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Fluorescence Enhancement of Aflatoxins Using Native and Substituted Cyclodextrins

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Abstract

Cyclodextrins have been used as fluorescence enhancers in HPLC to improve the detection limits of aflatoxins, cancerogens which can be found in several foodstuffs. In this work, a screening of several cyclodextrins has been performed in order to find the factors affecting the fluorescence enhancement. Evidence for formation of a 1 : 1 AF-CD inclusion complex has been achieved by titration and competitive experiments with adamantanecarboxylic acid and by fluorescence quenching by KI. Stability constants of the AF-CD complexes were evaluated.

Introduction

Aflatoxins B_1, B_2, G_1, G_2 (AFB_1, AFB_2, AFG_1, AFG_2, (Figure 1) are natural substituted coumarins produced by *Aspergillus flavus*, *A. nomius* and *A. parasiticus*, which can be found in foodstuffs supporting fungal growth, such as cereals, dried fruits, oil seeds, spices and pulses [1, 2]. These toxins are potent hepatocarcinogens and mutagen agents in man and other vertebrated, so that in 1993 they were declared as Group 1 carcinogens by International Agency for Research on Cancer (IARC) [3].

Aflatoxins have a rigid cyclic oxigenated structure with a high degree of conjugation which provides a low native fluorescence.

AFB_1 and AFB_2 are characterized by blue fluorescence (λ_{ex} = 365 nm, λ_{em} = 440 nm), while AFG_1 and AFG_2 give green fluorescence (λ_{ex} = 365 nm, λ_{em} = 460 nm) on account of a cyclopentenone ring *versus* an unsaturated δ-lactone ring in the structure. Differences are found also in the unsaturated or saturated terminal furan ring: the saturated derivatives, AFB_2 and AFG_2, have a higher fluorescence quantum yield than the unsaturated AFB_1 and AFG_1. Aflatoxin M_1 (AFM_1) is the 4-hydroxylated metabolite of AFB_1, produced by hepatic hydroxylation in mammalian species and it can be found in milk [4].

The analytical technique generally adopted for AF determination is HPLC with fluorescence detection, but due to their low natural fluorescence it is necessary to enhance the quantum yield, by using pre- or post-column derivatization systems, e.g., TFA, I_2 or Br_2 [5–8].

β-Cyclodextrins have also been reported to increase the fluorescence intensity as post-column derivatization reagents in HPLC [8] or added to the buffer in capillary electrokinetic chromatography [9, 10]. Hydroxypropyl-β-CD was also studied as solubility enhancer for aflatoxins [11].

In the past decade, spectroscopic and chromatographic studies were carried out to study the interactions between CDs and aflatoxins [12–16]. In particular, Vasquez *et al.* [12–14, 16] reported that the fluorescence quantum yield of AFB_1 and AFG_1 is enhanced in the presence of α- and β-CD in aqueous solution, whereas that of AFB_2 and AFG_2 is unaffected. Franco *et al.* [15] reported that fluorescence enhancement is evident for AFM_1 in aqueous solutions only in the presence of dimethyl-β-CD (DIMEB). The authors suggested that the change in the emission oscillator strength could be due to an interaction between the furan double bond and the inner or the outer surface of the cyclodextrin.

We have recently proposed a new RP-HPLC method for determining aflatoxins by using cyclodextrins as fluorescence enhancers directly added to the eluent: the use of β-CD or succinyl-β-CD allowed us to achieve detection limits more than one order of magnitude lower than those established as the EU legal limits [17].

The aim of this work is to investigate which factors affect the AF fluorescence enhancement in the presence of CDs and to gain evidence for the eventual formation of an AF-CD inclusion complex. In particular, we intend to study the effect of several unsubstituted, mono- and polysubstituted, neutral and charged CDs on the fluorescence properties of AFs in aqueous solution, as well as the nature of the AF-CD interactions by competitive experiments with adamantane-

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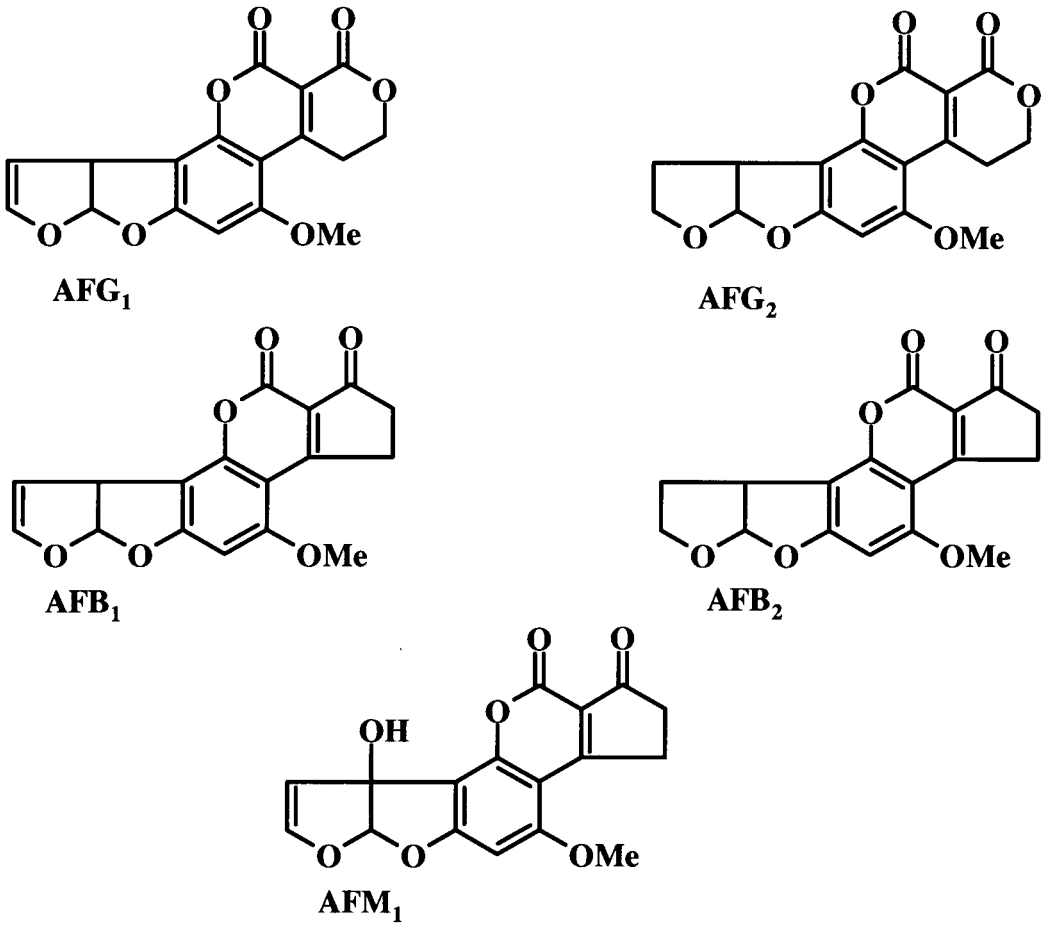

Figure 1. Chemical structures of aflatoxins.

carboxylic acid (ACA), which has a specific affinity for the β-CD cavity, and by luminescence quenching by KI. Stability constants of the AF-CD complexes will be evaluated by performing fluorescence titrations of aflatoxins with β-CD, succinyl-β-CD (β-CD-Su) and dimethyl-β-CD (DIMEB).

Experimental section

Reagents

All the aflatoxin standard solutions (benzene : acetonitrile 98 : 2) were from SIGMA (St. Louis, MO, USA). All solvents were LC grade from Carlo Erba (Milan, Italy) and bidistilled water was produced in our laboratory utilising an Alpha-Q System Millipore (Marlborough, MA, USA).

α-, β- and γ-CD were purchased from ACROS (Carlo Erba, Italy); heptakis(2,6-di-O-methyl)-β-CD (DIMEB), sulphated β-CD (S-β-CD; randomly substituted, substitution degree n = 7–10), tetra(6-O-(sulfo-n-butyl))-β-CD (SBE-β-CD; substitution degree n = 4), mono(6-δ-glutamylamino-6-deoxy)-β-CD (ZW-β-CD), O-(2-hydroxy-3-trimethyl-ammonio-n-propyl)-β-CD (TMA-β-CD), (2-hydroxypropyl)-β-CD (HP-β-CD), (2-hydroxypropyl)-γ-CD (HP-γ-CD) were purchased from ALDRICH (Steinheim, Germany); succinyl-β-CD (β-CD-Su; randomly substituted, substitution degree n = 4) carboxymethyl-β-CD (CM-β-CD; randomly substituted, substitution degree n = 4) were purchased from FLUKA (Buchs, Switzerland); heptakis (2,3,6-tri-O-methyl)-β-CD (TRIMEB) and 6-deoxy-6-amino-β-CD (MA-β-CD) were synthesized in our laboratory.

Adamantanecarboxylic acid and KI were from Aldrich (Steinheim, Germany).

Aflatoxin solutions: preparation and decontamination

Solutions of aflatoxins (10^{-7} M) were prepared from standard solutions by evaporating the organic phase under nitrogen and dissolving the residue in water. These solutions were stocked at -4 °C for a month. Decontamination of waste solutions and glassware was performed with sodium hypochlorite (10% aqueous solution) for 12 hours.

Spectroscopic studies

Fluorescence spectra were recorded on a PERKIN ELMER LS50 instrument in a 0.2 × 1.0 cm quartz cell. Due to the solvent effects on the absorption and luminescence spectra, the proper excitation wavelengths were selected by scanning the excitation spectra of the different aflatoxin solutions (10^{-7} M). The emission scan was performed in the wavelength range 380–600 nm. Both emission and excitation slits were 15 nm. Each spectrum was recorded in triplicate.

Table 1. Fluorescence enhancements of AFs in the presence of CDs

Cyclodextrins		$F_n = F/F_0$				
		AFB_1	AFG_1	AFM_1	AFB_2	AFG_2
N	β-CD	5.7 ± 0.1	6.5 ± 0.3	1.8 ± 0.6	1.0 ± 0.1	1.0 ± 0.1
	γ-CD	2.5 ± 0.3	1.7 ± 0.1	1.1 ± 0.1	1.05 ± 0.02	1.0 ± 0.2
	β-CD	11.6 ± 0.2	10.7 ± 0.2	1.7 ± 0.1	1.30 ± 0.03	1.06 ± 0.05
	TRIMEB	3.8 ± 0.7	2.4 ± 0.2	1.7 ± 0.1	1.1 ± 0.2	1.06 ± 0.06
	DIMEB	14.5 ± 0.5	21.0 ± 0.3	2.9 ± 0.1	1.32 ± 0.06	1.2 ± 0.1
	Hp-β-CD	12.3 ± 0.4	8.1 ± 0.6	1.1 ± 0.1	0.98 ± 0.07	1.28 ± 0.02
	Hp-γ-CD	1.5 ± 0.1	4.7 ± 0.5	2.5 ± 0.1	1.12 ± 0.02	1.0 ± 0.2
PC	MA-β-CD	7.5 ± 0.9	16.5 ± 0.7	3.6 ± 0.1	1.37 ± 0.04	1.11 ± 0.04
	TMA-β-CD	4.2 ± 0.2	12.1 ± 0.1	1.5 ± 0.1	1.13 ± 0.02	1.01 ± 0.01
NC	SBE-β-CD	6.5 ± 0.2	10.0 ± 0.6	1.1 ± 0.1	0.99 ± 0.03	1.15 ± 0.07
	S-β-CD	15.3 ± 0.5	3.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.1
	CM-β-CD	13.5 ± 0.4	8.3 ± 0.2	1.60 ± 0.04	1.26 ± 0.07	1.15 ± 0.01
	β-CD-Su	27.4 ± 0.5	13.9 ± 0.5	3.01 ± 0.03	1.21 ± 0.05	1.2 ± 0.2
	β-CD-Su (*)	63.0 ± 0.2	53.9 ± 0.5	10.1 ± 0.1	2.0 ± 0.1	2.24 ± 0.03
Z	ZW-β-CD	7.87 ± 0.1	17.4 ± 0.1	3.4 ± 0.2	1.4 ± 0.3	1.02 ± 0.02

Conditions: molar ratio AF-CD $1:10^5$, except (*) molar ratio AF-CD $1:10^6$; λ_{ex} = 365 nm AFB_1, AFG_1, AFG_2, AFB_2 and λ_{ex} = 360 nm for AFM_1; $\lambda_{MAX(em)}$ = 425 nm for AFB_1, $AFBG_1$, AFB_2, $AFBG_2$ and $\lambda_{MAX(em)}$ = 435 nm for AFM_1; both excitation and emission slits were 15 nm. The full names of cyclodextrins are reported in the Experimental Part. N = neutral CDs; PC = positively charged CDs; NC = negatively charged CDs; Z = zwitterionic CDs.

Measurements of fluorescence enhancements by CDs

The solutions of aflatoxins (10^{-7} M) and cyclodextrins (0.1 M or 0.01 M, according to the different solubilities) were prepared in water and the spectra of the aflatoxins alone (blank) and of the AF-CD solutions (molar ratio AF : CD = $1:10^5$ or, if possible, $1:10^6$) were recorded; the F/F_0 ratio was calculated, where F and F_0 are the fluorescence intensities in the presence (sample) or in the absence (blank) of the CDs, respectively (Table 1).

Competition experiment with ACA

0.5 and 0.1 M ethanolic solutions of ACA were prepared; 10^{-7} M aqueous solutions of each aflatoxin in 0.01 M aqueous solutions of β-CD or DIMEB or β-CD-Su were prepared. The reference spectra of each AF-CD solution (molar ratio AF : CD = $1:10^5$) and the spectra after addition of appropriate amounts of the ACA solution were recorded.

Quenching experiment with KI

Stock aqueous solutions of β-CD, DIMEB, β-CD-Su (0.01 M) and KI (0.1 M) were prepared. Appropriate aliquots of KI were added to the AF solutions (10^{-7} M), in the absence and in the presence of CDs (molar ratio AF : CD = $1:10^5$). The results were analysed applying the Stern–Volmer equation, by considering only dynamic (collisional) quenching:

$$F_n = 1 + K_q[\mathrm{KI}]_i,$$

where F_n is the fluorescence intensity measured after each KI addition and K_q is the quenching constant.

Determination of the complex formation constants

Aqueous solutions (0.01 M) of β-CD, DIMEB and β-CD-Su were prepared and diluted to the desired concentrations. Suitable aliquots of the CD solutions were added and the fluorescence spectra were recorded, measuring the change of the F/F_0 ratio as a function of the aliquots of CDs added. The fluorescence intensities were corrected for dilution effects.

The complex formation constants were calculated by assuming a 1 : 1 stoichiometric ratio, according to the Benesi–Hildebrand equation [19]:

$$\frac{1}{(F_i - F_0)} = \frac{1}{(F_\infty - F_0)K[\mathrm{CD}]_i} + \frac{1}{(F_\infty - F_0)},$$

where F_i and F_0 are the fluorescence intensities of AF in the presence and in the absence (blank) of CD, respectively; F_∞ is the fluorescence intensity of the complex and $[\mathrm{CD}]_i$ is the CD concentration after each addition.

Results and discussion

Screening of native and substituted CDs as fluorescence enhancers

In order to evaluate which cyclodextrin induced the highest AF fluorescence enhancement, a spectroscopic screening of native (α-CD, β-CD, γ-CD) and variously substituted CDs were carried out in aqueous solutions. The aim was that of investigating the role played by the CD cavity and by the nature of the substituents, generally on the upper rim (neutral, positively or negatively charged), on the AF fluorescence enhancement. Most of the tested CDs induced an

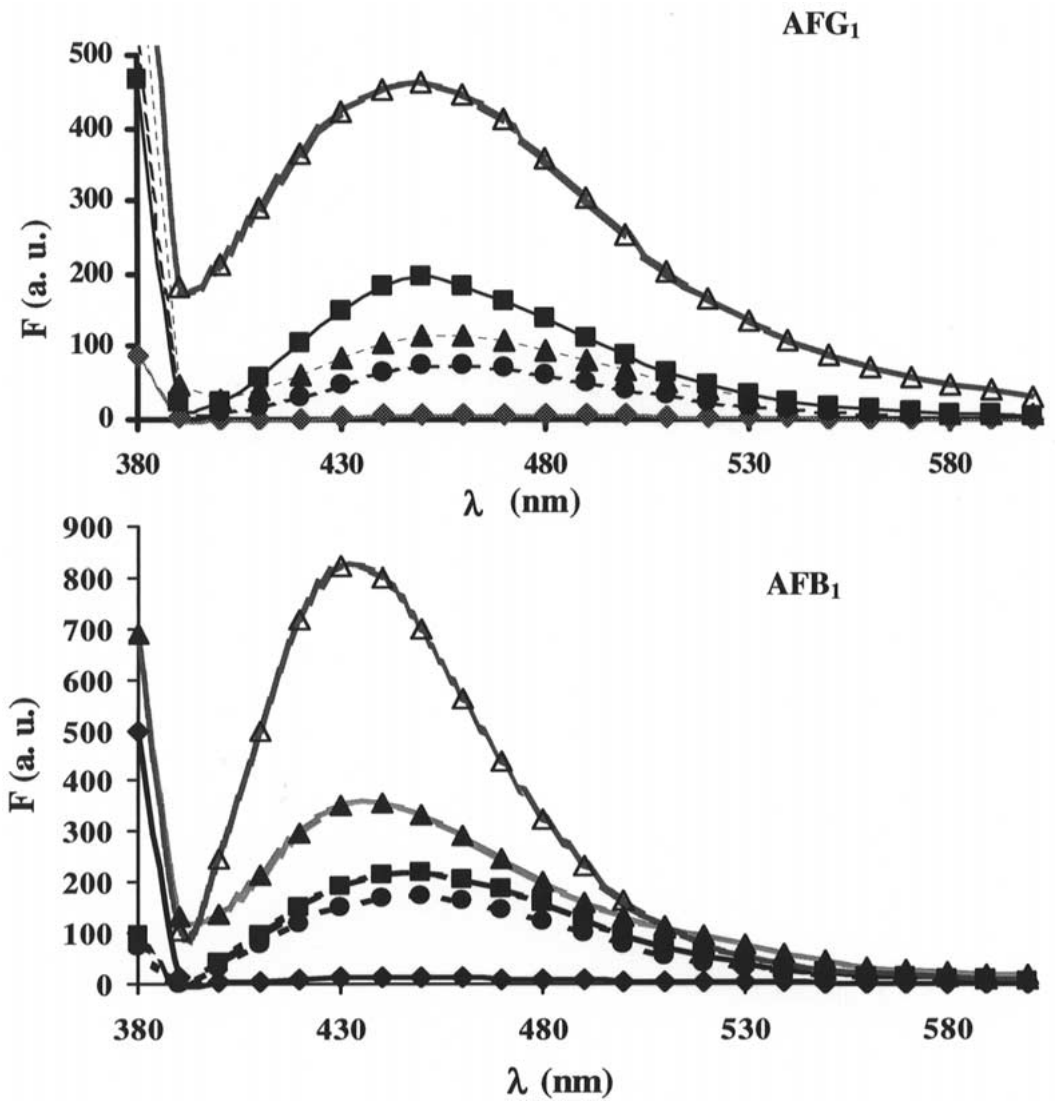

Figure 2. Emission spectra of AFB_1 and AFG_1 in the absence (♦) and in the presence of β-CD (●), DIMEB (■) and β-CD-Su (▲) at a molar ratio AF : CD = $1 : 10^5$ and β-CD-Su (△) at a molar ratio AF : CD = $1 : 10^6$.

enhancement in the fluorescence emission spectra of AFs and a blue shift in the emission maximum wavelength (from 440 nm to 435 nm), both phenomena suggesting a strong interaction between AF and CD. In particular, blue shifts of 3–6 nm in the emission maximum were similar to those recorded for AFs in media less polar than water, as methanol or ethanol, and were consistent with a lower polarity of the AF environment, thus suggesting an eventual inclusion phenomenon.

The AF fluorescence enhancements due to addition of CDs at a molar ratio AF : CD = $1 : 10^5$ are reported in Table 1.

The high natural fluorescence intensity of saturated AFB_2 and AFG_2 is not greatly affected by CDs (it becomes double only with β-CD-Su at high molar ratio), whereas the low emission intensity of the unsaturated AFB_1, AFG_1 are greatly enhanced by the addition of CDs (60 times) and that of AFM_1 is increased up to 10 times under the same conditions. Only β-CD seems to have an ideal cavity size (6.8 Å diameter) to accommodate aflatoxins, while the α-CD cavity (4.7 Å diameter) is too small and the γ-CD cavity (10 Å diameter) is too large and too flexible to give stable inclusion complexes. On the other hand, the functionalized CDs, expecially the neutral DIMEB, but also the positively or negatively charged CDs, induce fluorescence enhancement for AFB_1 and AFG_1. The highest increment was observed for succinyl-β-CD (β-CD-Su), at high concentration (0.1 M), probably on account of the increased solubility of the CD and of the relative complexes rather than to stronger molecular interactions. It was, therefore, interesting to compare the performance of the unsubstituted β-CD with that of the neutral DIMEB and the negatively charged β-CD-Su with AFs.

The emission spectra of AFG_1 and AFB_1 in the absence and in the presence of β-CD, DIMEB and β-CD-Su are reported in Figure 2. The highest fluorescence increment for both AFs was observed with β-CD-Su at high concentration (molar ratio AF : CD = $1 : 10^6$). At a lower concentration (AF-CD molar ratio $1 : 10^5$), the best performance was provided by DIMEB for AFG_1 and by β-CD-Su

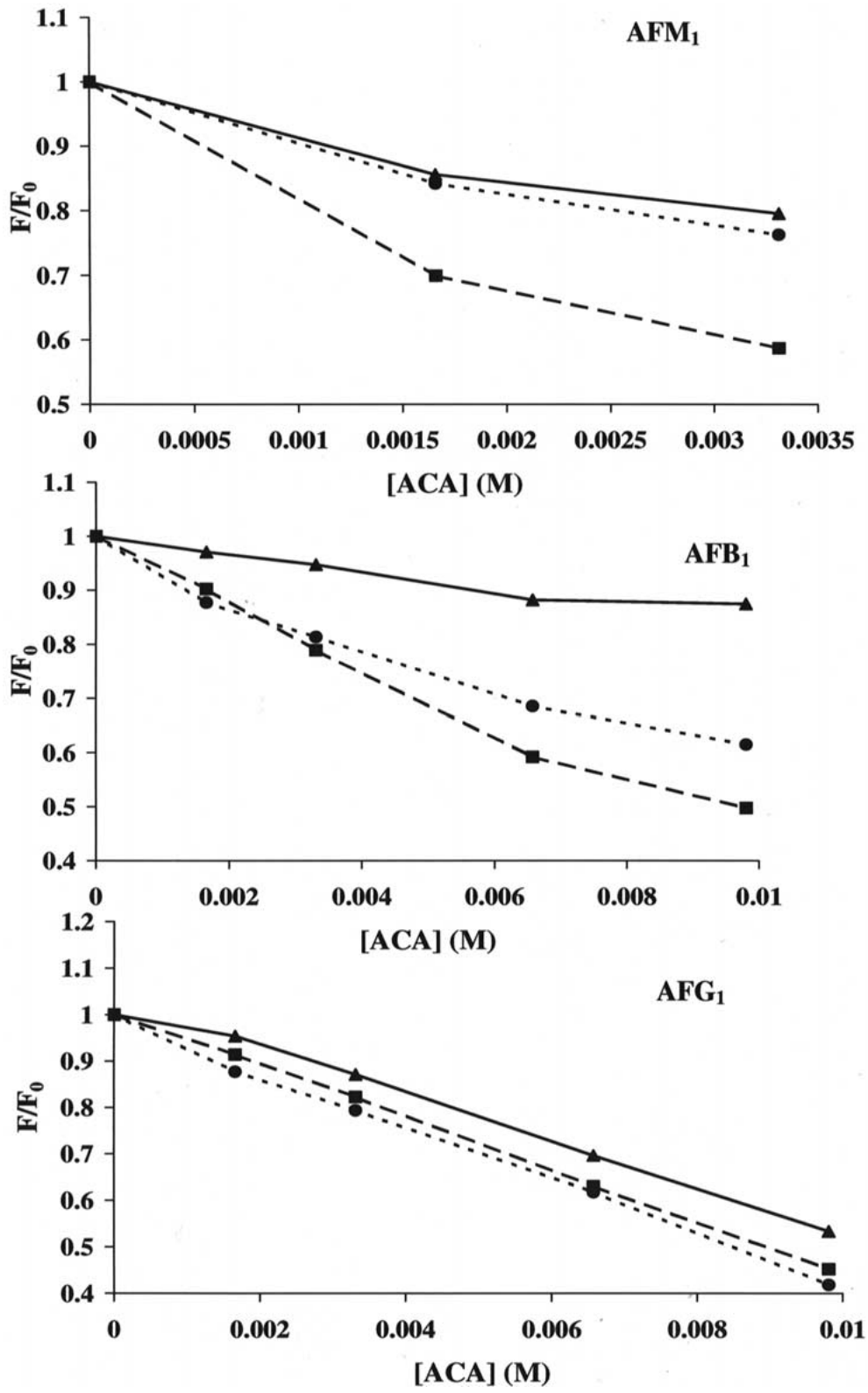

Figure 3. Variation of the fluorescence intensity of AFB_1, AFG_1 and AFM_1 (10^{-7} M) in the presence of β-CD (●), DIMEB (■) and β-CD-Su (▲) (10^{-2} M) upon addition of ACA.

for AFB_1. With the neutral CDs it was not possible to reach a higher concentration for lack of solubility.

Competitive experiments with adamantanecarboxylic acid (ACA)

In order to gain evidence for the formation of inclusion complexes, competitive experiments were carried out with ACA, which, for its great affinity for the β-CD cavity, is able to displace another guest from the CD cavity [18]. Indeed, addition of increasing amounts of ACA to the different AF-CD aqueous mixtures caused a progressive decrease of the fluorescence intensity of the AF emission, consistently with a greater exposure of the guest to the water solution. The decrease of the fluorescence intensity of the AFs in the presence of native and substituted β-CD by addition of increasing amounts of ACA is reported in Figure 3.

ACA was able to displace AFM_1 from DIMEB and less effectively from β-CD and β-CD-Su. The effect was more evident for AFB_1: in the presence of β-CD and DIMEB, ACA was able to displace the aflatoxin, whereas it was less efficient in the presence of β-CD-Su. In contrast, with AFG_1, in the presence of each CD, the fluorescence decreased almost linearly with the ACA concentration while no relevant differences were observed among the three CDs.

Accordingly, the ACA addition induced a red shift (3–4 nm) in the AF emission upon displacement of the AF from the CD-cavity. This effect was less evident with AFM_1 (2–3 nm), which probably gives rise to weaker complexes, on account of the steric hindrance exerted by the hydroxyl group at the 8-position.

Quenching experiments with KI

If the AFs are indeed included in the CD cavity, then they should be protected from the action of quenchers. Thus, we compared the quenching effect of KI on the AF fluorescence in the absence and in the presence of various CDs. KI was chosen since it does not form inclusion complexes with the CD cavity [18].

The quenching efficiency was evaluated by the Stern–Volmer equation as reported in the Experimental section. The Stern–Volmer plots for AFB_1 quenching in the absence and in the presence of β-CD, DIMEB and β-CD-Su are reported in Figure 4.

The plot of F°/F vs [KI] in the presence of CDs showed good regression coefficients (>0.99), which were lower (>0.95) in the absence of CDs. The same trend was observed for all the AFs (β-CD-Su > DIMEB > β-CD). On the basis of these data, we calculated the quenching constants (K_q) for all AFs in the presence (molar ratio AF : CD = $1 : 10^5$) and in the absence of CDs (Table 2).

We can reasonably conclude that the β-CD cavity protects the AF molecule from the quenching action of iodide, this effect being more evident for the β-CD-Su.

Determination of the complex formation constants

In order to calculate the binding constants and the stoichiometry of the host-guest complex AF-CD, fluorescence

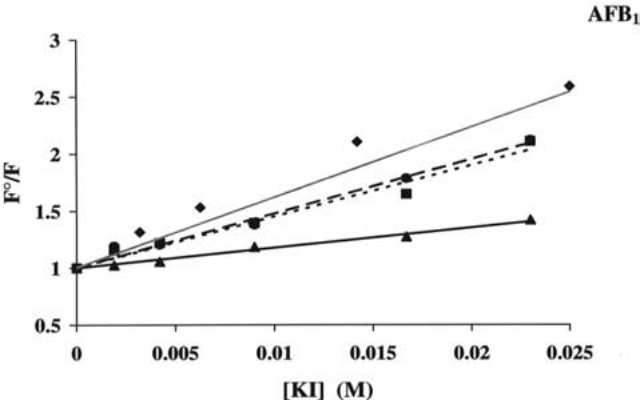

Figure 4. Quenching effect of KI on the fluorescence intensity of AFB_1 (10^{-7} M) in the absence (♦) and in the presence of β-CD (●), DIMEB (■) and β-CD-Su (▲) (10^{-2} M). F_0 native fluorescence, F measured florescence.

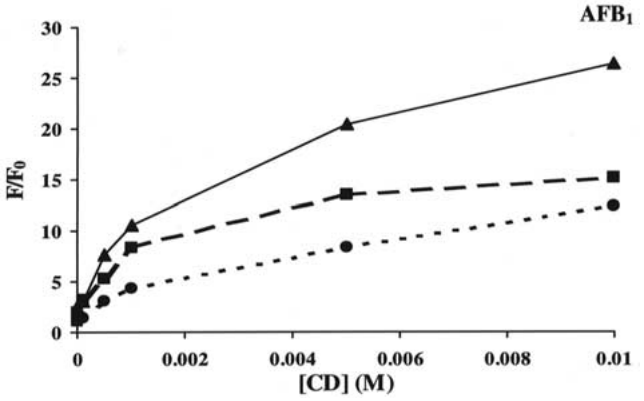

Figure 5. Fluorescence titrations of AFB_1 (10^{-7} M) by β-CD (●), DIMEB (■) and β-CD-Su (▲).

titrations of AFB_1, AFG_1, AFB_2 and AFG_2 with increasing amounts of β-CD, DIMEB and β-CD-Su were performed. The results obtained for AFB_1 are reported in Figure 5.

The binding constants of AFs with β-CD and DIMEB were recently calculated by Vasquez *et al.* [16] on the basis of chromatographic results and might be underestimated due to the dynamic nature of the system. With our method, the formation constants were calculated on the basis of fluorescence measurements, which were performed in a static system and were based on the Benesi–Hildebrand equation as reported in the Experimental section, assuming the formation of a 1 : 1 AF-CD inclusion complex [19].

The binding constants obtained for AF-CD complexes are reported in Table 3.

All the regression coefficients calculated for complexes of AFs with β-CD or DIMEB showed good correlation values (>0.99), which were lower (>0.94) for the AF/β-CD-Su complexes. This is probably due to the fact that for the titrations carried out in the presence of β-CD-Su a saturation point was not achieved. However, at least for β-CD and DIMEB, we can conclude that the experimental data are consistent with the formation of an AF : CD = 1 : 1 complex.

When comparing the formation constants (Table 3) and the fluorescence enhancements recorded in the presence of β-CDs, for each aflatoxin (Table 1), we can observe that

Table 2. Quenching constants (K_q) measured for AFs in the absence and in the presence of β-CDs

	K_q				
	AFB_1	AFG_1	AFM_1	AFB_2	AFG_2
Without CD	61.7 ± 0.3	79.2 ± 0.8	72.5 ± 0.5	98.3 ± 0.5	155 ± 1
β-CD	48 ± 1	71.4 ± 0.8	41.3 ± 0.6	72 ± 1	108.2 ± 0.9
DIMEB	45 ± 1	60 ± 1	36 ± 1	32.3 ± 0.7	72.2 ± 0.3
β-CD-Su	17.7 ± 0.3	30 ± 1	28.1 ± 0.8	27.2 ± 0.3	44.5 ± 0.7

Conditions: molar ratio AF-CD $1:10^5$, [KI] = 0.1 M; λ_{ex} = 365 nm for AFB_1, AFG_1, AFG_2, AFB_2 and λ_{ex} = 360 nm for AFM_1; $\lambda_{MAX(em)}$ = 425 nm for AFB_1, AFG_1, AFB_2, AFG_2 and $\lambda_{MAX(em)}$ = 435 nm for AFM_1; both excitation and emission slits were 15 nm.

Table 3. Binding constants (K) of AFs with β-CDs for a 1 : 1 inclusion complex

	K				
	AFB_1	AFG_1	AFM_1	AFB_2	AFG_2
β-CD	399 ± 65	238 ± 39	542 ± 82	273 ± 46	345 ± 57
DIMEB	892 ± 92	781 ± 88	554 ± 61	363 ± 66	577 ± 56
β-CD-Su	960 ± 55	658 ± 45	213 ± 37	533 ± 55	421 ± 45

the data are consistent only for AFB_1, AFG_1 and AFG_2, which give the higher stability constants with β-CD-Su and the higher fluorescence. Instead, the stability constants for the complexes of CDs with AFM_1 and AFB_2 are not correlated to the fluorescence enhancement. Actually, the variation of the fluorescence intensity upon complexation being very weak with these AFs, it is not possible to obtain a more reliable determination of the formation constants by this method.

Conclusions

In conclusion, all the data obtained suggest that aflatoxins and cyclodextrins give rise to a host-guest complexation, involving inclusion of the AF furan moiety inside the CD cavity, causing fluorescence enhancement.

Functional groups linked to the upper or lower rim of CDs greatly influence the intensity of the fluorescence emission, favouring the inclusion of AFs in the cavity. The higher fluorescence enhancements observed in the presence of β-CD-Su might be due to its higher solubility in water, so that it can be used at higher concentrations, thus increasing the amount of the complex formed.

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