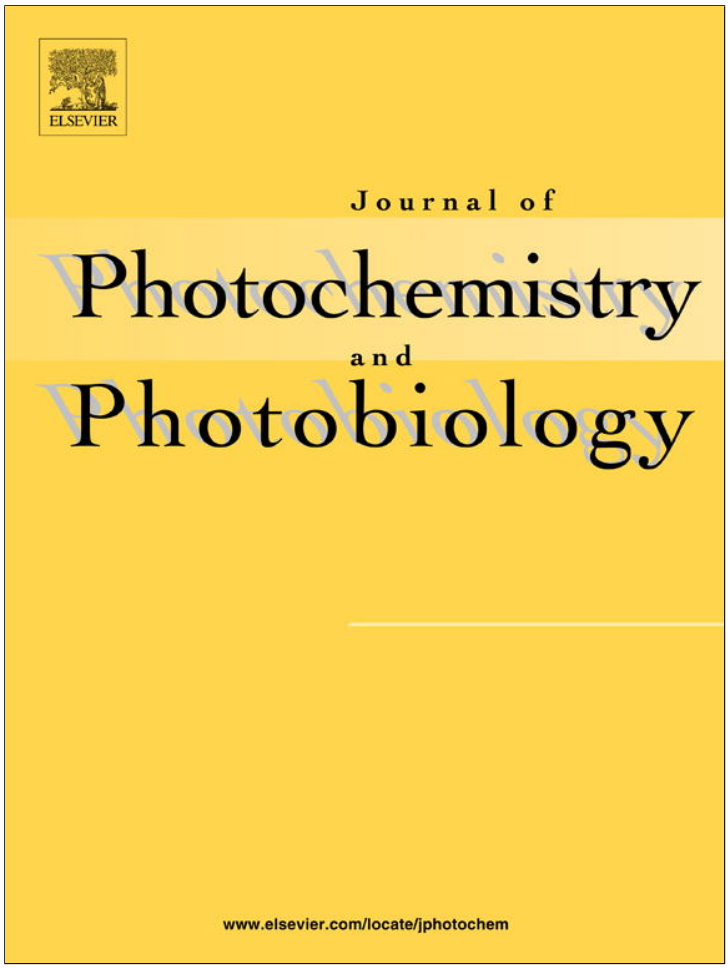

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Cyclodextrins nanocavities effects on basic and acid fluorescence quenching of hydroxy-indoles

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ABSTRACT

The cyclodextrins effects on the serotonin (5HT) and 5-hydroxy-3-indolyl acetic acid (5HIA) fluorescence emission in acid and alkaline quenching conditions were analyzed. From Stern–Volmer plots, the quenching constants (K_Q) were determined in the presence and absence of β-cyclodextrin (βCD) and hydroxypropyl-β-cyclodextrin (HPβCD). In both media a decrease in the values of K_Q was observed in the presence of CDs, as compared with their absence. In acid media, the decrease was: 36–39% with βCD and 48–54% with HPβCD for 5HIA and 5HT, respectively. In basic media, with the neutral receptors, these values are 69–29% with βCD and 56–43% with HPβCD for 5HIA and 5HT, respectively. Moreover, a complete quenching inhibition produced by ionized cyclodextrins was determined for both substrates. The results showed a net protective effect due to the guest inclusion into the cyclodextrin nanocavity. This study is particularly important for analytical determinations in such media.

1. Introduction

Tryptophan is the main amino acid residue which is responsible for fluorescence in proteins [1]. It has been studied in many media in order to predict and explain their emission in biological systems [2] and how the peptide structure affects the fluorescence emission [3]. This fluorescence comes mainly from the indole nucleus which is affected by many variables such as pH [4], solvents [5] and other organic molecules [6]. When tryptophan is metabolized, many indole derivates are synthesized as hydroxy-indole compounds. Hydroxy-indoles are important biological compounds as hormones, neurotransmitters or pharmaceutical products [7].

Serotonin (5-hydroxytryptamine, 5HT) (Fig. 1a) and 5-hydroxy-3-indolyl acetic acid (5HIA) (Fig. 1b), are derived from metabolism of tryptophan. 5HT is a neurotransmitter of the central nervous system, which is involved in a large number of physiological processes [8], and 5HIA is the metabolite of 5HT in urine.

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six (αCD), seven (βCD) or eight (γCD) units of α-D-glucose linked by α-(1,4) bonds. Among the derivatives of native CDs, hydroxypropyl-β-cyclodextrin (HPβCD) has higher solubility than its homologue in water and some analytical advantages [9]. These macrocycles have a nanocavity (internal diameter of βCD is 0.7 nm), which allows them to act as hosts, forming inclusion complexes with guest molecules in the solid state or in solution [10]. The acid–base property of CDs ($pK_a = 12.2$) [11], also provides either a neutral or an anionic receptor depending on the pH of the medium.

5HT and 5HIA have two acidic protons and each one of the three acid–basic species has a particular fluorescent characteristic. In our previous work, interactions of CDs with both hydroxy-indoles were studied at different pHs and on the basis of fluorescence changes the association constants of the complexes for each substrate species and their analytical applications were determined [12].

These hydroxy-indoles show fluorescence quenching in basic (pH > 12) and acid media (pH < 2) like other more simple indoles (mono acid or without any acidic proton) [13]. Based on the previous knowledge of indole quenching and their host-guest complex formation, the goal of this work was to study the effect of βCD and HPβCD on the fluorescence quenching of 5HT and 5HIA in acidic and basic aqueous solutions. The results have direct applications in analytical determinations in these media.

2. Experimental

Water was obtained using a Millipore apparatus. 5HT and 5HIA (99% purity, Sigma–Aldrich), βCD (Roquette), HPβCD (Cerestar, degree of substitution 5.5). The basic and acid solutions were prepared from concentrated solutions of NaOH (1 M) or HCl (4 M), respectively. All constituents of the buffers were commercial reagents of analytical grade.

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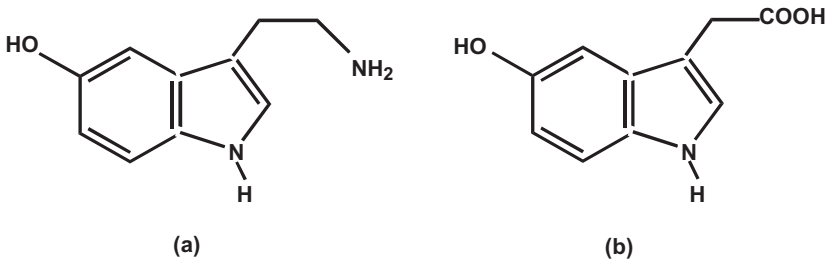


Fig. 1. Chemical structures: (a) 5HT and (b) 5HIA.

UV–vis and spectrofluorimetric determinations were carried out on a Shimadzu UV-2101 PC and a Jasco FP-777, respectively. The pH was measured using an Orion model 720 pH meter working at (25.0 ± 0.1) °C with a Ross combination pH electrode. The pH-meter was calibrated using standard buffers (pH = 2.932; 4.955; 6.994 and 9.155) prepared according to literature [14]. An ultrasonic bath (Testlab tb02) was used for the dissolution of the reagents.

A concentrated solution of substrate in water (2 mg/10 mL) was stored in the refrigerator (4 °C) for a maximum of 20 days. Stability of the stock solutions was periodically checked by spectrophotometry before preparing the appropriate dilute solutions for fluorimetric determinations. Water solutions were prepared by adding the stock solution of substrate to the reference buffer solution prepared as indicated above and diluting to the mark with water. All solutions were protected from light. For emission and excitation fluorescence measurements the photomultiplier gain was set to medium (acidic or neutral media) or high (basic media) and emission and excitation bandwidths were 10 nm. The samples were excited at a wavelength equal to the wavelength of maximum absorption. All the determinations were made at (25.0 ± 0.1) °C, and the temperature of the cell compartment was controlled with a Haake circulator. The ionic strength (μ) of all solutions was adjusted to 0.124 mol L^{-1} by adding NaCl when required.

For the analysis of the spectrofluorimetric data, the total area below the fluorescence spectrum (F) (Eq. (1)) and the fluorescence intensity at a fixed emission wavelength (F_λ) (Eq. (2)) were considered. In these equations B is a

$$F = B \sum \varepsilon_i \phi_i [i] \quad (1)$$

$$F_\lambda = B \sum \varepsilon_i \phi_i \gamma_i [i] \quad (2)$$

constant, which depends on the instrumental set-up, ε_i is the molar absorptivity at the λ^{ex}, ϕ_i is the fluorescence quantum yield, γ_i is the fraction of the total emission intensity at a given wavelength and (i) indicates the concentration of each fluorescent species i. In all cases the absorbance of the solution was <0.025, where Eqs. (1) and (2) are valid.

Stern–Volmer plots were used to evaluate the fluorescence quenching as pH was decreased below 2.00 and as pH was increased above 12.20. The Stern–Volmer quenching constants of 5HT and 5HIA in the presence and absence of βCD and HPβCD were determined taking into account the free and the complexed substrate in the corresponding media. In alkaline solutions, two molar fractions of complexed substrate had been considered, one with the neutral CD and the other one with the ionized cyclodextrin (CD^-) (βCD pK_a = 12.2) acting as receptor. Data analysis was performed with Sigma Plot (Scientific Graph system) version 8.00 (Jandel Scientific).

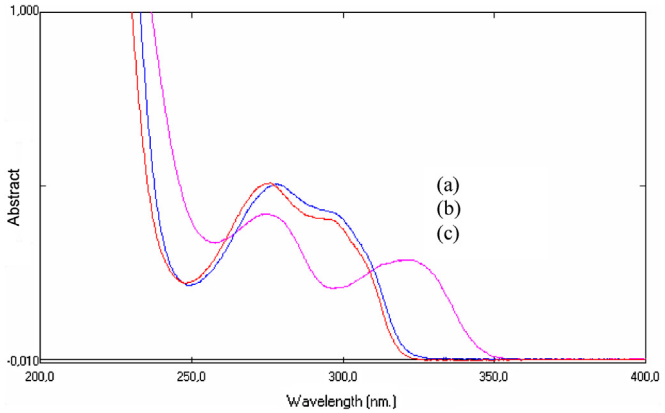


Fig. 2. Absorbance spectra of 5HIA at different pHs: (a) pH 2.00, (b) pH 7.00 and (c) pH 13.00 indicated a 300 nm.

3. Results and discussion

3.1. Influence of cyclodextrins on the acid-base equilibrium in the ground and excited state of hydroxy-indoles

The UV–vis absorbance spectra were measured for 5HT and 5HIA, and the same maximal absorption wavelength was determined for both substrates, since the chromophore is the same for 5HT and 5HIA and the specific substituent at position 3 of the indole in each case is not conjugated with the aromatic nucleus. This wavelength is centered at 275.0–278.0 nm at pH between 2.00 and 7.00. At pH 13.00 a second absorption band appeared at 322.0–323.0 nm caused by the phenol deprotonation of the aromatic moiety (Fig. 2 for 5HIA is representative). The presence of cyclodextrin (10 mM) has a negligible effect on the absorption spectra.

Based on the change of the absorbance when increasing the pH, the pK_a corresponding to the phenol deprotonation (pK_{a2}) for both hydroxy-indoles was determined. Considering the equilibrium involved between the mono acid species of the substrate (SH^{-1}) and the dibasic one (S^{-2}) (Scheme 1) and the additive Lambert Beer law, the acid constant was obtained fitting the data to Eq. (3). These values (pK_{a2}) were 11.12 ± 0.01 (at 322.0 nm) and 11.16 ± 0.03 (at 320.0 nm) for 5HT and 5HIA, respectively. These similar values for both 5-hydroxyindoles are higher than for 6-hydroxymelatonin (10.24 ± 0.01) [15] indicating the dependence

$$SH^{-1} \overset{K_{a2}}{\rightleftharpoons} S^{-2} + H^+$$

Scheme 1. Acid–base equilibrium for the phenol deprotonation for 5HIA or 5HT.

Table 1
Values of pK_a for the free and complexed substrate in the ground and excited state.

S	pK_{a1}	pK^*_{a1}	ΔpK^*_{a1}	pK_{a2}	pK^*_{a2}	ΔpK^*_{a2}
5HIA	4.74 ± 0.05	4.20 ± 0.50[b]	0.54	11.16 ± 0.03	10.72 ± 0.07[b]	0.44
5HIAβCD	4.63 ± 0.05[a]	4.20 ± 0.06[b]	0.43	11.55 ± 0.05[a]	10.9 ± 0.1[b]	0.25
5HIAHPβCD	4.76 ± 0.06[a]	4.2 ± 0.1[b]	0.54	11.46 ± 0.06[a]	11.3 ± 0.1[b]	0.16
5HT	~10.1[c] $-\log(K_{a1}K_{a2})$[d]	~9.5[c]	~0.6[c]	11.12 ± 0.03	10.26 ± 0.09[b]	0.86
5HT	21.22					
5HTβCD	20.90[a]					
5HTHPβCD	20.75[a]					

[a] Values obtained from the thermodynamic cycle.
[b] Values obtained from the Förster cycle.
[c] Estimated as explained in the text.
[d] In the presence of CDs it was not possible to estimate the K_A between the monoacid species of 5HT and the neutral CD.

of the acidity of the hydroxyl group with the position on the indole nucleus.

$$A_{pH} = \frac{A_{minimum} + A_{maximal}10^{(pH-pK_a)}}{10^{(pH-pK_a)} + 1} \quad (3)$$

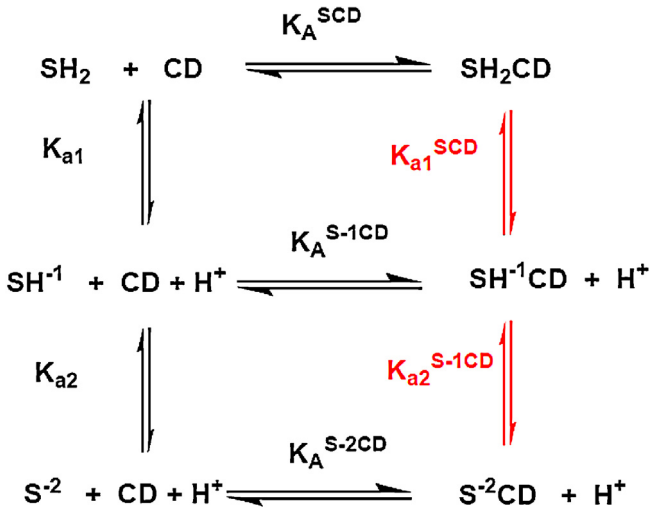
For 5HIA the pK_{a1} was determined from the fluorescence decrease (75%) produced from pH 7 to pH 2 employing an equation similar to Eq. (3) expressed in terms of *F*. This observation indicates that the carboxylic acid is much less fluorescent than the carboxylate species with a $pK_{a1} = 4.74 \pm 0.05$. In the case of 5HT it was not feasible to determine accurately the pK_{a1} for the amine deprotonation due to the proximity of pK_{a1} and pK_{a2} and the continuous decrease in *F* with the increase of the pH from 9 to 12. However, from careful experiments it was possible to estimate a pK_{a1} value of ~10.1. This value correlates with the previously obtained [13] for 5-methoxytryptamine ($pK_{a1} = 9.9 \pm 0.1$) [16] and tryptamine ($pK_{a1} = 9.5 \pm 0.2$) [17]. All the pK_a values are presented in Table 1.

Moreover, in order to study the effect of cyclodextrins on the acidity constants of the substrates, the pK_a of the complexes in the ground states (pK_{a1} for SH_2CD and pK_{a2} for SH^-CD) were calculated from the thermodynamic cycle based on the acid–base and the inclusion complex equilibriums represented in (Scheme 2). In this scheme, SH_2 is the completely protonated substrate (diacid species), SH^{-1} the monoacid (or monobasic) substrate, S^{-2} the dibasic substrate, and K_A^{SiCD} corresponds to the association constant for the species *i* of the substrate and CD. From this scheme and the K_A^{SiCD} determined previously in our laboratory [12], the acid constant values for the complexed substrates with βCD and HPβCD in the ground state were calculated (K_a^{SiCD}). Table 1 summarizes the pK_a^{SiCD} obtained.

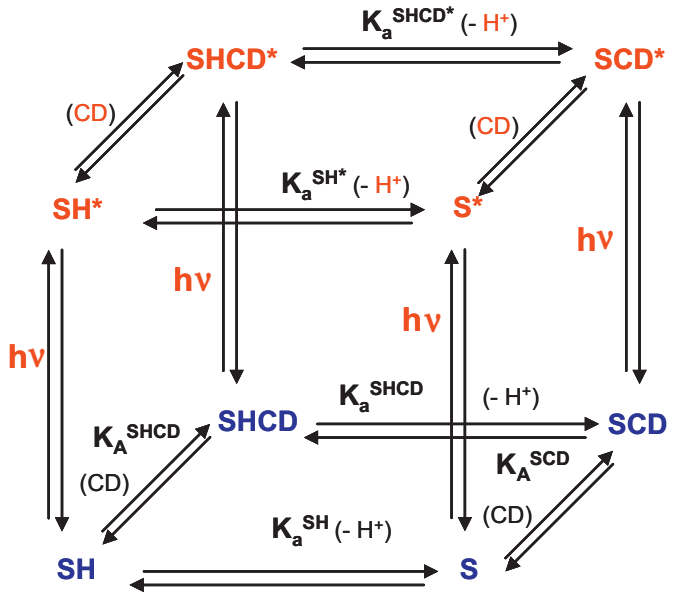
For 5HIA, the complex formation with both CDs practically does not affect the acidity of pK_{a1} due to the similarity of the K_A^{SiCD} values for the acid and basic species. Nevertheless, the pK_{a2}^{SiCD} values are higher with both CDs as a consequence of the higher affinity shown by CDs for the SH^{-1} form of the substrate than for S^{2-} ($K_A^{SH^-}/K_A^{S^{-2}} \sim 2$) [12]. This opposite effect results from the stronger association constants with a neutral aromatic nucleus than with a negative charged one, showing that the deprotonation of the carboxylic acid is not involved in the complex formation.

For 5HT-CD complexes, we were unable to calculate the pK_{a1}^{SiCD} and the pK_{a2}^{SiCD} due to our inability to determine the individual $pK_a^{SH^{-1}CD}$ because of the proximity of pK_{a1} and pK_{a2}. In this case from the thermodynamic cycle we estimated the values for $[-\log(pK_{a1}^{SiCD}pK_{a2}^{SiCD})]$ showed in Table 1. These values indicate major acidity for the complexes as result of the stronger affinity of dibasic form of the substrate (S^{-2}, anionic) than the diacid one (SH_2 cationic) ($K_A^{S^{-2}}/K_A^{SH^2} \sim 2$) [12].

The acidic constants in the excited state (pK_a^*) were determined from the Förster-cycle [18] (Scheme 3 and Table 1). The front face of the cube in Scheme 3 represents the equilibriums involved for the free substrates; meanwhile the back face of this scheme shows the situation for the complexed substrates. The $(\Delta pK_a^*)^{S-S^*}$ values showed a small increase in the acidic constants for the excited



Scheme 2. Acid–base and cyclodextrin complexation equilibriums involved for 5HIA and 5HT in the ground state.



Scheme 3. Representation of acid–base equilibriums in the ground and excited state for the free and complexed substrate with cyclodextrins.

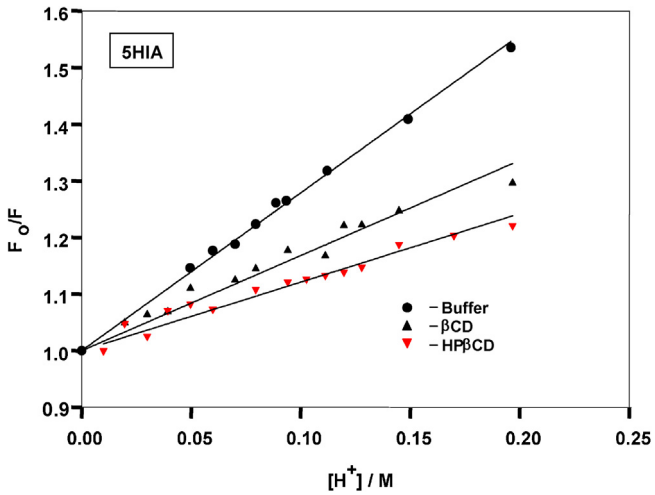


Fig. 3. Stern–Volmer plot for the fluorescence quenching by proton for 5HIA in presence of βCD (△), HPβCD (▽) and only in aqueous media (●).

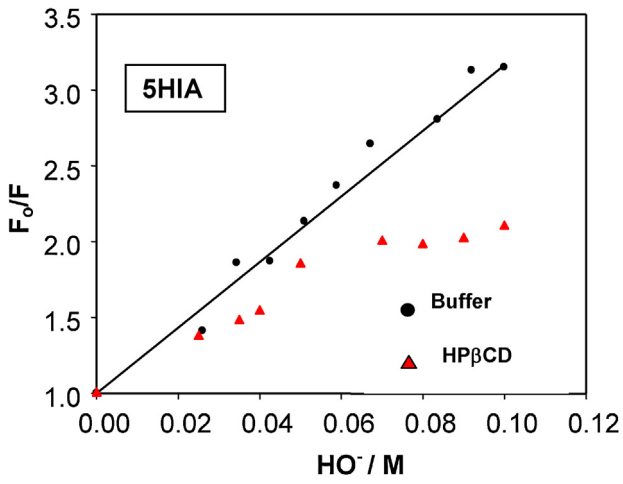


Fig. 4. Stern–Volmer plot for the fluorescence quenching by hydroxide ions for 5HIA free (●) and in presence of HPβCD (△).

states of free and complexed substrates (0.2–0.9) as observed for other indole derivates non-hydroxylated at position 5 [13,19].

Based on these results for both substrates, the pH conditions were selected in order that the fluorescence changes measured in the presence or absence of the host were only attributed to one acid–base fluorescent species.

3.2. Effect of proton and hydroxide ions on the fluorescence emission of serotonin and 5-hydroxy-3-indolyl acetic acid

It is known that the fluorescence quantum yield of simple indole compounds is independent of the pH in the range of 3–10 [20]. Dynamic quenching has been observed for these indolic compounds at pH < 3.00 and pH > 11.00 [4].

5HIA (pK_{a1} = 4.74) was studied at pH values smaller than 2.00 because at higher values the fluorescence of the carboxylate ion species (minority quantity with greater quantum yield) contributes significantly to the total fluorescence emission, although the carboxylic acid species is the majority (smaller quantum yield).

In alkaline media, the influence of hydroxide on quenching was studied at pH > 12.20 because below this value two acid–base species are found in the case of 5HT (pK_a = 11.10). For both substrates a decrease of the fluorescence was observed at pH < 2.00 and pH > 12.20 (Figs. 3 and 4). Such decrease was attributed to the quenching effect produced by the presence of H^+ and OH^-, respectively, as is reported for other indoles [4].

3.3. Determination of Stern–Volmer quenching constants in acid media

The plots of (F_0/F) versus [H^+] in the presence and absence of CD ([H^+] = 0.010–0.200 M) show a linear behavior (Fig. 3).

In the absence of CD, the experimental data were fitted by the general Eq. (4) taking into account only one fluorescent species (the free indole), where f_i is the fluorescent fraction of the species i (i: SCD for the complex and S for the free substrate) and K_Q^i the Stern–Volmer quenching constant for the i species.

$$\frac{F_0}{F} = \sum_{i=1}^{n} \left\{ \frac{f_i}{1 + K_Q^i[Q]} \right\}^{-1} \quad (4)$$

In presence of CD (βCD and HPβCD) two fluorescent species can be quenched by H^+, the free and complexed fluorophore. For this system, Eq. (4) considers these two species as expressed in Eq. (5). From this equation the Stern–Volmer constants (K_Q^i) for the complexed substrate were determined.

$$\frac{F_0}{F} = \left\{ \left(\frac{f_S}{1 + K_Q^S[Q]} \right) + \left(\frac{f_{SCD}}{1 + K_Q^{SCD}[Q]} \right) \right\}^{-1} \quad (5)$$

In all cases, a linear behavior was found. Table 2 displays the Stern–Volmer quenching constants obtained.

As for other indolic compounds, this effect was attributed to a collisional quenching by H^+ involving a proton transfer reaction in the excited state on the ring of the indole nucleus at positions 2 or 4 [20].

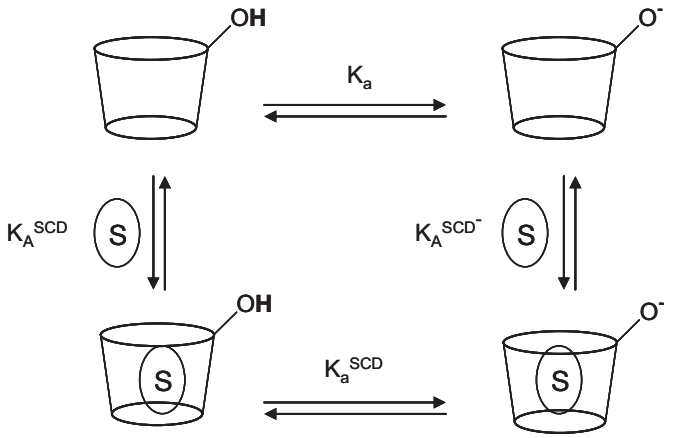
A decrease of the K_Q^{SCD} value compared to K_Q^S can be observed: from 36 to 39% with βCD and from 48 to 54% with HPβCD for 5HT and 5HIA, respectively. These decreases in the K_Q^{SCD} values for 5-hydroxy-indoles indicate that the CD nanocavity protects the excited state of the substrates from the quencher action. The formation of an inclusion complex produces a restriction for the quencher accessibility to the substrate, as observed previously for simple indoles in our laboratory [13]. Comparing the association constants (K_A^{CD}) with K_Q^{SCD}, a higher protective effect with HPβCD (K_Q^{SCD}) correlates with stronger K_A^{CD} for both substrates ($K_A^{S\beta CD}$ vs $K_A^{SHP\beta CD}$: 56 M^{-1} vs 148 M^{-1} for 5HIA and 170 M^{-1} vs 340 M^{-1} for 5HT) [12]. In addition, comparing different indolic substrates, for example 5-hydroxy-indoles vs non-substituted at position 5 homologous or other simple non-protic indoles (for example methylindole and melatonin), the data also correlate very well since for a higher K_A^{CD} a minor K_Q^{SCD} is found [13].

Moreover, if the K_Q of the hydroxy-indoles are only compared with the non-substituted at position 5 homologous (3-indolyl acetic acid, 3IA, K_Q^{3IA}: 34.4 M^{-1} and tryptamine, T, K_Q^T: 33.3 M^{-1}), the values obtained for the 5-hydroxy-derivatives are ≥5 times

Table 2
Values of Stern–Volmer fluorescence quenching constants by proton for free and complexed substrates.

Substrate i	K_Q^i (M^{-1})
5HIA[a]	(2.02 ± 0.06)
5HIAβCD[b]	(1.29 ± 0.07)
5HIAHPβCD[b]	(1.05 ± 0.03)
5HT[a]	(6.0 ± 0.1)
5HTβCD[b]	(3.69 ± 0.05)
5HTHPβCD[b]	(2.77 ± 0.05)

[a] Values obtained fitting Eq. (4), considering the free substrate only.
[b] Values obtained fitting Eq. (5), considering the free and complexed substrate.



Scheme 4. Acid–base equilibrium of the substrate (S) and CD-complex at basic pHs.

smaller; and comparable or lower than with 5-methoxytryptamine, 5MT ($K_Q^{5MT} \approx 10\,M^{-1}$) [13].

3.4. Determination of Stern–Volmer quenching constants in alkaline media

The effect of the hydroxide ion (concentration intervals: 0.016–0.100 M) on the fluorescent emission of 5HT and 5HIA in the presence and absence of βCD and HPβCD was measured. In both cases, the free indole fluorescence in the pH range 12.20–13.00 shows a linear decrease. On the other hand, non-linear plots were obtained in the presence of CDs (Fig. 4).

In order to explain these experimental results in the presence of CDs, a model taking into account the variation of the molar fractions of the two CDs species is herein proposed. In the hydroxide concentration interval studied, the ratio between neutral ($pK_a \sim 12$) [11] and ionized CD (CD/CD$^-$) changes (Scheme 4).

At pH = 12.20 ([OH$^-$] = 0.016 M), the fluorescence expression (F_0) is represented with Eq. (6). At pH > 12.20 where the concentration of quencher ([Q]) increases, the fluorescence equation (F) is shown in Eq. (7).

$$F_0 = cte \cdot (\phi_S X_S + \phi_{SCD} \cdot X_{SCD} + \phi_{SCD^-} X_{SCD^-}) \quad (6)$$

$$F = cte \cdot (\phi_S^Q \cdot X_S^Q + \phi_{SCD}^Q \cdot X_{SCD}^Q + \phi_{SCD^-}^Q \cdot X_{SCD^-}^Q) \quad (7)$$

From the ratio between Eqs. (6) and (7), Eq. (8) is obtained for the data fitting:

$$\frac{F_0}{F} = \left\{ \left[\left(\frac{\phi_S^Q}{\phi_S} \right) \left(\frac{X_S^Q}{X_S} \right) f_S \right] + \left[\left(\frac{\phi_{SCD}^Q}{\phi_{SCD}} \right) \left(\frac{X_{SCD}^Q}{X_{SCD}} \right) f_{SCD} \right] + \left[\left(\frac{\phi_{SCD^-}^Q}{\phi_{SCD^-}} \right) \left(\frac{X_{SCD^-}^Q}{X_{SCD^-}} \right) f_{SCD^-} \right] \right\}^{-1} \quad (8)$$

where (ϕ_i^Q/ϕ_i) is the quantum yield of species i (i: S; SCD and SCD$^-$); X_i = molar fraction of species i; and X_{Qi} = molar fraction of species i in the presence of quencher; f_i is the fluorescence fraction of the species i. Replacing (ϕ_i^Q/ϕ_i) in Eq. (8), the equation for the experimental data fitting (Eq. (9)) is finally obtained:

$$\frac{F_0}{F} = \left\{ \left[\left(\frac{1}{1 + K_Q^S[Q]} \right) \left(\frac{X_S^Q}{X_S} \right) f_S \right] + \left[\left(\frac{1}{1 + K_Q^{SCD}[Q]} \right) \left(\frac{X_{SCD}^Q}{X_{SCD}} \right) f_{SCD} \right] + \left[\left(\frac{\phi_{SCD^-}^Q}{\phi_{SCD^-}} \right) \left(\frac{X_{SCD^-}^Q}{X_{SCD^-}} \right) f_{SCD^-} \right] \right\}^{-1} \quad (9)$$

In order to fit the data of the alkaline quenching plots, different situations and an iterative procedure were used. First, at pH 13.00 ([OH$^-$] = 0.01 M), Eq. (9) has two terms taking into account two species i: SCD$^-$ and S. At this pH $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ were estimated. Second, at pH 12.20, considering all the species i: SCD$^-$; SCD and S

Table 3
Values of Stern–Volmer fluorescence quenching constants by ion hydroxide for free and complexed substrates.

Species i	K_Q^i (M^{-1})
5HIA[a]	(22.6 ± 0.5)
5HIAβCD[b]	(7 ± 1)
5HIAHPβCD[b]	(10 ± 1)
5HT[a]	(14 ± 0.2)
5HTβCD[b]	(10 ± 1)
5HTHPβCD[b]	(8 ± 3)

[a] Values obtained fitting a lineal model, considering the free substrate.
[b] Values obtained fitting Eq. (9). This equation takes into account the variation of the molar fractions of the two CD species with the pH.

and using the value estimated for $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ at pH 13.00, K_Q^{SCD} was determined. From the value of K_Q^{SCD}, the $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ value for each hydroxide concentration was calculated. At [OH$^-$] > 0.05 M (pH > 12.6) the values of $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ were found to be constant. This observation indicates that the third term of Eq. 9 is practically constant and independent of the [OH$^-$] concentrations, so $K_Q^{SCD^-}$ must be null. For this reason, an average of the $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ values was calculated at higher hydroxide concentrations. The average values of $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ are the following: for 5HT, 0.596 with βCD and 0.553 with HPβCD; for 5HIA, 0.357 with βCD and 0.576 with HPβCD.

Using the $(\phi_{SCD^-}^Q/\phi_{SCD^-})$ average value calculated and K_Q^S in Eq. (9), the experimental data were fitted obtaining the final K_Q^{SCD}. Table 3 exhibits the K_Q^{SCD} values estimated for both substrates.

The protective effect of the cyclodextrin nanocavity was also observed for both CDs (higher for HPβCD). Both ionized CDs (βCD$^-$ and HPβCD$^-$) were better protectors than neutral receptors ($\boldsymbol{\Phi}_{SCD^-}/\boldsymbol{\Phi}_S$) > ($\boldsymbol{\Phi}_{SCD}/\boldsymbol{\Phi}_S$) of the indole excited state [12]. The higher effect obtained with ionized cyclodextrin nanocavities indicate that the substrate fraction bonded to CD$^-$ is not accessible to the quencher, producing the downward curvature measured [13].

The values of K_Q^{SCD} obtained are smaller than those for K_Q^S: a 29% and 43% decrease for 5HT, and a 69% and 56% decrease for 5HIA, with βCD and HPβCD respectively. The decrease is provoked by a cyclodextrin protective effect on the fluorescence guest, due to a decrease in the accessibility of the quencher into the inclusion complex. At [OH$^-$] > 0.05 M (pH > 12.6), the ionized CD is the major species and its complex with the substrate has a total repulsion effect against the hydroxide ion diffusion into the CD cavity.

These values are similar to those from other indole compounds studied by time-resolved techniques in the order of picoseconds in alkaline media. This process is controlled by diffusion with values of kinetic constants around $k_Q \cong 26 \times 10^9$ for indole at T = 26 °C [21]. Comparing K_Q for 5HT and 5HIA with the non-substituted at position 5 homologous (T and 3IA), the ratios $(K_Q^T/K_Q^{5HT}) = 5$ and $(K_Q^{3IA}/K_Q^{5HIA}) = 3$ show that the non-substituted compounds are intrinsically more affected by hydroxide ions. However, in the

presence of CD the effective quenching protection is similar for each pair of homologous. The decrease percentage of K_Q^{SCD} compared with K_Q^S is 30% for 5HT-βCD and 40% for T-βCD; 70% for 5HIA-βCD and 72% for 3IA-βCD; and larger values were measured for HPβCD complexes.

Cyclodextrin complexes have shown a protective quenching effect also in acid and alkaline media for aromatic compounds (1-metoxynaphthalene [22]; 2-naphthol [23] and 2-naphthylamine [24]) and a kinetic increase on the deprotonation of carbazole [25].

In both media studied, a CD protective effect was found, and it is known that in acid media the quenching process is a protonation at position 2 or 4 of the indole nucleus; meanwhile, the alkaline quenching is mediated by the deprotonation of the pyrrolic-NH. The results presented in the present work agree with the mechanism proposed in literature for acid and basic quenching of indoles and with the mode of inclusion proposed for 5HIA and 5HT in a previous report [12].

4. Conclusions

The pK_a values in the ground state of hydroxy-indoles complexed with cyclodextrins did not show any substantial change compared with those of the free substrates, whereas in the excited state the acidity increased for free and complexed substrates. The decrease in fluorescence measured is due to collisional quenching by H^+ and OH^-. Cyclodextrins, βCD and HPβCD, showed a quenching protective effect in these media. The decrease of K_Q^{SCD} values indicates that the CD protects the excited state of the substrate to the accessibility of the quencher action by restriction in the host guest complex. This can be confirmed by comparing the association constants (K_A) with K_Q^{SCD}. A higher protective effect with HPβCD correlates with stronger K_A for both substrates.

These results with hydroxy-indoles allow us to conclude that the quenching protection by CDs and the quenching inhibition by ionized CDs is a general behavior for indole compounds, since similar effects have been previously reported by us for simple indoles. In conclusion neutral, cationic or anionic indoles, singly or doubly charged, are partial or totally protected by CDs or ionized CDs, respectively, from the action of quenchers.

This protective quenching effect by cyclodextrins highlights the importance of supramolecular chemistry applied to fluorescence quenching protection for future analytical determinations in acid and basic strong media.

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