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Interaction between cyclodextrins and aflatoxins Q_1, M_1 and P_1 Fluorescence and chromatographic studies

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Abstract

The fluorescence properties of the aflatoxins M_1, Q_1, P_1 in solution and the effect of various cyclodextrins (α-, β-, γ-, hydroxypropyl-β- and α-β-heptakis-di-*O*-methyl-β-) on their fluorescence emission were studied. Among the aflatoxins, a substantial enhancement of the fluorescence emission of aflatoxin Q_1 in the presence of aqueous solutions of α-, β-, hydroxypropyl-β, and α-β-heptakis-di-*O*-methyl-β-cyclodextrin, was observed. On the contrary, γ-cyclodextrin proved to be inefficient to enhance the fluorescence properties of this compound. No important fluorescence enhancement was found for aflatoxins P_1 or M_1 for any of the cyclodextrin derivatives tested. The complex formation constant (K_f) of these compounds with β-cyclodextrin was chromatographically determined, and from the results obtained, we can conclude that K_f cannot be used alone to explain the fluorescence increase. Thermodynamic studies showed that ΔH and ΔS parameters, associated with the partition of aflatoxins in RP-HPLC, increased when β-cyclodextrin was added to the eluent.

Keywords: Thermodynamic parameters; Complexation; Fluorescence detection; Aflatoxins; Cyclodextrins

1. Introduction

Aflatoxins Q_1, M_1 and P_1 (Fig. 1) (AFQ_1, AFM_1 and AFP_1) are relevant hydroxylated metabolites produced by the liver of several animal species, including humans after consumption of food contaminated with aflatoxin AFB_1 [1–4]. The latter is produced by the widespread moulds *Aspergillus flavus* and *Aspergillus parasiticus* [5,6]. Aflatoxins are powerful carcinogenic and mutagenic agents [7], and some of the studied metabolites have been explored as urinary biomarkers for hepatocellular carcinoma [8,9]. Additionally, health problems associated with milk and dairy products contaminated

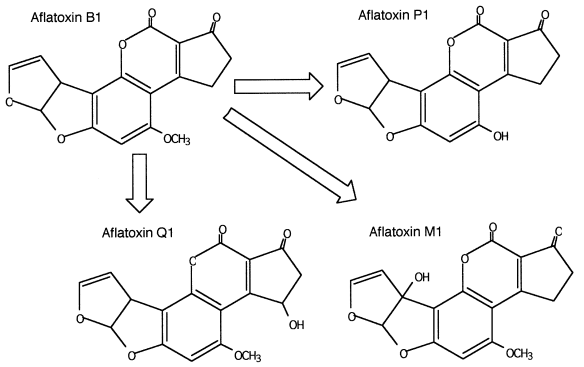

Fig. 1. Structural formulae of the four aflatoxins studied. AFQ_1, AFM_1 and AFP_1 are hydroxylated derivatives from AFB_1 metabolism.

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with AFM_1 are well known [10]. All these reasons have focused the efforts of analytical chemists in studying highly sensitive methods to detect the metabolites mentioned above.

AFM_1 and AFQ_1 are structural isomers characterized by the change in the position of the hydroxyl group on the aflatoxin skeleton (see Fig. 1). Besides, AFP_1 can be considered as demethylated AFB_1 exhibiting thus a phenolic hydroxyl group on the benzenic ring of the coumarinic moiety. As pentaheterocyclic and highly conjugated compounds, aflatoxins exhibit native fluorescence. This is the reason why sensitive analytical methods for the detection of AFB_1 based either on its native emission properties, or on enhanced fluorescence after chemical derivatization have been published specially using halogenation (I_2 or Br_2) of the 8–9 unsaturated double bond [11–13]. Likewise, the use of cyclodextrins (CDs) in a post-column reaction system has been developed to improve the detection limit of this important fungal toxin, due to the high fluorescence enhancement obtained with β-CD derivatives [14,15].

Concerning AFM_1, methods using thin-layer chromatography [16], immunoassays [17] and reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection [18] have been published. Methods for the detection of AFQ_1 are based on liquid chromatography with post-column chemical derivatization using potassium bromide [19,20]. As for AFB_1 determination, the use of the halogenation leads to several problems, such as the instability of the halogen species in solution. Less analytical work has been carried out for the detection of AFP_1 though the existing reports are also based on fluorimetric detection [21]. Moreover, the use of bromine to enhance the fluorescence of AFP_1 with a post-column reaction mode produces an extinction of the native fluorescence of this compound [19,20]. Therefore, the detection of this latter compound has an added interest. To our knowledge, no studies on the spectroscopic properties of these hydroxylated metabolites (i.e., AFQ_1, AFM_1 and AFP_1) in the presence of cyclodextrins were done until now.

Consequently, this work was performed to collect emission data for AFQ_1, AFM_1 and AFP_1 in solvents usually used for their chromatographic separation, in absence and in presence of different cyclodextrins. The aim was to explore some further possible analytical applications, especially in the liquid chromatographic area. Likewise, chromatographic studies have shown to allow the determination of the complex association constants (K_f) of these compounds with β-CD. The variations of thermodynamic parameters (enthalpy and entropy) were also chromatographically determined with and without β-CD since important variations were observed in the retention of the solutes in presence of β-CD. Finally, an effort was made to correlate the chromatographic and spectroscopic data in order to get a better insight in the complexation of the studied compounds with cyclodextrins.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade. The purity of the organic solvents and ultra-pure water (Milli-Q quality) was checked via fluorescence prior to use. Aflatoxins M_1, AFQ_1, AFP_1 and AFB_1 were obtained from Sigma (St. Louis, MO, USA) α-CD, γ-CD and heptakis-di-*O*-methyl-β-cyclodextrin (DIMEB) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Aldrich; β-CD was purchased from Roquette (Lestrem, France).

2.2. Solutions

Stock standard solutions of aflatoxins were prepared using acetonitrile, as this solvent was recommended to avoid rapid degradation [21]. Stock concentrations were of $3\cdot10^{-4}$ *M* for AFQ1; $3\cdot10^{-5}$ *M* for AFM_1; $1.5\cdot10^{-5}$ *M* for AFP_1 and $3\cdot10^{-3}$ *M* for AFB_1. Each solution was protected against light (aluminum sheet), and kept at 4°C for no more than seven days. Successive dilutions were performed with methanol and then with the mobile phase in order to yield working concentrations of $1.5\cdot10^{-5}$ *M*; $3\cdot10^{-7}$ *M*; $1.5\cdot10^{-6}$ *M* and $3\cdot10^{-6}$ *M* for AFQ_1, AFM_1, AFP_1 and AFB_1, respectively.

Stock aqueous solutions (10^{-2} *M*) of the cyclodextrins cited above were prepared daily and maintained at room temperature before use.

2.3. Spectroscopy

Absorbance spectra were recorded with a Model 2100 UV–visible spectrometer (Shimadzu, Japan) using a 10-mm optical path length quartz cuvette. All emission measurements were made at 25±2°C with an LS 50B Model luminescence spectrometer (Perkin-Elmer, USA); both excitation (ex) and emission (em) slits were set at 5 nm and all measurements were performed in triplicate in a 10-mm optical path length quartz cuvette. The relative fluorescence quantum yields (ϕ_F) were established according to Parker and Rees [22] using 0.005 *M* sulphuric acid quinine bisulphate as standard. The first singlet-state energy levels (E_{S1}) were calculated as the 0–0 band level on the corresponding frequency spectra.

2.4. Chromatography

All liquid chromatography measurements were made using a Shimadzu LC-10AS metering pump equipped with a Rheodyne Model 7125 20 μl loop injector (Cotati, CA, USA) and the mobile phase flow-rate was 1 ml min^{-1}. The analytical column was a Kromasil 5 μm, 100 Å, reversed-phase C_8 (150×4.6 mm I.D.) from Touzart and Matignon (France). A Jones Chromatography (UK) Model 7955 column oven was used to keep the column temperature constant with an accuracy of ±0.1°C. For temperature effect studies (enthalpy and entropy calculations for the studied aflatoxins using chromatography), nine temperatures from 30 to 54°C were investigated. When each temperature was reached, the temperature of the column was carefully stabilized for up to 30 min before the injections were performed. A Perkin-Elmer LC240 fluorescence detector was programmed with the following excitation and emission wavelengths: λ_{ex}=365 nm, λ_{em}= 466 nm for AFQ_1 and AFM_1; λ_{ex}=365 nm, λ_{em}= 504 nm for AFP_1; and according to Ref. [23], λ_{ex}= 360 nm, λ_{em}=435 nm for AFB_1. The chromatograms were recorded on a Shimadzu CR5A Chromatopac integrator.

Throughout this study a basic methanol–water (35:65, v/v) mobile phase was used after degassing with helium and filtering (0.45 μm) (Whatman).

For the determination of the inclusion complex formation constant (K_f), different mobile phases, characterized by an increasing concentration of β-CD in the range from $2.5 \cdot 10^{-4}$ *M* to $8 \cdot 10^{-3}$ *M*, were used. The appropriate amount of β-CD was dissolved in water and then methanol was added to yield the final desired concentration.

With each change in the composition of the mobile phase or in temperature, the void volume (as t_{Ro}) was simply verified by injection of a methanol solution of sodium nitrite (UV detection at 220 nm with a Shimadzu SPD 6A UV detector), and the mean of three measurements of t_{Ro} was used in subsequent calculations of the capacity factor (k').

All capacity factor measurements and all the experiments reported here are referred to the average of these three measurements, with an estimated error lower than 0.5%.

Enthalpy and entropy changes in the absence (ΔH, ΔS) and in presence of β-CD (ΔH_β, ΔS_β) were calculated according to Van't Hoff equations and using a value for *R* of 1.99 cal K^{-1} mol^{-1} (1 cal=4.184 J).

3. Results and discussion

3.1. Spectroscopic studies

3.1.1. In the absence of cyclodextrin

Table 1 shows the excitation and emission maxima, the Stokes shifts ($\upsilon_A - \upsilon_f$), the relative quantum fluorescence yields (ϕ_F) and the energy levels of the first singlet state (E_{0-0}) for AFQ_1, AFM_1 and AFP_1 in the most common solvents used in RP-HPLC. It can be observed that the absorption band is slightly affected by the increase in the polarity, with a red shift for AFQ_1 and AFM_1. This solvent effect on the absorption band reflects a π–π nature of the electronic transition. Nevertheless, this effect is more significant than those which were observed with the parent compounds AFB_1, AFG_1 (and also with AFB_2 and AFG_2) under the same experimental conditions [23]. These differences in the absorption band seem to be correlated with the presence in each tested compound of a hydroxyl group on the aflatoxin skeleton which certainly entails an increase in the solvent–solute interaction due to the dipolar and strong hydrogen-donor charac-

Table 1
Photophysical parameters at room temperature and solvent effects on the three hydroxylated aflatoxins (AFM_1, AFQ_1, AFP_1)

Aflatoxin	Acetonitrile					Methanol					Water				
	λ_{ex} (nm)	λ_{em} (nm)	ϕ_F	$\upsilon_A-\upsilon_f$ (cm^{-1})	E_{0-0} (eV)	λ_{ex} (nm)	λ_{em} (nm)	ϕ_F	$\upsilon_A-\upsilon_f$ (cm^{-1})	E_{0-0} (eV)	λ_{ex} (nm)	λ_{em} (nm)	ϕ_F	$\upsilon_A-\upsilon_f$ (cm^{-1})	E_{0-0} (eV)
AFM_1	352	456	0.009	4824	3.10	357	430	0.134	4755	3.05	361	433	0.168	4606	3.14
AFQ_1	357	441	0.061	5336	2.80	365	460	0.043	5658	3.04	370	466	0.004	5568	2.90
AFP_1	338	506	0.020	9823	3.52	332	437	0.091	7185	3.67	333	435	0.018	7042	3.08

λ_{ex}=Wavelength of the maximum fluorescence excitation spectrum; λ_{em}=wavelength of the maximum of the emission spectrum. ϕ_F=Relative fluorescence quantum yield; $\upsilon_A-\upsilon_f$=Stokes shift; E_{0-0}=energy of the lowest singlet state.

ter of the hydroxyl group. It should be noted that AFP_1 does not exhibit a trend in the displacement of its absorption band equal to that for the AFM_1 and AFQ_1, which may be due to the presence of the phenolic OH.

Concerning the emission band (Table 1) a strong blue shift can be observed for AFM_1, associated with an increase of ϕ_F with the increase of solvent polarity. This suggests an important solvent–solute interaction in polar medium lowering the S_1 excited state. On the contrary, AFQ_1 exhibits a nearly opposite behaviour (i.e., a red shift of the emission and a decrease of ϕ_F) with the increase in solvent polarity. This could be related to the vicinity of the OH group attached to the cyclopentanoic ring, with the methoxy group on the aromatic ring, which sterically makes possible the occurrence of a pseudo-cycle involving an intramolecular hydrogen bond. AFP_1 seems to exhibit a specific behaviour with a considerably increased Stokes shift in acetonitrile and a highest ϕ_F in methanol (Table 1). This suggests that the emitting species is certainly different in dipolar solvent (acetonitrile) and in protic solvent (water and methanol). In contrast to AFB_1, AFB_2, AFG_1 and AFG_2, [23] there is here no clear correlation between Stokes shifts, E_{0-0} and ϕ_F. The fluorescence of the studied compounds is weak (highest ϕ_F for AFM_1 in water) but it is relatively similar to the parent compound (AFB_1) [23]. The case of AFP_1 is different, due to its phenolic character. The phenolic nature of AFP_1 leads to a different behaviour depending on the dissociating and alkaline character of the environment. Then, two emission peaks can be recorded if dissociated and non-dissociated species of the compound coexist in the medium. That is why other authors have performed the fluorimetric detection in HPLC of this compound with different λ_{em} (i.e., 430 nm or 500 nm) with best sensitivity using the latter wavelength [21]. Therefore, a limited study was undergone on the location of the emission maximum for this derivative as a function of pH. It was found that in acidic aqueous solution (pH 3), the emission maximum is located at 510 nm, while at pH 7.5 two maxima can be recorded at 436 nm and 508 nm, respectively, with a loss in fluorescence intensity. Therefore, it should be logical to consider that the 436 nm emission is associated with a more dissociated form of the phenolic OH than the 508 nm emission maximum. Recently, methods have been developed for the detection of other fungal phenolic metabolite based on the enhancement of its fluorescence emission at low pH [24,25].

3.1.2. In the presence of cyclodextrin

No significant changes of excitation and emission maxima for the three hydroxylated aflatoxins were recorded in the presence of 10^{-2} *M* of cyclodextrins, whatever the cyclodextrins tested. No important fluorescence enhancement was found for AFP_1, or AFM_1 for any of the cyclodextrin derivatives tested (i.e., α-CD, β-CD, γ-CD, HP-β-CD, DIMEB) in aqueous solution (10^{-2} *M*). Although the emission of AFM_1 is higher in the presence of certain cyclodextrins, (i.e., α-CD, β-CD, HP-β-CD and DIMEB) this enhancement is of limited analytical interest with a maximum $F/F_o=5$ for DIMEB (F_o= fluorescence in water; F=fluorescence in the presence of cyclodextrin derivative). On the other hand, a substantial enhancement in the luminescence of AFQ_1 (up to $F/F_o=39$) was observed (Fig. 2). As shown elsewhere [23] for AFB_1, AFG_1, AFB_2 and AFG_2, the higher increase was obtained with DIMEB, then with β-CD and HP-β-CD. Finally, the

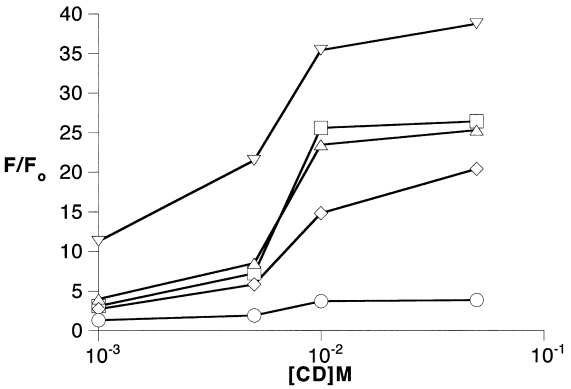

Fig. 2. Effect of cyclodextrin derivatives on the fluorescence of the AFQ_1 ($3\cdot10^{-6}$ *M*, $\lambda_{ex}=365$ nm, $\lambda_{em}=466$ nm, both em. slit and ex. slit were set at 2.5 nm). F_o=Fluorescence in water; F=fluorescence in the presence of cyclodextrins (in arbitrary units). □=β-CD; △=HP-β-CD; ▽=DIMEB; ○=γ-CD; ◇=α-CD.

smallest fluorescence increase was achieved with α-CD, certainly due to its limited cavity size (5 Å). No increase in the emission was observed with the larger γ-CD. As the larger γ-CD cavity (8 Å internal diameter) does not lead to fluorescence improvement, we can assume that the fit of the inclusion complex is superior using β derivatives. In summary, the degree of enhancement is not a clear function of the size of the internal cavity or of the chemical nature of the outer surface of cyclodextrins.

The relationship between the fluorescence enhancement of aflatoxins and the presence of cyclodextrins involves the problem of the formation constant of the inclusion complex (K_f) and the stoichiometry of the association reaction. A chromatographic approach has been used in order to explain this aspect. The study was carried out adding β-CD in the mobile phase.

3.2. Chromatographic studies

3.2.1. Effect of adding β-CD in basic eluent

Methanol was chosen as organic modifier of the mobile phase because it interacts less than acetonitrile with β-CD due to the relatively higher polarity [26]. However, the complex formation constant of methanol with β-CD ($K_m=0.32$ l mol^{-1} [26]) must be taken into account due to its high molar concentration in the eluent. Then, if the interaction between β-CD and the apolar stationary phase is neglected, it can be stated that:

$$[CD_T]=[CD_m]+[CD\cdot M]_m$$

where $[CD_m]$ is the available β-CD concentration in the mobile phase, $[CD_T]$ is the total concentration of β-CD in the mobile phase, and $[CD\cdot M]_m$ is the methanol-complexed cyclodextrin in the mobile phase. Then, from the equilibrium constant K_m between complexed and non-complexed methanol, we can calculate $[CD_m]$ as follows [27,28]:

$$[CD_m]=[CD_T]\left(\frac{1}{K_m[M]+1}\right) \quad (1)$$

Therefore, methanol contents in the mobile phase were selected as low as possible to minimize its interference in the determination of K_f values for the metabolites here studied. In previous studies with the main aflatoxins AFG_2, AFG_1, AFB_2 and AFB_1 [27] a dramatic decrease of the K_f of the aflatoxin–β-CD inclusion complex was shown when methanol content was increased in the mobile phase, due to competition of the organic solvent with the aflatoxins for the cyclodextrin cavity. Thus methanol content as low as 40 ml in 100 ml of mobile phase or less is required for a good determination of K_f of the inclusion complex [27]. Likewise, the use of a high water ratio in the mobile phase allowed us to use molar concentrations of β-CD as high as used in this study without problems of cyclodextrin precipitation.

Using an eluent spiked with β-CD, and after the injection of the solute (i.e., aflatoxins), the complexation involves a decrease in the measured capacity factor owing to the modification of the mass transfer process in comparison with the same eluent without cyclodextrin [27,29]. Therefore, it can be shown that the formation constant can be obtained from the following equation [27,28,30,31].

$$1/k'=1/k'_o(1+K_f[CD_m]) \quad (2)$$

where k' is the capacity factor of the solute in the presence of a concentration $[CD_m]$ of available β-CD (Eq. (1)), k'_o is the capacity factor in the same eluent without addition of β-CD and $K_f=[CD\cdot S]_m/([CD]_m\cdot[S]_m)$, $[CD\cdot S]_m$ being the concentration of solute complexed in the eluent and $[S]_m$ the concentration of free solute. Thus, Eq. (2) implicitly

Table 2
Equations of: $1/k'$ versus equilibrium available concentration of β-CD [CD_m], ln k' versus $1/T$ (K) in the absence of β-CD in the mobile phase, and ln k'_β versus $1/T$ (K) in the presence of $8\cdot10^{-3}$ *M* β-CD in the mobile phase (for detailed chromatographic conditions, see Section 2.4)

Aflatoxins	$1/k'$	ln k'	ln k'_β
Q_1	$0.19965+0.0033353$ [CD_m], $r=0.98$	$-4.0358+1.7231$ $(1/T)$, $r=0.99$	$-2.6802+1.2494$ $(1/T)$, $r=0.99$
M_1	$0.15974+0.0019431$ [CD_m], $r=0.96$	$-4.057+1.7958$ $(1/T)$, $r=0.99$	$-3.1691+1.4952$ $(1/T)$, $r=0.99$
P_1	$0.12092+0.0034581$ [CD_m], $r=0.98$	$-6.2586+2.5619$ $(1/T)$, $r=0.99$	$-4.4528+1.9246$ $(1/T)$, $r=0.99$
B_1	$0.070483+0.001485$ [CD_m], $r=0.98$	$-4.18+2.0799$ $(1/T)$, $r=0.99$	$-2.5982+1.5219$ $(1/T)$, $r=0.99$

assumes a 1:1 stoichiometry in inclusion complex formation between the solute and β-CD [32]. In the present study, AFB_1 has been included for comparison with the hydroxylated toxins of interest.

The equations obtained for $1/k'$ versus [CD_m] for AFQ_1, AFM_1, AFP_1 and AFB_1 at 30°C, with added β-CD ranging from $2.5\cdot10^{-4}$ to $8\cdot10^{-3}$ *M* (i.e., from $2\cdot10^{-4}$ to $2.1\cdot10^{-3}$ *M* available β-CD) are shown in Table 2. As can be observed, the linear plot of $1/k'$ versus [CD_m] fits fairly well with Eq. (2) with *r* values of 0.98 for AFQ_1, AFP_1 and AFB_1 and 0.96 for AFM_1. Hence, the assumption of a 1:1 stoichiometry seems reasonable. The 1:1 stoichiometry for these compounds is in agreement with other studies on complexation processes of other aflatoxins with β-CD [27], being the most common complexation ratio found between cyclodextrins and other compounds [33], though other ratios (i.e., 2:1, 1:2, 2:2 or 1:1:1) have also been reported [33].

The values of K_f obtained from Eq. (2) [$K_f(1/k'_o)$ is the slope of plots, $(1/k'_o)$ being the intercept] are shown in Table 3. The K_f found now for AFB_1 using a RP C_8 column, is close to that reported by us, using a RP C_{18} column for this metabolite (258 l mol^{-1}) in a previous work [27]. Therefore, the reproducibility of this chromatographic approach seems to be good, and the differences found are probably due to the partitioning difference due to the two stationary phases used. The lowest K_f found for AFM_1 can be interpreted from a structural point of view, due to the position of the hydroxyl group in the furanic part of the molecule (Fig. 1). The importance of the furanic part of the aflatoxin skeleton has been rationalized towards the inclusion process in β-CD [27]. The fact that AFM_1 exhibits the lowest K_f among the studied compounds is certainly associated with a steric hindrance due to the presence of the hydroxyl located in a part of the molecule which was demonstrated to be included in the internal cavity of the β-CD, generating thus a poor fit between the host (β-CD) and the guest (AFM_1). It should be pointed out that this comment appears in good agreement with the poor quality of the fit obtained for AFM_1 ($r=0.96$) in the $1/k'=$ f$[CD]_m$ plot (Table 2).

On the other hand, the elution order $AFQ_1<AFM_1<AFP_1<AFB_1$ seems to be related to the polarity of the molecules. The hydroxylated metabolites are less retained by the apolar stationary phase than the less polar AFB_1.

From a fluorimetric point of view, K_f does not seem to be related to any emission enhancement.

Table 3
K_f of aflatoxins–β-CD complex, ΔH, ΔS, ΔH_β, ΔS_β, $(\Delta H_\beta-\Delta H)$ and $(\Delta S_\beta-\Delta S)$ for AFQ_1, AFM_1, AFP_1 and AFB_1 (for experimental conditions, see Section 2.4)

Aflatoxin	K_f (l mol^{-1})	Without β-CD		With β-CD		$\Delta H_\beta-\Delta H$ (kcal mol^{-1})	$\Delta S_\beta-\Delta S$ (cal mol^{-1} K^{-1})
		ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔH_β (kcal mol^{-1})	ΔS_β (cal mol^{-1} K^{-1})		
AFQ_1	167	−3.43	−7.21	−0.944	7.49	2.48	14.70
AFM_1	122	−3.57	−7.25	−0.595	7.79	2.97	15.04
AFP_1	286	−5.10	−11.63	−1.270	7.66	3.83	19.29
AFB_1	211	−4.14	−7.50	−1.111	7.50	3.03	15.00

This can be easily stated from this work as well as others [27], i.e., AFP_1 shows the highest K_f, although this toxin does not show increase of its emission in the presence of β-CD; in contrast, AFQ_1 with lower K_f, than that of AFP_1, exhibited a high increase of its emission, as mentioned in the spectroscopic part. In the following sections we attempt to explain these aspects using a thermodynamic approach.

3.3. Effect of the temperature

3.3.1. In the absence of cyclodextrin

In RP-HPLC, when the temperature increases, the partition coefficient (K) decreases according to [34].

$$\ln K = -\Delta G/RT \quad (3)$$

so

$$\ln K = -\Delta H/RT + \Delta S/R \quad (4)$$

where ΔG is the Gibbs free energy for solute–stationary phase interaction, R is the gas constant, T is temperature (K) and ΔH and ΔS are the enthalpy and entropy changes, respectively, associated with the transfer of a solute from the mobile to the stationary phase [34].

As the capacity factor (k') of the solute is related to K by $k' = \phi K$ where ϕ is the ratio of the stationary to the mobile phase ($\phi = V_s/V_m$), the Van't Hoff equation describes the relationship between the capacity factor and the temperature of the chromatographic system:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \quad (5)$$

According to Refs. [35,36] and with the assumption that V_m can be obtained from a non-retained solute, e.g., sodium nitrite (see Section 2.4), $\ln \phi$ can be calculated under our conditions to be equal to -0.413. Table 2 shows the results obtained for the plot of Eq. (5). Therefore, the enthalpy changes associated with the chromatographic partition process can be obtained from the slope ($-\Delta H/R$) and the entropy change from the intercept ($\ln \phi + \Delta S/R$). The results for ΔH, ΔS are shown in Table 3.

Concerning the enthalpy change, it can be expected that the more efficient the transfer of the solute to the stationary phase, the smaller the enthalpy change. As shown here, (Table 3) not all the data agree satisfactorily with the model for the four aflatoxins tested. As shown in the spectroscopic part, due to the uncontrolled pH of the eluent (see Section 2.4) the occurrence of dissociated and undissociated form of AFP_1 can not be excluded. With this presumption, the calculated ΔH value for this toxin as well as all the other thermodynamic parameters should be considered with extreme caution. The ΔH value obtained for AFB_1 agrees with that reported by us in a previous paper [27].

According to the literature [32], ΔS can be associated with the change in ordering of the solute during its transfer between the mobile phase and the stationary phase. The ΔS values shown in Table 3 let us classify the studied compounds into two groups. First, AFQ_1, AFM_1, and AFB_1 with very similar entropy values and regular variation (i.e., the more retained is the solute, the lower the entropy); second, the AFP_1 which exhibits largely the lowest ΔS, and, in contrast with the other studied compounds ΔS of AFP_1 derivatives does not follow the elution order ($AFQ_1 < AFM_1 < AFP_1 < AFB_1$). This can be interpreted in a similar way to ΔH, i.e., the partitioning of AFQ_1, AFM_1 and AFB_1 leads to a similar organization change in both stationary and mobile phases, whereas AFP1 leads to higher ordering, probably due to the interactions of the phenolic hydroxyl with the eluent as suggested before.

3.3.2. In the presence of β-CD

In the presence of β-CD the aflatoxin–β-CD inclusion complex formation constant, K_f must be taken into account. Taking logarithms on Eq. (2) obtains:

$$\ln k_\beta = \ln k' - \ln K_f - \ln [1/(K_f + [CD_m])] \quad (6)$$

where k'_β is the capacity factor of the solute in the presence of β-CD. Combining Eqs. (5) and (6), the variation of the capacity factor in the presence of β-CD can be evaluated by:

$$\ln k'_\beta = (\Delta H_\beta - \Delta H)/RT + (\Delta S - \Delta S_\beta)/R + \ln \phi - \ln \{1/(K_f + [CD_m])\} \quad (7)$$

where ΔH_β and ΔS_β are the standard enthalpy and entropy changes of the solute associated with its transfer from the mobile to the stationary phase on addition of β-CD to the eluent.

Table 2 shows results obtained for the plot of ln k'_{β} versus $1/T$ according to Eq. (7). The data clearly show that the linear relationship between ln k' and $1/T$ remains valid upon addition of β-CD. Table 3 summarizes also the influence of added β-CD on ΔH and ΔS (ΔH_{β} and ΔS_{β}). In order to evaluate the effect of β-CD on k' upon temperature variation, for a fixed methanol content, a concentration of $8 \cdot 10^{-3}$ M was chosen (i.e., $2.1 \cdot 10^{-3}$ M available β-CD). From Table 2, it can be stated that if AFM_1 and AFP_1 are co-eluted with β-CD ($8 \cdot 10^{-3}$ M), as seen with the use of high temperature, a change in the elution order can be obtained. Therefore this aspect must be taken into account due to the probability of simultaneous presence of both derivatives in biological specimens [2].

As can be seen, the introduction of β-CD into the eluent led for all compounds, to a significant enthalpy increase. This can be interpreted as a decrease in the interaction between the solute and the stationary phase, probably because of the competition for the dissolved cyclodextrin in the mobile phase. If we consider the K_f of the compounds the data agree rather well with this hypothesis, i.e., the higher enthalpy increases were those for AFP_1 and AFB_1, which exhibit higher K_f in comparison with AFQ_1 and AFM_1. Moreover, no correlation between the values of ΔH_{β} and k'_{β} was observed, which may be due to the same perturbing effect of β-CD on the transfer process.

The entropy of the system in the presence of β-CD (ΔS_{β}) was positive in contrast to data obtained without cyclodextrin (ΔS) in the eluent for all the metabolites tested. From Eq. (7) it can be stated that: $(\Delta S - \Delta S_{\beta})/R + \ln \phi - \ln \{1/(K_f + [CD_m])\}$ corresponds to the intercept in Table 2. So, the term $-\ln \{1/(K_f + [CD_m])\}$ has a positive contribution to the entropy if $(K_f + [CD_m])$ is higher than 1, which is the case. Then, the introduction of β-CD has a positive contribution to the whole entropy (i.e., disorder) of the system. In other words, β-CD introduction in the eluent with complexable solutes involves a greater disorder in the transfer process. It should be noted that this increases in the entropy values are in good agreement with other reported data on aflatoxins interaction with β-CD [27] as well as with data on the interaction of hydroxyaromatic compounds and β-CD [32].

On the other hand, when both enthalpy and entropy of the inclusion complex between cyclodextrin and other solutes are negative, the inclusion complex formation is said to be ''enthalpy-driven'' [33]. As the classical hydrophobic interaction is widely accepted to be entropy-driven [33]; for the compounds here studied, the hydrophobic contribution should be of reduced importance. Nevertheless, this reasoning has to be considered with extreme caution as hydrophobic interaction should be involved in the inclusion of furanic part of the toxins in the cavity of the cyclodextrin.

As it was noted that K_f cannot be used alone to explain the fluorescence increase, an attempt has been undergone to use $\Delta H_{\beta} - \Delta H$ and $\Delta S_{\beta} - \Delta S$ differences. Table 3 shows these different values. There is a strict correlation between $\Delta(\Delta H)$, $\Delta(\Delta S)$ and the elution order of the hydroxylated aflatoxins. This clearly demonstrates that the increase in retention is associated with the increase in enthalpy (ΔH) and disorder (ΔS). In contrast, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ of AFB_1 are not correlated with its derivatives (AFQ_1, AFP_1 and AFM_1). This strongly suggests that thermodynamic data are discriminant chromatographic descriptors only for structurally close compounds, (i.e., exhibiting the same functional groups) as shown by the non-hydroxylated AFB_1 parameters [27].

On the other hand, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ upon cyclodextrin addition failed to explain the possible fluorescence increase. Thus, a clear distinction should be made between thermodynamic data and spectroscopic measurements in this area of inclusion of guest compounds within cyclodextrins. This latter point shows that quantitative approaches such as enthalpy–entropy compensation method, and K_f calculation, are not precise enough to understand the exact mechanism involved in the fluorescence increase upon β-CD complexation.

Based on these insights, the application of cyclodextrins to the analytical determination of hydroxylated AFB_1 derivatives, is currently in progress.

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