{-1}$	Method tested only with standard solutions of aflatoxins. Short response time (2 min). Antibodies required.	[41]
Monoclonal affinity clean-up column (Aflatest^R test kit)	5–300 $\mu g\,kg^{-1}$ LOD = 1 $\mu g\,kg^{-1}$	The Aflatest^R used for determination of AFB1, AFB2, AFG1, and AFG2 in feeds, food, grain and nuts and AFM1 in dairy products. Performed in less than 10 minutes. Monoclonal antibody affinity columns are very expensive.	[38, 42]

The sample extracts (5 mL) were then treated according to the following optimised clean-up procedure:

Aqueous phase: PBS + 65% methanol
Organic solvent: toluene
Ratio aqueous phase/organic solvent: 3:1
Clean-up time: 30 min
Finally, the fluorescence was measured in the recovered aqueous phase using $\lambda_{exc} = 350$ nm.

As seen from Table 3, the efficiency of extraction of aflatoxin B_1 from barley samples does not exceed 46% when the extraction is done with chloroform, while with methanol the efficiency is over 86%. This explains the wide use of methanol for the extraction of aflatoxins from different food matrices as reported in several studies.

Similarly, spiked milk samples were successfully extracted with chloroform (10 and 15 mL successively) as has been done with barley. The data obtained (Table 4) with a spiked milk sample with aflatoxin M_1 showed high recoveries between 95 and 100%. The present method could be an effective method for the analysis of aflatoxin M_1 in real milk samples since it is the only aflatoxin which can occur in milk.

Table 5 compares the characteristics of direct fluorimetric assay that have been reported for the determination of aflatoxins. While the method here has the limitation of non-specificity, this is also an issue for many antibody-based systems. On the other hand, the simplicity and cost savings constitute an important advantage.

Conclusions

The proposed method based on the two-phases clean-up is relatively rapid and effective for the detection of aflatoxins in foods. However, it is limited in that the fluorescence analysis is unable to provide selective determination of the different aflatoxins based on their excitation wavelength. The developed clean-up technique needs to be coupled to an analysis method which can offer individual determination of each aflatoxin component.

The limit of detection of this method is equal to 0.3 $\mu g\,kg^{-1}$, which is lower than the maximum admissible level of aflatoxin B_1 (2 $\mu g\,kg^{-1}$).

Data obtained from the analysis of real samples showed that recoveries of AFM_1 in spiked milk extracted using chloroform were over 95%, while for the barley matrix satisfactory results were obtained when the original extraction was done with methanol. In these two foods the results suggest that the clean-up excludes interference coming from the sample matrix and thus has the potential to be used as a screening method and without the need of column extraction based on clean-up and derivatization steps.

Acknowledgements. The authors would like to thank the European Community and the Italian M.P.A. for financial support of the EU project "Rosepromilk" and the national project "Aflarid", respectively. The authors acknowledge the financial support obtained from the scientific and technological cooperation between the Republic of Italy and the Kingdom of Morocco (2004–2006).

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