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Journal of Chromatography A, 937 (2001) 31–40

JOURNAL OF CHROMATOGRAPHY A

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New reversed-phase liquid chromatographic method to detect aflatoxins in food and feed with cyclodextrins as fluorescence enhancers added to the eluent

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Received 15 June 2001; received in revised form 10 September 2001; accepted 19 September 2001

Abstract

The effect of succynil–β-cyclodextrin (β-CD–Su), dimethyl-β-cyclodextrin (DIMEB) and β-cyclodextrin (β-CD) on the fluorescence of aflatoxins B_1, B_2, G_1, G_2 and M_1 (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1) was studied: β-CD–Su promoted the largest fluorescence enhancement for AFB_1 and AFM_1 while DIMEB showed better results for AFG_1. On the basis of the fluorescence enhancement, a new RP-HPLC method for detecting aflatoxins B_1, B_2, G_1, G_2 and M_1 was developed using cyclodextrins directly dissolved in the LC eluent. Aflatoxins B_1, B_2, G_1 and G_2 were resolved using a MICRA NPS ODS-1 column using methanol–water as mobile phase to which 6×10^{-3} *M* β-CD–Su or β-CD were added. Chromatographic responses of AFB_1 and AFG_1 achieved using β-CD dissolved in the mobile phase were enhanced, respectively, 8 and 12 times, and 10 and 15 times with β-CD–Su. Detection limits lower than 0.3 μg/kg were achieved for all the four aflatoxins. Aflatoxin M_1 was analysed using a Spherisorb S3 ODS-2 Narrow Bore column and methanol–water as mobile phase with added 2×10^{-3} *M* β-CD–Su. An area enhancement of 1.5 was detected for the toxin and the detection limit achieved under these analytical conditions was lower than 0.0005 μg/kg. Both methods were statistically validated showing a linear response for all the aflatoxins tested ($R^2\geq0.99$), and applied to the analysis of spiked and naturally contaminated food samples.

Keywords: Food analysis; Aflatoxins; Cyclodextrins

1. Introduction

Aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) are the main toxins produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* [1,2] (Fig. 1).

They may be present in any foodstuff or animal feed which can support fungal growth, although the main production has been reported in maize, peanuts, Brazil or pistachio nuts, copra and cottonseeds.

Aflatoxins are carcinogenic, mutagenic, teratogenic and immunosuppressive to most animal

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01300-0

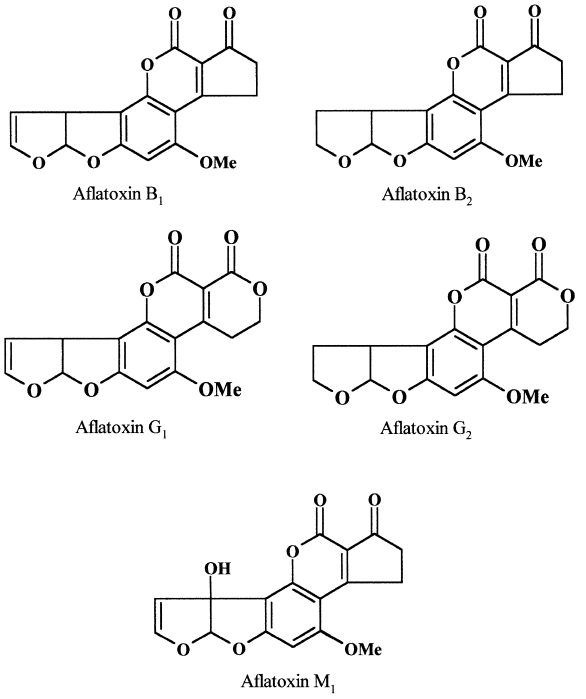

Fig. 1. Structures of aflatoxins AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1.

species. The order of toxicity, $AFB_1 > AFG_1 > AFB_2 > AFG_2$, indicates that the terminal furan moiety of AFB_1 is the critical point for determining the degree of biological activity of this group of mycotoxins [3–6]. The International Agency for Research on Cancer (IARC) has classified all four aflatoxins as Group 1 carcinogens [7].

Aflatoxin M_1 (AFM_1) is the main metabolic derivative of aflatoxins in several animal species (Fig. 1). It is produced by hydroxylation of AFB_1 in the liver of lactating animals, including humans [8]. AFM_1, also known as 'milk toxin', is much less carcinogenic and mutagenic than AFB_1 although its acute toxicity is similar to that of the other aflatoxins [9]. It has been classified by IARC as a Group 2 carcinogen [7]. AFM_1 can generally be found in milk and milk products such as dry milk, whey, butter, cheese, yoghurt and ice cream, together with AFM_2, which is the analogous metabolic derivative of AFB_2.

Legal limits for aflatoxins in food and animal feed have been established in approximately 60 countries. Values vary widely and tend to be higher in sub-tropical countries, which produce susceptible agricultural commodities, and lower in consumer nations with temperate or colder climate [10]. The ECN 1525/98 Regulations have established common legal limits in peanuts and cereals for direct human consumption, for the total AFB_1, AFB_2, AFG_1 and AFG_2 (4 μg/kg) and for AFB_1 singularly (2 μg/kg), and for AFM_1 in milk (0.05 μg/kg), for all EC member states [11].

Recently there has been an increase in aflatoxin contamination of food causing concern among producers and consumers: hence, the availability of reproducible and sensitive methods for the screening of foodstuffs is essential. Different analytical methods are currently used: immunoassay methods such as ELISA may be well suited for the rapid, routine diagnostic application of aflatoxin detection, although they show low reproducibility, particularly for the detection of AFM_1 when present in concentrations very close to the EC legal limits [12]. Chromatographic methods, in particular RP-HPLC with fluorescence detection, are currently the most commonly used, the latter offering greater versatility in the analysis of complex matrices such as cereal extracts and mixed feed [13,14]. In order to improve the detection limits, the native fluorescence of AFG_1 and AFB_1 has been enhanced by pre-column formation of hemiacetal derivatives with trifluoracetic acid [15] or by post-column derivatization with bromine or iodine [16,17]. However, both methods present several disadvantages. The use of post-column in-line photochemical derivatization of AFG_1 and AFB_1 has also been reported [18], the method requiring a sophisticated irradiation system.

Cyclodextrins (CDs), mainly β-CD, have been used as fluorescence enhancers, usually as post-column additives [19–21], in order to improve methods for the detection of aflatoxin contamination. A substantial enhancement of the fluorescence emission has been reported for those aflatoxins with an unsaturated furan ring (AFB_1, and AFG_1), while the emission properties of aflatoxins with a saturated furan ring (AFB_2 and AFG_2) have been shown to remain practically unchanged [22].

Franco et al. [23] have investigated fluorescence properties of AFM_1 and two other AFB_1 metabolic derivatives, aflatoxin P_1 and aflatoxin Q_1, in the presence of aqueous solutions of the CDs mentioned

above, reporting that the highest increases were obtained with DIMEB.

To our knowledge, only one paper by Vasquez et al. [24] has been published on AFB_1, AFB_2, AFG_1 and AFG_2 detection using RP-HPLC with β-cyclodextrin directly dissolved in the mobile phase: in this paper capacity factors (k) for aflatoxins at different concentrations of β-CD were used for calculating the complex formation constants. In this case, no analytical validation (detection limits, calibration curves, etc.) and application to foods have been neither performed nor envisaged. A RP-HPLC method was developed by Seidel et al. [25] for the simultaneous determination of other mycotoxins such as ochratoxin and zearalenone, using β-cyclodextrin as a mobile phase additive.

In this work we propose a new, highly sensitive and reproducible RP-HPLC method for aflatoxin detection in food by adding different CDs to the eluent in order to enhance the native fluorescence of AFB_1, AFG_1, AFB_2, AFG_2 and AFM_1.

Fluorescence measurements of aflatoxins in the absence and in the presence of aqueous solutions of succinyl, DIMEB and β-CD were first carried out in order to compare the relative fluorescence response.

Chromatographic studies were performed in order to establish the optimal conditions for the fluorescence enhancement using columns which allowed a good resolution of aflatoxins, at short retention times and with minimal CD consumption.

The method has been statistically validated and applied to animal feed in order to detect the aflatoxin concentrations below the legal limits and to naturally or artificially contaminated milk samples in order to detect AFM_1 at concentrations near zero.

The mechanism of fluorescence enhancement has been simultaneously studied with the CDs here considered and it has been found to be consistent with an inclusion phenomenon [34].

2. Experimental

2.1. Reagents

The aflatoxin standard solutions (benzene–acetonitrile, 98:2; concentration of each aflatoxin solution: 3 μg/ml) were obtained from Sigma (St. Louis, MO, USA). All solvents used were of LC grade from Carlo Erba (Milan, Italy); bi-distilled water was produced in our laboratory utilising an Alpha-Q system from Millipore (Marlborough, MA, USA). β-CD (purity ≥99.0%) and β-CD–Su (randomly substituted, substitution degree 3.5) were purchased from Aldrich (Steinheim, Germany), dimethyl-β-CD (DIMEB; purity ≥98.5%) from Acros, Carlo Erba (Milan, Italy). The immunoaffinity columns AFLA M_1™ and AFLATEST P™ were obtained from VICAM (Watertown, MA, USA).

2.2. Fluorescence

Fluorescence spectra were recorded on a Perkin-Elmer LS 50 instrument (Perkin-Elmer, Beaconsfield, England); both excitation and emission slits were set at 15 nm; all measurements were performed in triplicate in a 1×1 cm optical length quartz cell. Concentrated solutions of CDs (10^{-2} M) were prepared in aqueous medium, then diluted to the desired concentrations.

Working solutions of aflatoxins (10^{-7} M) were prepared drawing 100 μl from the commercial standard solutions, evaporating to dryness the organic phase and dissolving the residue in 10 ml of bi-distilled water. These aqueous solutions were stored in amber-coloured vials at −18°C for no longer than a month.

For the fluorescence experiments, increasing amounts of CD solutions (10^{-2} M) were added to the AF solution (10^{-7} M) in order to obtain molar ratio AF/CD in the range 1:1–1:10^5. Fluorescence intensities were corrected for dilution effect. Results were reported in Stern–Volmer graphs by plotting the ratio F_0/F of the fluorescence intensity, at the maximum emission, vs. the CD concentration.

2.3. HPLC analysis

Chromatographic analyses were performed with Waters Model 510 pumps (Waters, Milford, MA, USA) equipped with a Rheodyne Model 7161 injector (20 μl loop) (Cotati, CA, USA), a Waters Model CHM column oven and a Model 474 fluorescence detector (λ_{ex}=365 nm and λ_{em}=425 nm for AFB_1, AFB_2, AFG_1 and AFG_2, λ_{ex}=360 nm and λ_{em}=435

nm for AFM_1). The system was computer controlled by a MAXIMA 820 Chromatography Workstation for data handling.

A Spherisorb ODS-2 Narrow Bore column (150×2.1 mm, 5 μm particles) for AFM_1 analyses and a MICRA NPS ODS-1 column (33×4.6 mm, 1.5 μm particles) (MICRA, Northbrook, IL, USA) for AFB_1, AFB_2, AFG_1 and AFG_2 analyses were used. Both columns were thermostated at 40°C.

The mobile phase was prepared daily by dissolving the appropriate amount of cyclodextrin in water and by adding methanol to give the desired final concentration. The methanol–water eluent was 12:88 v/v for β-CD–Su, 10:90 v/v for β-CD on a MICRA NPS ODS-1 column, and methanol–water 42:58 v/v on a Spherisorb ODS-2 column; it was filtered and degassed under reduced pressure on HPLC filters (0.45 μm). In order to equilibrate the system, the mobile phase was allowed to flow through the column for approximately 60 min at a flow-rate of 0.2 ml/min. The flow-rate was 0.6 ml/min for the MICRA NPS ODS-1 column analyses and 0.2 ml/min for the Spherisorb ODS-2 column analyses. Working solutions of the aflatoxins were prepared daily from standard solutions, by evaporating the appropriate amount of the organic phase under nitrogen and dissolving the residue in bi-distilled water to the desired concentrations.

Calibration curves were based on the analysis of standards at four concentration levels (three determinations at each level have been performed). Linear calibration graphs were obtained by plotting the peak area against the aflatoxin amounts injected (from 0.1 to 5.0 ng for all aflatoxins) at the conditions mentioned above. The significance of linear regression and intercept were calculated by SPSS 8.0 statistical software with the ANOVA model and the Student *t*-test, respectively. Quantitation of AF was performed by comparing the peak areas with the calibration curve.

The detection limits (LOD) were calculated by using a signal-to-noise ratio of 3:1, the quantitation limits (LOQ) by using a signal-to-noise ratio of 10:1. The precision of the procedure was obtained from standard deviations and variation coefficients for three replicate injections of known amounts of aflatoxin.

2.4. Sample preparation and immunoaffinity clean-up procedures

2.4.1. Feed

The procedure was based on Trucksess' method [26], modified as follows: 50 g of ground sample and 5 g of NaCl were extracted by mechanical stirring with 100 ml of methanol–water (80:20) for 15 min followed by filtration through prefolded paper. A 10 ml volume of filtrate was diluted with 40 ml of bi-distilled water and filtered again. The filtrate was applied to an immunoaffinity column at a flow-rate of 2–3 ml/min. The column was washed twice with 10 ml of bi-distilled water, then eluted with 1 ml of methanol. The eluate was evaporated to dryness under nitrogen, redissolved in 500 μl of bi-distilled water and filtered through a 0.45 μm filter membrane. It was again evaporated to dryness under nitrogen and redissolved in 100 μl of bi-distilled water before HPLC injection. The procedure was modified for the peanut meal sample as follows: 25 g of ground sample with the addition of 5 g of NaCl were extracted under mechanical stirring with 125 ml of methanol–water (60:40) for 15 min and filtered through prefolded paper. Twenty milliliters of filtrate were diluted with 20 ml of bidistilled water and filtered again. The filtrate was applied to an immunoaffinity column and treated as described above.

Recovery experiments were performed on AF free samples (three determinations at each concentration level have been performed). The volume of the spiking solution required to obtain feed containing 0.25–1.00 μg/kg was directly added to the ground sample. After an hour, the sample was extracted as described before and the AF concentration determined using the protocol previously described.

2.4.2. Milk

The procedure was based on Hansen's method [27], modified as follows: 50 ml of milk were centrifuged at 4000 rpm for 15–20 min to separate the fat and applied to an immunoaffinity column at a flow-rate of 2–3 ml/min. The column was washed twice with 10 ml bi-distilled water, then eluted with 1.25 ml acetonitrile–methanol (3:2) and 1.25 ml bi-distilled water. The eluate was collected in a vial and evaporated to dryness under nitrogen, redis-

solved in 500 μl bi-distilled water and filtered through a 0.45 μm filter membrane. It was again evaporated to dryness under nitrogen and redissolved in 100 μl bi-distilled water before HPLC injection.

Recovery experiments were performed on AFM_1 free samples (three determinations at each concentration level have been performed). The volume of the spiking solution required to obtain milk containing 0.002–0.010 μg/kg was directly added to the sample and the AFM_1 concentration determined using the protocol previously described.

3. Results and discussion

3.1. Spectroscopic studies

In order to develop a RP-HPLC method using CDs as mobile phase additives for increasing the fluorescence emission of aflatoxins, we performed spectroscopic measurements of the fluorescence emission of all aflatoxins in the presence of charged and uncharged CDs and compared our results with those previously reported in the literature [22,23].

The ratios F/F_0, where F is the fluorescence intensity of each aflatoxin in the presence of CD and F_0 is the fluorescence intensity of each aflatoxin in aqueous solution, measured for all aflatoxins with each CD are shown in Table 1.

A strong fluorescence enhancement was observed for the furan-unsaturated aflatoxins, in particular AFB_1 and AFG_1 with all the CDs. No significant enhancements were observed for saturated AFG_2 and AFB_2. Actually, with β-CD–Su, due to its higher water solubility, it was possible to reach a CD/AF ratio of 10^6 which could not be reached with the other CDs: in these conditions very high fluorescence enhancements were obtained. AFM_1 showed a moderate enhancement with these CDs and a ten-fold increase at the highest concentration of β-CD–Su.

The ratio F/F_0 for AFB_1 was also reported as a function of the CDs concentrations (Fig. 2). A similar trend was also observed for the other aflatoxins.

Significant enhancement of the fluorescence intensity was observed for all the aflatoxins when the CD/AF ratio reached a value $>10^3$ confirming the data previously reported in the literature [22]. The mechanism responsible for aflatoxin fluorescence enhancement by CDs involves the formation of inclusion complexes and is discussed elsewhere [34].

Table 1
The response ratio (F/F_0)[a] for aflatoxins (10^{-7} *M* aqueous solution) recorded using β-CD, DIMEB and β-CD–Su

	F/F_0 [a]				
	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1
β-CD[a]	11.6	1.3	10.2	1.1	1.7
DIMEB[a]	14.5	1.3	21.0	1.2	2.9
β-CD–Su[a]	27.5	1.2	13.9	1.2	3.0
β-CD–Su[b]	63.0	2.0	54.0	2.2	10.1

[a] F_0 native aflatoxin fluorescence in water; F fluorescence in the presence of CD. Experimental conditions: $\lambda_{ex}=365$ nm for AFB_1, AFG_1, AFB_2 and AFG_2 and $\lambda_{ex}=360$ nm for AFM_1; CD concentration 1×10^{-2} *M* unless stated otherwise.

[b] 1×10^{-1} M.

3.2. Chromatographic studies

3.2.1. AFB_1, AFB_2, AFG_1 and AFG_2 determination

Preliminary studies were carried out in order to establish the best chromatographic conditions for the

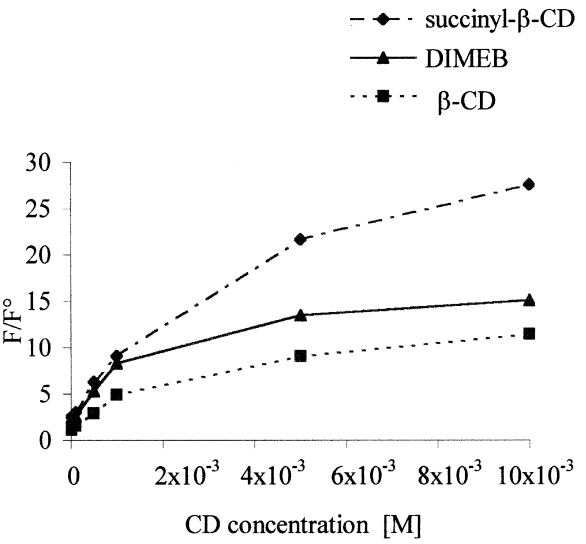

Fig. 2. Effect of increasing amounts of CDs on the AFB_1 fluorescence (10^{-7} *M* aqueous solution). (F_0: native aflatoxin fluorescence in water; F: fluorescence in the presence of CD).

aflatoxin separation using columns that allowed rapid analysis, thus minimising consumption of the cyclodextrin dissolved in the mobile phase.

A MICRA NPS ODS-1 column (33×4.6 mm) was used for AFB_1, AFB_2, AFG_1 and AFG_2 analyses using a methanol–water solution (12:88 v/v) as mobile phase at a flow-rate of 0.6 ml/min. Under these experimental conditions, the analysis was performed in less than 10 min, maintaining the column temperature at 40°C in order to obtain sharp chromatographic peaks. The elution order obtained was $AFG_2 < AFG_1 < AFB_2 < AFB_1$ when using β-CD or β-CD–Su dissolved in the mobile phase, while no aflatoxin separation was obtained using DIMEB on this column.

The natural fluorescence of AFB_1 and AFG_1 was enhanced considerably by increasing the concentration of β-CD or β-CD–Su dissolved in the mobile phase, as shown in Figs. 3 and 4, where the chromatograms recorded in the absence and in the presence of β-CD and β-CD–Su, respectively, are reported.

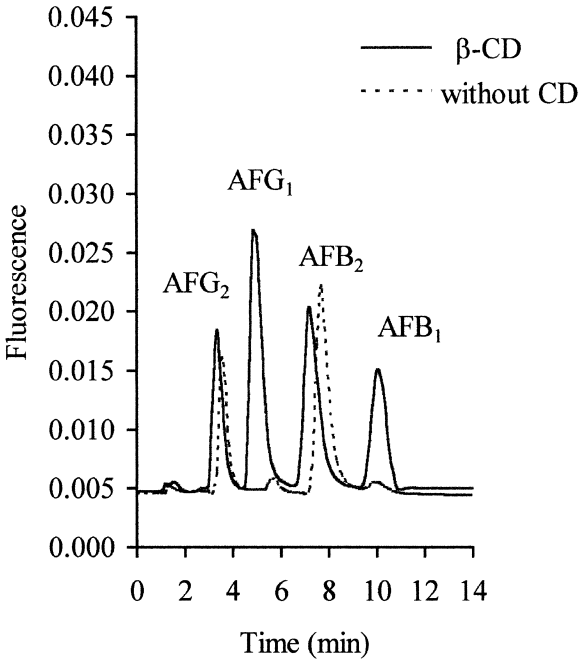

Fig. 3. Chromatographic separation of an aflatoxin standard mixture in the absence and in the presence of β-CD. Chromatographic conditions: MICRA NPS ODS-1 column; mobile phase, methanol–water (10:90 v/v); flow-rate 0.6 ml/min; column temperature 40°C; fluorescence detector (λ_{ex}=365 nm and λ_{em} 425 nm).

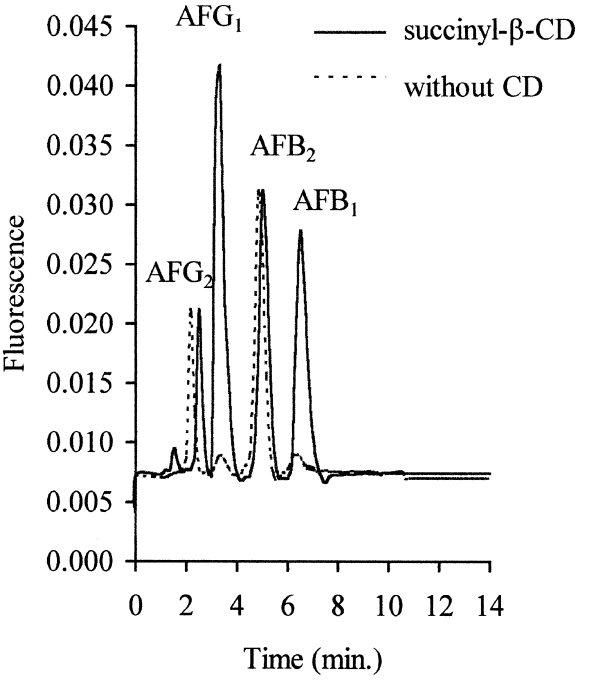

Fig. 4. Chromatographic separation of an aflatoxin standard mixture in the absence and in the presence of β-CD–Su. Chromatographic conditions: MICRA NPS ODS-1 column; mobile phase, methanol–water (12:88 v/v); flow-rate 0.6 ml/min; column temperature 40°C; fluorescence detector (λ_{ex}=365 nm and λ_{em}= 425 nm).

The response ratios recorded for each aflatoxin in the presence (A_{CD}) and in the absence (A_0) of β-CD and β-CD–Su are reported in Table 2.

The use of β-CD–Su at a concentration of 6×10^{-3} *M* gave the best results, significantly increasing the response of the furan-unsaturated aflatoxins. No significant fluorescence enhancement was recorded for AFG_2 and AFB_2, in agreement with the spectroscopic studies previously described. A good separation of the four aflatoxins was also obtained with a Spherisorb ODS-2 column, using a methanol–water mixture (42:58 v/v) as mobile phase, at a flow-rate of 0.2 ml/min. AFB_1 and AFG_1 chromatographic responses in the absence of CD were higher with this column than the ones recorded by the MICRA NPS ODS-1 column, where a lower methanol content (methanol–water 12:88 v/v) was used in the mobile phase. This phenomenon could be probably related

Table 2
Area ratios (A_{CD}/A_0)[a] for the aflatoxins at different β-CD and β-CD–Su concentrations in HPLC

	AFB_1	AFB_2	AFG_1	AFG_2
$A_{\beta\text{-CD}}/A_0^a$ [2×10^{-3} M]	2.36	1.00	3.06	1.02
$A_{\beta\text{-CD}}/A_0^a$ [6×10^{-3} M]	7.60	1.12	12.30	1.25
$A_{\beta\text{-CD-Su}}/A_0^a$ [2×10^{-3} M]	4.80	1.00	6.50	1.00
$A_{\beta\text{-CD-Su}}/A_0^a$ [6×10^{-3} M]	10.40	1.10	15.40	1.30

Chromatographic conditions: MICRA NPS ODS-1 column; mobile phase: methanol–water (β-CD: 10:90 v/v; β-CD–Su: 12:88 v/v); flow-rate 0.6 ml/min; column temperature: 40°C; $\lambda_{ex}=365$ nm, $\lambda_{em}=425$ nm). Injected amount 0.50 ng for AFB_1 and AFG_2, 0.12 ng for AFB_2 and 1.00 ng for AFG_1; values reported are the average of three injections.

[a] A_{CD} = peak area in the presence of CD; A_0 = peak area in the absence of CD.

to the spectroscopic properties of AFB_1 and AFG_1, both showing a higher fluorescence emission in methanol than in water [28].

In contrast, the responses obtained in the presence of β-CD or β-CD–Su with the Spherisorb ODS-2 column were lower than those observed with the MICRA NPS ODS-1 one, in agreement with the fact that methanol may compete with aflatoxins for the CD cavity, as already reported [24]. The proposed method (MICRA NPS ODS-1 column) was applied for the analysis of several samples of raw ingredients for animal feed such as maize, maize pellets, and nut pellets. The chromatogram obtained for a contaminated peanut meal sample is reported in Fig. 5. The four aflatoxins were well separated in less than 10 min and no interfering peaks were detected during the analysis.

Calibration graphs were obtained as reported in the experimental section. All the graphs were linear in the range studied, showing correlation coefficients ≥ 0.99 for all aflatoxins. The calculated statistical significances for linear regressions gave values $\geq 99.9\%$ for all aflatoxins.

The fit parameters of the linear regression performed for each aflatoxin using β-CD and β-CD–Su are reported in Table 3.

The repeatability of the method was studied by three repeated injections of a standard mixture of the four aflatoxins: the relative standard deviations were ±1.7% for AFB_1, AFB_2 and AFG_2 and 3% for AFG_1. Variation coefficients (RSD) were less than 6% for most samples analysed.

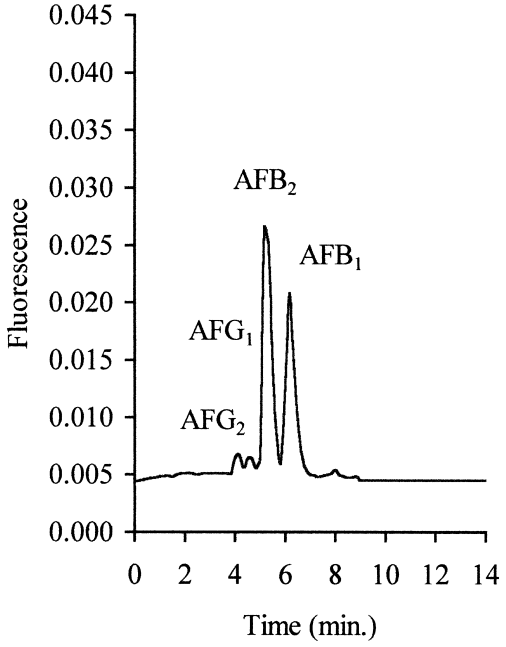

Fig. 5. Chromatogram of a contaminated peanut meal (contamination levels for aflatoxins: $AFB_1=13.5\pm0.3$ ppb, $AFB_2=4.05\pm0.09$ ppb, $AFG_1=6.7\pm0.2$ ppb, $AFG_2=4.57\pm0.08$ ppb). Chromatographic conditions: MICRA NPS ODS-1 column; mobile phase, methanol–water (12:88 v/v), β-CD–Su added 6×10^{-3} M; flow-rate 0.6 ml/min; column temperature 40°C; fluorescence detector ($\lambda_{ex}=365$ nm and $\lambda_{em}=425$ nm).

Mean recoveries for all AF of 76.5±1.1, 75.2±5.3 and 73.2±6.4% were obtained by spiking feed samples with an aflatoxin standard mixture of 0.25, 0.50 and 1.00 μg/kg, respectively (three determinations at each level have been performed).

Limits of detection (LOD) and limits of quantitation (LOQ) for each aflatoxin were determined (Table 4). LOD and LOQ values obtained with β-CD–Su in the mobile phase for all aflatoxins were lower than those obtained with β-CD or without CD, particularly for AFB_1 and AFG_1, as expected, on the basis of the strong fluorescence enhancement.

No significant differences were recorded for the furan-saturated aflatoxins.

Detection limits shown in Table 4 were lower than those obtained by Cepeda et al. [20] using CDs as post-column additives. Recently, Vasquez et al. [21] have obtained a detection limit of 0.006 ng for AFB_1 with 20 μl of standard samples, using a 2.0 mm I.D. column and DIMEB as a post-column reagent at a

Table 3
Parameters of linear regression[a] measured for AFB_1, AFB_2, AFG_1 and AFG_2 using 6×10^{-3} *M* β-CD and β-CD–Su and for AFM_1 using 2×10^{-3} *M* β-CD–Su in HPLC

β-CD	AF	Slope (*a*)	Intercept (*b*)	R^2
	AFB_1	1.00×10^{6}	1.36×10^{5}	0.992
	AFB_2	1.00×10^{7}	1.00×10^{6}	0.991
6×10^{-3} *M* β-CD	AFG_1	4.29×10^{5}	1.36×10^{5}	0.994
	AFG_2	1.00×10^{6}	2.59×10^{4}	0.992
	AFB_1	1.50×10^{6}	1.03×10^{6}	0.997
	AFB_2	1.00×10^{7}	6.67×10^{5}	0.998
6×10^{-3} *M* β-CD–Su	AFG_1	4.32×10^{6}	3.00×10^{3}	0.998
	AFG_2	1.02×10^{6}	3.24×10^{4}	0.998
2×10^{-3} *M* β-CD–Su	AFM_1	3.00×10^{6}	9.08×10^{4}	0.999

Chromatographic conditions for AFB_1, AFB_2, AFG_1 and AFG_2: MICRA NPS ODS-1 column; mobile phase: methanol–water (10:90 v/v β-CD; 12:88 v/v β-CD–Su); flow-rate 0.6 ml/min; column temperature 40°C; $\lambda_{ex}=365$ nm, $\lambda_{em}=425$ nm. Chromatographic conditions for AFM_1: Spherisorb ODS-2 Narrow Bore column; mobile phase: methanol–water (42:58 v/v); flow-rate 0.2 ml/min; column temperature 40°C; $\lambda_{ex}=360$ nm, $\lambda_{em}=435$ nm.

[a] $y=ax+b$; y=peak area, x=ng injected (0.1, 0.5, 1.0, 2.5, 5.0 ng), R^2=regression coefficient.

1×10^{-2} *M* concentration, higher than the one used here.

The detection limits in feed samples (maize, maize pellets, and nut pellets), obtained with 6×10^{-3} *M* β-CD–Su, were 0.05 μg/kg for AFB_1, 0.003 μg/kg for AFB_2, 0.125 μg/kg for AFG_1 and 0.075 μg/kg for AFG_2. These values are much lower than the EC Regulatory limits and still lower than those obtained by the pre- or post-column derivatization methods previously reported [15–17].

Therefore, the method here proposed is very useful not only for determining aflatoxins at levels below the EC legal limits, but also for detecting very small traces in foodstuff or feed, thus providing an easily accessible quality control tool.

3.2.2. AFM_1 determination

AFM_1 surveillance in dairy products requires a quantitative and extremely sensitive analytical method [29–31]. EC legal limits for the AFB_1 metabolic derivative is fixed at 0.05 μg/kg, although proposals have been advanced to diminish maximum tolerance limits to 0.01 μg/kg, as already existing in Italy for infant formula [32]. Detection limits obtained by the official method (pre-column derivatization with trifluoracetic acid) are in the range of 0.005–0.025 μg/kg [33].

With the aim of further lowering the AFM_1 detection limits, we investigated fluorescence enhancement of AFM_1 by β-CD, DIMEB and β-CD–Su added to the mobile phase.

AFM_1 analyses were carried out using a Spherisorb ODS-2 Narrow Bore column because of the presence of interfering peaks, recorded during contaminated milk analyses with the MICRA NPS ODS-1 column. A methanol–water mixture was

Table 4
Limits of quantitation (LOQ) and limits of detection (LOD) in the absence and in the presence of β-CD and β-CD–Su added to the mobile phase

AF	LOQ (ng)			LOD (ng)		
	Without CD	β-CD	β-CD–Su	Without CD	β-CD	β-CD–Su
AFB_1	3.75×10^{-1}	4.00×10^{-2}	2.00×10^{-2}	1.25×10^{-1}	2.00×10^{-2}	1.00×10^{-2}
AFB_2	3.00×10^{-3}	1.50×10^{-3}	1.50×10^{-3}	1.00×10^{-3}	0.75×10^{-3}	0.50×10^{-3}
AFG_1	1.50	1.00×10^{-1}	5.00×10^{-2}	5.00×10^{-1}	5.00×10^{-2}	2.50×10^{-2}
AFG_2	4.00×10^{-2}	3.00×10^{-2}	3.00×10^{-2}	2.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}

Chromatographic conditions: MICRA NPS ODS-1 column; mobile phase: methanol–water (β-CD: 10:90, v/v; β-CD–Su: 12:88 v/v); flow-rate 0.6 ml/min; column temperature: 40°C; $\lambda_{ex}=365$ nm, $\lambda_{em}=425$ nm).

utilized as mobile phase (42:58 v/v at a flow-rate of 0.2 ml/min) rather than the more commonly used acetonitrile–water, in order to decrease the quenching of AFM_1 natural fluorescence by acetonitrile and to separate AFM_1 from AFM_2.

The response ratios obtained for AFM_1 in the presence (A_{CD}) and in the absence (A_0) of β-CD, DIMEB and β-CD–Su are reported in Table 5.

The addition of 2×10^{-3} *M* β-CD–Su to the mobile phase produced an area enhancement of 1.50 for AFM_1 while the increase recorded using the other CDs at the same concentration was less noticeable, as expected according to the results of the spectroscopic studies previously reported. The use of a higher concentration (6×10^{-3} *M*) of β-CD–Su or DIMEB did not improve further the AFM_1 chromatographic response.

A chromatogram of a spiked raw milk sample (0.002 μg/kg), recorded by adding 2×10^{-3} *M* β-CD–Su to the mobile phase, is shown in Fig. 6.

A calibration graph was obtained as reported for the other aflatoxins. The graph was linear in the range studied showing a correlation coefficient ≥0.99. The calculated statistical significance for linear regression gave values of ≥99.9%. The repeatability was proved by three repeated injections of AFM_1; the calculated standard deviation was less than 2%. Coefficients of variation (RSD) were less than 6% for all the samples analysed. Mean recoveries of AFM_1, added at 0.002, 0.005 and 0.010 μg/kg to raw milk samples were 101±5.0, 92.5±7.5 and 92.7±2.0%, respectively (three determinations at each concentration level have been performed). The detection limit was <0.0005 μg/kg, largely below the 0.010 μg/kg, which is the legal limit demanded by some EC countries.

Table 5
Area ratios (A_{CD}/A_0)[a] for AFM_1 using β-CD, DIMEB and β-CD–Su at different concentrations in HPLC

	AFM_1
$A_{\beta\text{-CD}}/A_0^a$ [2×10^{-3} M]	1.02
$A_{\beta\text{-CD–Su}}/A_0^a$ [2×10^{-3} M]	1.50
$A_{\beta\text{-CD–Su}}/A_0^a$ [6×10^{-3} M]	1.55
A_{DIMEB}/A_0^a [2×10^{-3} M]	1.20
A_{DIMEB}/A_0^a [6×10^{-3} M]	1.30

Spherisorb ODS-2 Narrow Bore column; mobile phase: methanol–water (42:58 v/v); flow-rate 0.2 ml/min; column temperature 40°C; λ_{ex}=360 nm, λ_{em}=435 nm. Injected amount 0.5 ng, values reported as average of three injections.

[a] A_{CD}=peak area in the presence of CD; A_0=peak area in the absence of CD.

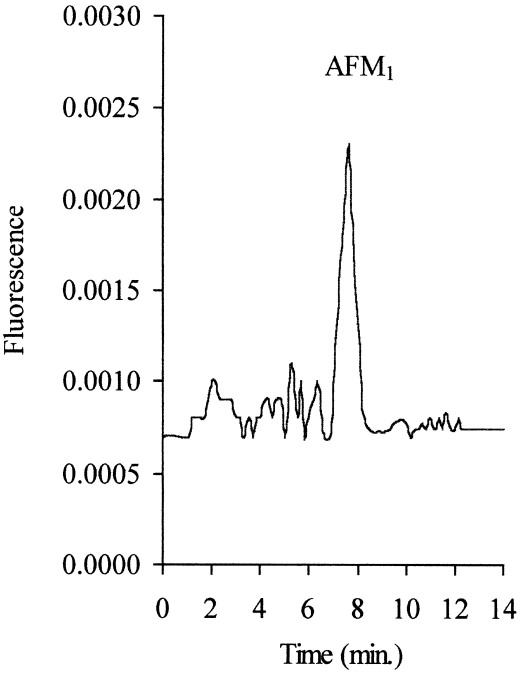

Fig. 6. Chromatogram of a spiked raw milk sample (0.002 μg/kg of AFM_1). Chromatographic conditions: Spherisorb ODS-2 Narrow Bore column. Mobile phase, methanol–water (42:58 v/v); β-CD–Su added, 2×10^{-3} M; flow-rate 0.2 ml/min; column temperature 40°C; fluorescence detector (λ_{ex}=360 nm and λ_{em}=435 nm).

4. Conclusions

In conclusion, an accurate investigation of the spectroscopic properties allowed us to obtain very satisfactory results in the HPLC separation of the four aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 with the addition of β-CD and β-CD–Su to the eluent. The detection limits were lowered for AFB_1 and AFG_1.

In principle, the detectability of AFM_1 could also be greatly improved by using high concentrations of β-CD–Su, as shown in the fluorescence experiments. However, limiting the concentration of β-CD–Su to a reasonable amount (2×10^{-3} *M*) it is still possible to improve the detection limits in HPLC.

Acknowledgements

This work has been financially supported by Ministero della Ricerca Scientifica e Tecnologica (MURST, Rome, Italy): Supramolecular devices.

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